**CRANFIELD UNIVERSITY**

**NKWONTA CHIKERE GODWIN**

**PhD THESIS**

# BIOCHEMICAL AND BIOMEDICAL STUDIES ON AFRICAN WALNUT (*TETRACARPIDIUM CONOPHORUM* -MULL. ARG.) – A POSTHARVEST PERSPECTIVE

**ACADEMIC YEAR: 2011 – 2015**

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# ABSTRACT

African Walnut (*Tetracarpidium conophorum*- Mull. Arg) is a perennial climbing shrub which grows mainly in the Western region of Africa. It is found mainly in Nigeria, Gambia, Sierra Leone, Gabon, Equatorial Guinea and Cameroon as well. The nuts are encased in pods which may contain between 2 to 5 nuts. The seed is enclosed in a hard shell-like case. The nuts are commonly processed by boiling or roasting and consumed as a snack or used as soup thickener. In ethnobotanical medicine, the nut extract is extensively used in decoctions for treatment and/or management of common and chronic ailments such as malaria, dysentery, high blood pressure, diabetes and cancer. The nuts are generally exposed to high temperatures (25 – 37 °C) and relative humidity (RH) which increases susceptibility to fungal contamination and nutrient degradation, hence, raising concerns over product quality and safety. Experiment simulating the common retail postharvest storage and processing practices was conducted to: (i) determine the effects on the fatty acid profile; (ii) assess the impact on the fungal population contaminating the nut shells at different maturity stages, and potential mycotoxigenic implications; (iii) evaluate the cytotoxicity of four extract of the nut on lung cancer (A549) cells; and finally (iv) assay the total phenolic content and profile potential individual phenolic components of the nut.

Results indicated the presence of essential and non-essential fatty acids namely; palmitate, oleate, stearate, linoleate, arachidate and α-linolineate with α-linolineate being the most abundant (1.1 – 8.2 mg/g freeze-dry weight). Boiling and roasting generally improved the concentration of the fatty acids best when nuts are cold stored at 5 °C for maximum of 10 days.

Potential mycotoxigenic species - *Aspergillus section Nigri, Aspergillus flavus/Parasiticus, Fusarium* spp. and *Penicillium* spp. - were frequently isolated from cultured shell pieces of stored nuts. When compared with unprocessed nuts, roasting completely prevented fungal contamination in shell pieces from nuts in the non-stored (NSN) group at early maturity stage, while boiling significantly reduced the level of contamination to about 58 % ($P < 0.05$). Simulating open market conditions caused 100

% fungal contamination in all boiled samples and roasted samples at early maturity. Mycotoxin analysis using Yeast Extract agar (YES) and High Performance Liquid Chromatography (HPLC) - Fluorescence detector (FLD) showed that Aflatoxins - G1 (AFG1), B1 (AFB1), G2 (AFG2), and B2 (AFB2) were produced by 20 isolates with both AFG1 and AFB1 being predominant at concentration ranges 4 – 32,200 and 4 – 22,700 ng/g plug weight, respectively. No Ochratoxin A (OTA) was detected.

Phenolic component analysis indicated unprocessed (20.79 ± 1.0 mg gallic acid equivalent per gram freeze-dry weight – GAE/g FDW) samples showed the highest value for total phenolics while both boiling (9.90 ± 1.8 mg GAE/g FDW), and roasting (9.32 ± 2.7 mg GAE/g FDW) reduced the amount by more than 50 % when compared with unprocessed. Potential individual phenolic compounds were unambiguously separated using high performance liquid chromatography – diode array detector (HPLC-DAD). There were no differences between chromatograms of defatted and non-defatted unprocessed, roasted and boiled samples. Cytotoxicity evaluation showed no decrease in cell densities in plates treated with extracts from unprocessed nuts at all concentrations. Diethyl ether-ethyl acetate (10 µg/mL) and n-butanol (1000 and 500 µg/mL) extracts of roasted nuts as well as dichloromethane and water (1, 10 µg/mL) of boiled nuts caused a non-significant decrease of < 10 % in cell densities when compared with the phosphate buffered saline-media control. However, all extracts showed no cytotoxic effect on the A549 cells

African walnut is basically produced at subsistence level in Nigeria, but considering the presence of desirable fatty acid profile and phenolic compounds, need for increased industrial scale production is herein recommended. Although fungal attack and potential mycotoxin risk on the nut may be high, retail processing by roasting has prospects to greatly accentuate the risk. Cold storage of the nut may help to improve the shelf life although it may not be cost effective for local farmers in Nigeria and Africa, however, it provides opportunity for export business. Although the nut extracts showed no cytotoxic effect on A549 lung cancer cell lines, there is need to investigate further to confirm it non-cytotoxicity activity on other cancer lines and normal cell lines.

# EXECUTIVE SUMMARY

African Walnut (*Tetracarpidium conophorum*- Mull. Arg) is a perennial climbing shrub which grows mainly in the Western region of Africa. It is found mainly in Nigeria, Gambia, Sierra Leone, Gabon, Equatorial Guinea and Cameroon as well. The nuts are encased in pods which may contain between 2 to 5 nuts. The seed is enclosed in a hard shell-like case. The nuts are commonly processed by boiling or roasting and consumed as a snack or used as soup thickener. In ethnobotanical medicine, the nut extract is extensively used in decoctions for treatment and/or management of common and chronic ailments such as malaria, dysentery, high blood pressure, diabetes and cancer. As a result, it is usually on high demand during harvest and costly when compared with the prices of other nuts. The nuts are generally exposed to high temperatures and relative humidity (RH) which increases susceptibility to fungal/microbial contamination and nutrient degradation, hence, raising concerns over product quality and safety. In spite of promising potentials of this nut in the field of nutrition and medicine, only a few studies have been conducted on it. Most of these studies have been done using non-sophisticated equipment and techniques. In contemporary science, it is quite pertinent that claims on the activities of this nut are verified and solutions to its potential mycotoxin contamination proffered in order to maximise the use in industries.

Effects of postharvest storage and processing techniques on the fatty acid profile of African walnut were investigated. The nut is sold within 1 – 7 days to consumers through the open market system. During processing, storage and distribution, they are typically exposed to high temperatures raising concerns over nutrient quality and safety. Although African walnut, like several other nuts, contains high amount of oil, there is no study reporting on how the processing methods (boiling and roasting) affect the fatty acid profile. Nut samples ($n$ = 702) at both early and late maturity were harvested and stored at 5°C for 0, 10 and 20 days, afterwards, they were grouped according to treatments - boiling, roasting and unprocessed. Nuts were then held for 3 and 7 days at either 25°C or 37°C to simulate normal retail practices. Oil was extracted and analysed as fatty acid methyl esters using gas chromatography flame ionization detection and gas chromatography coupled mass spectrometry. Retention times were compared with known

standards. This is the first documented analysis of fatty acid contents of African walnut harvested from Nigeria. Results indicated the presence of essential and non-essential fatty acids which are generally sought for in edible oils. These are palmitate, oleate, stearate, linoleate, arachidate and α-linolineate with α-linolineate being the most abundant (1.1 – 8.2 mg $g^{-1}$ dry weight). In general, boiled and roasted nuts subjected to simulated retail practices (viz. 25°C or 37°C) for either 3 or 7 days significantly increased concentrations of the fatty acids (> 50%) in nuts samples stored for 10 days compared to unprocessed. The abundance of mono and poly–unsaturated fatty acids in the profile suggest that the use of the oil in daily cooking may convey potential nutritional benefits on consumers. The study also showed that the current retail postharvest practice of boiling, roasting and storage (25 - 37 °C) improves the fatty acid profile by increasing concentrations of major fatty acids. However, cold storage (up to 10 days) is not a common practice by retailers and may be challenging to undertake in a large scale giving the instability of electricity in west and central Africa. Consequently, it creates an opportunity for export business. Currently, African walnut is basically produced at subsistence level in Nigeria, however, considering the need for increased affordable plant sources of healthy edible oil in developing nations, industrial scale production of African walnut is herein recommended.

The impact of postharvest processing on the fungal population contaminating African walnut shells at different maturity stages, and potential mycotoxigenic implications was also assessed. This assessment was as a result of the understanding that exposure of the nuts to both high temperatures and high relative humidity in open markets predisposes them to fungal growth. Hence, the dangers of spore inhalation and resultant mycosis cannot be over-emphasized as retailers and consumers are always in direct contact with these nuts during harvest, processing and consumption. So far, there is no reported research on potential mycotoxin contamination of African walnut and whether this risk might be accentuated by processing. African walnut, at early and late maturity stages, were processed by roasting, boiling or left unprocessed before being stored at 25°C and 37°C, respectively under controlled relative humidity (78%) for 7 days. Nuts were cracked and shell pieces cultured in Malt Extract Agar (MEA) and Dichloran Glycerol 18 (DG18) media and incubated at 25°C for 7 days. Results revealed that potential mycotoxigenic species - *Aspergillus section Nigri, Aspergillus flavus/Parasiticus, Fusarium* spp. and *Penicillium* spp. - were frequently isolated. When compared with

unprocessed nuts, roasting completely prevented fungal contamination in shell pieces from nuts in the non-stored (NSN) group at early maturity stage, while boiling significantly reduced the level of contamination to about 58 % ($P < 0.05$). In general, simulating open market conditions caused 100 % fungal contamination in all boiled samples and roasted samples at early maturity. However, contamination in roasted samples at late maturity were raise to 90 and 70 % at 25°C in DG 18 and MEA, respectively, while at 37°C contamination was 40 and 60 % in DG 18 and MEA, respectively. Mycotoxin analysis using Yeast Extract agar (YES) and High Performance Liquid Chromatography (HPLC) - Fluorescence detector (FLD) showed that Aflatoxins - $G_1$ ($AFG_1$), $B_1$ ($AFB_1$), $G_2$ ($AFG_2$), and $B_2$ ($AFB_2$) were produced by 20 isolates with both $AFG_1$ and $AFB_1$ being predominant at concentration ranges 4 – 32,200 and 4 – 22,700 ng/g plug weight, respectively. No Ochratoxin A (OTA) was detected out of 23 isolates analyzed. From these findings, it is suggested that roasting of nuts is a safer processing option in terms of prevention of possible fungal growth on nut shells and risk of mycotoxin contamination. The current postharvest handling of unprocessed African walnuts may favour growth and development of mycotoxigenic fungi species as well as promote aflatoxin contamination on the nuts. This poses a great potential health risk yet to be considered by policy makers in Nigeria and other African nations where the nut is consumed. Further research is needed to establish whether the kernels are contaminated with mycotoxin.

As the nut extracts are constantly mixed in decoctions used in management of chronic diseases such as diabetes and cancer (various types) in ethnobotanical medicine, the cytotoxicity of four extract of African walnut on lung cancer (A549) cells was evaluated. Currently, there is no research report documenting the *in vitro* cytotoxicity potentials of the nut extracts on cancer cells. Four extracts (diethyl ether/ethyl acetate-diEt/EA, dichloromethane- DCM, n-butanol- n-but, and water) of unprocessed, boiled and roasted African walnut were investigated in A549 lung cancer cell lines using standard cell culture aseptic techniques. Cell were grown in media containing Dulbecco's modified eagle media (DMEM/F12), foetal calf serum (FCS 10 %) and penicillin/streptomycin mix (pen-strep 1 %) - Life Technologies UK. Cells at 90 % confluence were seeded into 96 well flat bottom nunclon plates at 5,000 and 10, 000 cells/well seeding densities, and incubated 48 h in a humidified cell incubator set at 37 °C and 5 % RH. The incubated

plates were treated with 1, 10, 100 and 500 µg/mL of each extract together with PBS-media and 5-fluorouracil as negative and positive controls respectively, in triplicates. These were incubated for another 48 h under previous conditions. Cytotoxicity was assessed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The results showed no decrease in cell density in plates treated with extracts from unprocessed nuts at all concentrations. DiEt/EA (10 µg/mL) and n-butanol (1000 and 500 µg/mL) extracts of roasted nuts as well as DCM and water (1, 10 µg/mL) of boiled caused a decrease of < 10 % in cell densities when compared with the PBS-media control. However, these decreases were non-significant. In general, all extracts showed no cytotoxic effect on the A549 cells. The study suggests that the nut had no lethal effect on the A549 cells regardless the postharvest processing method. Hence, it can be said that the use of the nut extract in ethnobotanical medicine for management of chronic diseases such as cancer may be for the purpose of general body nourishment as against the notion of direct chemotherapeutic effect on the cancer cells.

The choice to analyse the potential phenolic components in the African walnut samples was based on the hypothesis that most plant materials contain phenolics that have antioxidant activities in biological systems. The total phenolic content of defatted African walnut samples was assessed and the potential individual phenolic components profiled in order to give insight on how they may be affected by the common postharvest retail processing methods. Unprocessed (20.79 ± 1.0 mg Gallic acid equivalent per gram dry weight – GAE/g FDW) samples showed the highest value for total phenolics while both boiling (9.90 ± 1.8 mg GAE/g FDW), and roasting (9.32 ± 2.7 mg GAE/g FDW) reduced the amount by more than 50 % when compared with unprocessed. Analysis of defatted and non-defatted samples of unprocessed, boiled and roasted African walnut respectively, using high performance liquid chromatography – diode array detector (HPLC-DAD) showed unambiguous separation of potential individual compounds. There were no differences in the chromatogram profile of compounds detected in the different samples analysed. Compounds were detected in wavelengths 280, 332, 355 nm commonly used in analysis of phenolics thus suggesting that African walnut contains several individual potential phenolic components which may be in low concentrations.

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# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# 1 Chapter one: Introduction

## 1.1 Background

Nuts have remained a major part of the diet for humans since pre-agricultural times (King, Blumberg, Ingwersen, Jenab & Tucker, 2008). They are consumed either as snacks or part of a meal. They can be eaten whole (fresh or roasted), in spreads (peanut butter, almond paste) or part of commercial products such as sauces, baked goods, oils etc. (Rajaram & Sabate, 2006). Some of the recognised and popular edible nuts include almonds, cashew nuts, peanuts, hazel nuts, macadamias, pecans, pistachios, Brazil nuts and English walnut (Bolling, Chen, McKay & Blumberg, 2011; Gonzalez 2006). In many developing nations such as Nigeria, there is a continuous search for alternatives to animal protein which is fast getting out of reach for many citizens as a result of poor governance, inflation, political turbulence, ethnic and guerrilla wars. Great attention is being turned to plant sources of which nuts are of paramount interest. Several nuts exist in the wild in African forests and many are yet to be documented. Some that have been brought to the lime light are still to be thoroughly researched. Many of these nuts are consumed daily either raw or processed. The nuts are also often sold in the open markets (Figure **2**) by table display or hawking. This practice exposes them to high temperatures and relative humidity which may be detrimental to food quality and may trigger fungal growth or even Maillard reactions leading to rancidity. Despite concerns over mycotoxin contamination, there is a scarcity of research on potential contamination of these nuts and how risks can be accentuated by postharvest handling.

African Walnut (*Tetracarpidium conophorum*- Mull. Arg) is a perennial climbing shrub which grows mainly in the Western region of Africa. It is found mainly in Nigeria, Gambia, Sierra Leone, Gabon, Equatorial Guinea and Cameroon (Amaeze, Ayoola, Sofidiya, Adepoju-Bello, Adegoke & Coker, 2011). The nuts are encased in pods which may contain between 2 to 5 nuts. The seed is made of two cotyledons which are enclosed in a hard shell-like case. The nuts mature and are harvested between the months of June and September. In Nigeria, the nuts are basically processed by boiling in water or roasting in hot sand before consumption as snacks (Nkwonta, Ezeokonkwo, Obidoa & Joshua, 2010). The flour is used as a soup thickener especially by the Yoruba tribe. In ethnobotanical medicine, the nut is extensively used in decoctions for treatment of several

ailments such as malaria, male sterility dysfunctions, dysentery, constipation, abdominal cramps and general fever, as well as in management of chronic diseases such as diabetes, cancer and high blood pressure (Aladeokin & Umukoro, 2011; Amaeze, Ayoola, Sofidiya, Adepoju-Bello, Adegoke & Coker, 2011). However, it is not certain as to the main role of the nut extract in the decoctions; whether it is to affect a direct impact on the disease causing agent either by preventive or curative processes, or simply to contribute to general body nourishment and hence boost the immune system of the patient. Another key challenge is that the nut is very susceptible to fungal infestation. It grows mouldy within few days of harvesting as the pods decay to release the nuts. Although the nuts may be processed, the fungal contamination is noticed when the shells are cracked as the cotyledons become slimy. This affects the sensory qualities of the nut as well as the sale by retailers. In spite of the promising potentials of the African walnut in the field of nutrition and medicine, very limited peer reviewed study reports have been documented.

**Figure** 2 Typical display of nuts and fruits in Nsukka open market - Nigeria

### *1.1.1 Nutrient content of nuts*

Nuts are nutrient-dense foods since they contain a high total fat content which is associated with high caloric index. They are generally perceived by the public as having fattening potentials which is a risk for certain health conditions, however, the perception has been changed as research reports show that the fatty acid composition of nuts is more beneficial than harmful (Bes-Rastrollo, Sabaté, Gómez-Gracia, Alonso, Martínez & Martínez-González, 2012). The saturated fatty acid content is generally low, and they contain much of mono- (oleic acids) and poly-unsaturated fatty acids such as linoleic acid, α-linolenic acid, as well as ω–3 (omega-3) fatty acids (Ros & Mataix, 2006). Furthermore, Bes-Rastrollo, Wedick, Martinez-Gonzalez, Li, Sampson & Hu, (2009) showed that nut consumption is associated with reduced body mass index (BMI). Ros (2009) noted that, fatty fraction of nuts also contains sizeable amounts of phytosterols, which are known to have both antioxidant and cholesterol-lowering properties. Nuts are good sources of protein with high L-arginine content which is important in vasodilation processes. They also contain several other bioactive macronutrients such as flavonoids (Huynh & Chin, 2006). Nuts are also a good source of dietary fibre, contain sizeable amounts of folate and are rich sources of antioxidant vitamins such as tocopherols and phenolic compounds (Blomhoff, Carlsen, Frost & Jacobs, 2006; Ros, 2009). Almonds and English walnuts are rich in α-tocopherol, although the later contain significant amounts of its isomer - γ - tocopherol. Both α-tocopherol and its isomer are recognized as relevant anti-atherogenic molecules (Ros, 2009; Wagner, Kamal & Elmadfa, 2004). When nuts are compared with similar food sources such as vegetables, they show optimal nutritional density with minerals such as calcium, magnesium, and potassium and their sodium content are usually infinitesimal (Ros, 2009; Segura, Javierre, Lizarraga & Ros, 2006).

Although nuts are composed of healthy nutrients and bioactive compounds as enumerated above, it cannot be conclusively said that consumption of nuts in whole or as part of diet will guarantee healthy state of individuals. This is because the bioavailability of nutrients and bioactive substances are influenced by genetic variation. Lots of individuals and people groups are believed to process various nutrients in different ways depending on the information encoded in their genes as a result of polymorphism. In addition, the various methods of processing, storage and other postharvest activities before

consumption, as noted by Terry and Thompson (2011), play significant roles in modifying the quality and quantity of these multipurpose nutrients. Extensive studies are therefore required to establish correlation between human genetic interactions and delivery of these nutrients from nut sources, as well as to ascertain the biophysiological changes that arise as a result of postharvest processes.

## 1.2 Aim and objectives

The aim of this research is to identify and quantify specific nutrients and bioactives contained in African walnut; study the impact of postharvest processing and storage on these substances; examine potential fungal infestation and mycotoxin contamination and finally investigate possible effects of these bioactives/nutrients as contained in the extracts on cell lines of chronic diseases such as cancer.

### *1.2.1 Specific objectives*

1) To identify and quantify fatty acids contained in African walnut and study the impact of postharvest processing and storage, as well as shelf-life on its profile using gas chromatography with flame ionization detector (GC-FID) and single - quadruple gas chromatography coupled mass spectrometer (GC-MS) techniques.
2) Examine potential fungal infestation and mycotoxin contamination on the nut shells of African walnut at different maturity stages using controlled temperature and relative humidity techniques, as well as classical culture methodologies
3) Investigate cytotoxic effects of sequential solvent extracts of the nut on cell lines of chronic diseases such as cancer (lung cancer cell line - A549 cells).
4) Identify possible phenolic bioactives in the nut extract and carryout targeted metabolomics studies on the compounds identified using ultra high definition accurate-mass quantitative time of flight liquid chromatography/mass spectrometry (UPLC-Mass QToF) techniques.

## 1.3 Thesis Structure

This study report is grouped into seven chapters with different sections and sub-sections. Chapter one gives the background and considerations for undertaking the study as well as specifies the aim and objectives of the research. Chapter two sections 2.1 to 2.3 details on review of literature relating to nutritional and health benefits of nuts in relation to specific health conditions. Further discussions in section 2.3 were based on the main bioactive/phytochemicals commonly found in nuts. The review also elaborated in section 2.4, on African walnut description, agro-geographic distribution, ethnobotanical uses, and survey of various published research studies on the nut since its discovery. The methods normally used in processing and preserving nuts and their effects on nutrient contents were reviewed in sections 2.5 and 2.6. The last section of chapter reviews the factors that affect nut fungal invasion and mycotoxin contamination.

Chapter three explains the general experimental design, sampling regime, experimental factors considered, and the general materials and methods used in treatment of samples. The chapters begins with a brief introduction explaining the basic postharvest retail handling practice of local farmers on African walnut. It further explains the rational for conducting only one major experiment during one harvest season of the nut. The rest of the chapters are based on different analyses carried out on the nut samples whilst assessing the different effects of the experimental factors. They all follow similar pattern of brief introduction, specific materials and methods, results, discussion and conclusion

Chapter four presents quantitative and qualitative profiling of main fatty acids in oil extracted from African walnut and the effect of postharvest storage and retail processing methods on these fatty acids. Chapter five captures the study on the fungal infestation and mycotoxin contamination of African walnut which was a follow-up mini experiment on the main experiment. Chapter six focuses on the preliminary analysis of potential phenolic compounds in African walnut methanolic extract as well as the cytotoxicity test of four different extracts of the sample on A549 cancer cell line. It includes sections for both assay of total phenolic content and high performance liquid chromatography flame ionization detector.

Chapter seven is the general discussion on the entire work on its contribution to the world of science, in the areas of nutrition, food security, crop protection, agriculture and

medicine. This chapter is followed by a list of literature cited and appendices of ANOVA tables used for statistical inferences and front pages of peer reviewed publications resulting from this thesis, list of relevant doctoral training centre trainings attended and certificates of participation/presentations in international conferences attended during the course of this work.

# 2 Chapter Two: Literature Review

## 2.1 Nuts and health related conditions

A nut is considered botanically as a simple dry fruit with one seed (or more sometimes) and a hardened ovary wall at maturity. The seed remains attached or fused to the ovary wall. They are indehiscent- do not break open at maturity. The ovary may be simple or compound. All nuts that fall into this category are known as true nuts. Some examples of true nuts include those from the order *Fagales* such as walnuts, hazelnut, chestnut, wingnut, beech, etc. (Alasalvar & Shahidi, 2008). However in culinary sense, nuts are widely categorised and the term is applied to many seed that are not botanically accepted as nuts. It includes oily kernels found within a shell that are used in food. Some examples include pistachios, almonds, Brazil nuts, cashew, pine nuts, groundnuts, etc. (O'Neil, Keast, Fulgoni III & Nicklas, 2010; Sathe, Monaghan, Kshirsagar & Venkatachalam, 2009).

Several studies (especially observational studies) often show an inverse relationship between nut consumption and cardiovascular diseases, some kinds of cancer, diabetes and several other biological disorders (Allen, 2008; Hoevenaar-Blom, Nooyens, Kromhout, Spijkerman, Beulens, van der Schouw, Bueno-de-Mesquita, Verschuren, 2012). Proximate analyses of nuts portray that they contain the classes of food namely proteins, carbohydrates, fats (unsaturated fatty acids in particular), vitamins and minerals in varying amounts, however, in addition to these they also contain numerous phytochemicals that may contribute to promoting health and reducing the risk of chronic diseases (Chen & Blumberg, 2008; McCullough, Peterson, Patel, Jacques, Shah & Dwyer, 2012). These phytochemicals are known as bioactive substances and many of them are yet to be fully identified and characterised. Currently, the available evidence that inversely correlates nut consumption with incidence of certain diseases has compelled United States, Canada and Spain to incorporate nuts into the recommended dietary guideline (Chen and Blumberg, 2008).

### *2.1.1 Nuts and cardiovascular/coronary heart disease (CHD)*

According to a review by Kris--Etherton, Zhao, Binkoski, Coval & Etherton, (2001); Mukuddem-Petersen, Oosthuizen & Jerling, (2005);and Kelly & Sabaté (2006), the

results of observational studies in large cohorts have consistently suggested that regular nut consumption reduces the risk of cardiovascular disease by between 30 and 60 %, an effect that has been observed in several population groups and is independent of other lifestyle factors. Nut-containing diets, low in saturated fat and cholesterol, while high in poly- and mono-unsaturated fatty acids, has been shown to have beneficial effect on plasma lipids and lipoproteins (McKay, Chen, Yeum, Matthan, Lichtenstein & Blumberg, 2010). Kelly & Sabaté (2006) have also reported that a pooled analysis of results from four major cohorts namely, Nurses' Health Study (NHS), the Iowa Women's Health Study (IWHS), Adventist Health Study and Physician' Health, showed that in comparison to little or no nut intake, nut consumption of greater than four (4) times per week may reduce the risk of death from coronary heart disease (CHD) by 37%. Other investigations involving hyperlipidemic subjects mainly, have added credence to these findings by linking nut consumption to reduced serum cholesterol levels. Reviews by Mukuddem-Petersen, Oosthuizen & Jerling, (2005) and; Griel & Kris-Etherton (2006) have also shown that the intake of 40-100 grams of nuts, five (5) or more times per week, can reduce low density lipoprotein-cholesterol (LDL-C) 3-19% in comparison to Western or lower fat diets. These finding were supported as well by Phung, Makanji, White & Coleman, (2009) following their meta-analysis which showed the intake of 25-168 grams per day of almonds lead to significant reductions in total cholesterol (TC) and LDL-C ($p = 0.03$ and $p = 0.05$, respectively). Apart from lowering LDL-C, some nuts are known to also improve the blood lipid profile in terms of CHD risk. For instance, three clinical trials demonstrated that 50-100 grams per day of pistachio nuts significantly improved high density lipoprotein-cholesterol (HDL-C) (Aksoy, Aksoy, Bagci, Gergerlioglu, Celik & Herken, 2007; Sheridan, Cooper, Erario & Cheifetz, 2007).

The cholesterol lowering effect of nuts is very much being attributed to the soluble fibre content (viscous fibre) and arginine (Jenkins, Kendall, Axelsen, Augustin & Vuksan, 2000). Salas-Salvado, Bulló, Pérez-Heras & Ros (2006) purports that since the viscous fibre is often well-hydrated, it does make the intestinal contents more viscous as well. This reduces the rate of absorption and spreading of the nutrient load over time. Other factors involved include resistance to bulk diffusion due to the increased viscosity of the luminal contents and increased resistance of the unstirred water layer that lines the absorptive surface of enterocytes. These factors together with the binding of bile acids to

the fibre in the distal ileum increasing its faecal loss are noted as key mechanism by which nut fibre reduces serum cholesterol. Increase in serum cholesterol has often been implicated in cardiovascular diseases (Gonzalez, 2006; Salas-Salvadó Bulló, Pérez-Heras & Ros. 2006).

Another aspect of a nut diet associated with reduction of cardiovascular disease is the fact that it does not contribute to increase in body weight. There has been a common perception that fatty foods provides excess energy and thus promote obesity. This has had negative effect on nuts since they contain lots of fats, however, a review by Rajaram & Sabaté (2006) as well as Onge (2005), showed that there is considerable evidence suggesting that frequent nut consumption (peanuts, walnuts, almonds and pecans) is not associated with weight gain. There are epidemiological studies showing a neutral or even negative association between nut intake and biological mass index - BMI (Rajaram & Sabate, 2006; Vadivel, Kunyanga & Biesalski, 2012). Secondly, interventional trials in free-living individuals have shown no weight gain or a tendency to lose weight in those assigned to nut diets compared with control diets. Earlier epidemiological studies have often associated low rate of obesity with increase in nut (walnuts, hazelnuts and peanuts) consumption in developing countries (Bes-Rastrollo, Sabaté, Gómez-Gracia, Alonso, Martínez & Martínez-González, 2012; Newby, Tucker & Wolk, 2005), however, current reports show increase in obesity in developing nations (Popkin, Adair & Ng, 2012; Popkin, Paeratakul, Zhai & Ge, 2012) despite the fact that there may be continued intake of nuts as part of normal diet. One may wonder if the earlier observations were solely due to high consumption rate of nuts and fibre foods or was it in conjunction with other less obvious factors such as increase in physical activities associated with local farming methods and lack of facilities that encourage sedentary lifestyle. It is pertinent therefore, that further studies incorporating these factors are carried out. The results will provide a solid ground for conclusive inference on the influence of nut nutrition on weight gain.

### *2.1.2 Nuts and Diabetes (Type 2)*

Over the past three decades, there has been a dramatic increase in the prevalence of diabetes (Kendall, Esfahani, Truan, Srichaikul & Jenkins, 2010). The prevalence of diabetes in adults is expected to increase by 69% between 2010 and 2030 in developing nations and by 29% in developed nations (Kendall, Esfahani, Truan, Srichaikul &

Jenkins, 2010; Shaw, Sicree & Zimmet, 2009). These alarming rates of increase in diabetes especially in developing nations have been associated with the fact that urbanization has led to the adoption of sedentary lifestyle as well as increase in prevalence of obesity. Diets have also gradually moved away from traditional whole food and become more reliant on processed foods (Shaw, Sicree & Zimmet, 2009; Kendall, Esfahani, Truan, Srichaikul & Jenkins,, 2010). Within the above mentioned dietary components, tree nuts are of great interest, because of their unique macro and micronutrient profile. Frequent consumption of nuts has been linked to lower risk of diabetes in the Nurses' Health Study (Jiang, Manson, St\mpher, Liu, Willett & Hu, 2002; Tchankou, Tchiégang, Barbé, Nicolas & Guéant, 2009).

Type 2 diabetes is insulin resistant and hence will mean that biological components capable of increasing the sensitivity of insulin will have a positive effect regarding prevention or treatment of the ailment. Several studies have shown that a high intake of monounsaturated and polyunsaturated fat improves insulin sensitivity while a higher intake of polyunsaturated fats is associated with a lower risk of Type 2 diabetes (Jiang, Manson, St\mpher, Liu, Willett & Hu, 2002). Other components of nuts such as the fibre and magnesium content decrease insulin demand and resistance.

In the Nurses' Health Study (Jiang, Manson, Stampher, Liu, Willett & Hu, 2002), it was reported that peanut butter consumption was inversely associated with the risk of developing type 2 diabetes after adjusting for multiple confounding variables, including body weight and intake of other dietary components. Women who consumed a 28g serving of nuts five times per week had a lower risk (25%) of developing type 2 diabetes compared with women who never ate nuts. According to Lovejoy (2005), nuts are relatively high in total calories and fat and could potentially contribute to positive energy balance and weight gain but interestingly, most studies do not find that nut consumption is associated with increased body weight. In fact, in the population based study which examined nut (peanuts) intake and prevention of diabetes in women (Jiang, Manson, St\mpher, Liu, Willett & Hu, 2002), there were no differences in weight gain in sixteen (16) years of follow-up based on quantity of nut consumption (ranging from never to five times per week). Similarly, in a cross-sectional study of 777 girls (in puberty) in Spain (Soriguer, Garcia-Garcia, Santiago & Millon, 2005), there was no effect on amount of

nuts consumed and body weight, although there was an association between nut and seed intake and age of menarche, which tends to be related to body size.

### *2.1.3 Nuts and cancer*

Cancer is a disease that is characterized by the loss of genetic control over cell growth and proliferation, mainly as a result of the exposure to environmental factors. In this sense, it is considered a genetic disease (Gonzalez & Salas, 2006). It is known to be the second most important cause of death in Europe, in both men and women. In their report to mark the world cancer day, the World Cancer Research Fund (W.C.R.F. 2012) organization announced that 396, 000 new cases of cancer are expected by 2030 in United Kingdom as against 304, 000 in 2008. In 2006, it was reported that about one out of every three men and one out of every four women in Europe will be diagnosed as having cancer at some point in their lives (Gonzalez & Salas, 2006). Nuts are generally a source of proteins, unsaturated fatty acids, vitamin E, phenolic compounds, selenium, fibre, folic acid and phytosterols. The concentrations of these compounds usually vary among different nuts (Sabate & Wien, 2010). The mechanisms of action of these components of nuts which have the potential to intervene in the prevention of cancer have not been totally elucidated. Some of them are related with antioxidant activity, the regulation of cell differentiation and proliferation, the reduction of tumour initiation or promotion, the repair of DNA damage, the regulation of immunological activity and inflammatory response, the induction or inhibition of metabolic enzymes and hormonal mechanisms, and the supply of fibre and monounsaturated fatty acids (Greenwald, Clifford & Milner, 2001; Kris-Etherton, Hecker, Bonanome, Coval, Binkoski, Hilpert, Griel & Etherton, 2002). Allen (2002) in his review, recognised the study of nuts and cancer as one of the major emerging area of research and currently, very few studies showing relationship between nuts and cancer exist. Therefore there is need for further scientific research to clarify the possible roles of nuts on different types of cancer.

## 2.2 Nut fatty acids and human health

Fatty acids are nutrients necessary for human health and development. They form major constituents of cell, organs and tissues playing strategic structural and functional roles in the human biological system. Several of these fatty acids such as palmitic acid, stearic acid, arachidic acid, oleic acid etc. can be synthesized by the human body cells and these

are designated non-essential fatty acids whereas, a few important ones such as linoleic acid, arachidonic acid , α – linolenic acid, gamma linolenic acid, eicosapentanoic acid etc. cannot be synthesized *de novo* in humans and they are known as essential fatty acids. This implies that they must of necessity be ingested as part of diets or as food supplements (Melariri, Campbell, Etusim & Smith, 2012). Three of these essential fatty acids namely, linoleic acid, gamma linolenic acid and α – linolenic acid are found mainly in vegetable oils, seed and nut oils. Linoleic acid and gamma linolenic acid belong to the omega-6 (ω – 6) fatty acid family while α – linolenic acid belongs to the omega-3 (ω – 3) family.

These essential fatty acids are polyunsaturated and research has shown that they have great medicinal properties and health benefits. Melariri, Campbell, Etusim & Smith (2012) reported that linoleic acid and linolenic acid both inhibited the growth of rodent malaria parasite, *Plasmodium berghei,* by 64% and 70 % respectively whilst a combination of both showed 96% inhibition using the 4-day suppressive test. The methyl esters were also shown to have the same effect with no significant difference. In the recent times, studies have shown that these polyunsaturated fatty acids and their derivatives have strong influences on chronic diseases including neurodegenerative, neoplastic and inflammatory disorders (Calder, 2013; Fahrmann, Ballester, Ballester,

Witte, Salazar, Kordusky & Boskovic, 2013). Several reports have given recognition to the omega-3 family as being more beneficial than the omega-6 group. In fact it was initially believed that the activities of omega-6 fatty acids alone generally enhances the incidence and progression of these diseases however recent reports indicate that gamma linolenic acid and conjugated linoleic acids have potential beneficial effects (Serini, Piccioni, Merendino & Calviello, 2009). More so, arachidonic acid has been reported to exert pro-apoptotic and anti-neoplastic action when its oxidative metabolism is inhibited and there is intracellular accumulation in an unesterified form (Serini, Piccioni, Merendino & Calviello, 2009). In their review, Bougnoux, Hajjaji, Maheo, Couet & Chevalier, (2010) noted that polyunsaturated fatty acids, namely decosahexanoic acid (DHA) sensitizes breast malignant tumours only to chemotherapy and radiotherapy while naturally occurring conjugated linoleic acid have been shown to have the potential to prevent tumor re-growth. The ratio of omega-6 and omega-3 fatty acids intakes are also considered of great importance in maximizing the health benefits of fatty acids. This is

supported by reports which have shown that eicosanoids produced by omega-6 and omega-3 dietary fatty acids are involved in the regulatory pathway of platelet aggregation, inflammation, vasodilation and vasoconstriction and these eicosanoids and the ω-6 arachidonic acid generally compete for the enzymes (cyclooxygenase and lipoxygenase) involved in these pathways (Riediger, Othman, Suh & Moghadasian, 2009). This ratio is also considered important since there are more abundant dietary omega-6 fatty acids than omega-3 fatty acids.

## 2.3 Bioactive compounds in nuts and their classification

In their special article on bioactive compounds, Biesalski, Dragsted, Elmadfa, Grossklaus, Müller, Schrenk & Weber,(2009) gave the basic definition as essential and non-essential compounds such as vitamins and phenolics that occur in nature, which are part of food chain and can be shown to have effect on human health. These compounds are also referred to as *Nutraceuticals* is a term that reflects their existence in the human diet and their biological activity. They are natural constituents in food that provide health benefits beyond the basic nutritional value of the product. In the introduction to their book - Health promoting properties of fruits and vegetables- Terry & Thompson (2011) referred to these bioactive substances as phytochemicals which are also abundant in different species and cultivars of fruits and vegetables of which only a limited number has been evaluated in terms of their effect on human health. Bioavailability and metabolism are key factors that influence the effect of bioactive compounds in an individual apart from the abundance of the target analyte (Terry & Thompson, 2011).

The term "bioactive compounds" is synonymous with "phytochemicals" and are often used interchangeably. They have been classified in different ways. According to Jaganath and Crozier (2010), they are classified into four major groups: Nitrogen-containing alkaloid, phenolics and polyphenolics, sulphur-containing compounds and terpenoids. Chen & Blumberg (2008) in their review article on Phytochemical composition of nuts, showed the major classification of the bioactive compounds as; Alkaloids, Carotenoids, Organosulfurs, Phenols and Phytosterols.

### *2.3.1 Alkaloids*

"Alkaloid" is a term linguistically derived from the Arabic word *al-qali*, which simply indicates the plant from which soda was first obtained (Kutchan, 1995). They are nitrogenous compounds that constitute the pharmacologically active "basic principle" although not exclusively, of flowering plants. Alkaloids belong to the broad category of secondary metabolites. The isolation of alkaloids dates back to nineteenth century. The use of modern methods and instrumentation has also enabled the elucidation of their structures. Alkaloids are generally well-defined crystalline substances which react with acids to form salts. They may exist in the free-state in plants as salts or as *N*–oxides. They contain the elements carbon, hydrogen and nitrogen, as well as oxygen (Figure 3). A few, such as *coniine* from hemlock and *nicotine* from tobacco, are oxygen-free and are liquids (Evans, Trease & Evans, 2002). The functions of alkaloids in plants has remained a debatable issue although a key suggestion has been that it could serve a role in the defence of the plant against singlet oxygen which causes damage to living tissues. The toxic nature of alkaloids has also been suggestive that it acts as defence chemical against attacks by plant microorganisms and herbivores. In nuts, they often occur in small amounts (< 0.09%). Arecolin, arecaine and guvacine have been identified in Areca nut. Only arecoline have been shown to possess medicinal properties (Evans, Trease & Evans, 2002).

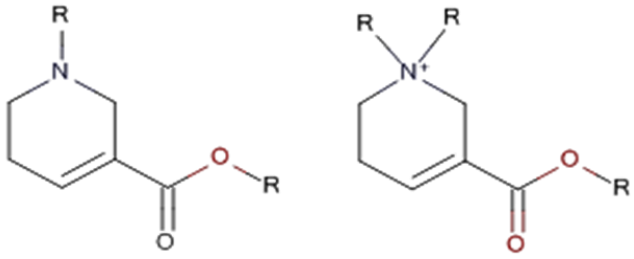



**Figure 3** Structure of Arecoline (left) and Arecoline methiodide (right). R = $CH_3$ showing structure of alkaloids.

### *2.3.2 Carotenoids*

Carotenoids are polyisoprenoid (Figure 4) organic compounds that are naturally occurring in plants (Chen & Blumberg, 2008; Nancy, Tyler, Moran & Jarvik, 2010). Carotenoids serve two key roles in plants; they absorb light energy for use in photosynthesis, and they protect chlorophyll from photo damage. There are over 600 known carotenoids that have been isolated from natural sources and characterised. They are classified into two major groups namely, xanthophylls (these contain oxygen in their structure) and carotenes (these are purely hydrocarbons, and contain no oxygen in their structure). These isoprenoid compounds are biosynthesized by tail-to-tail linkage of two $C_{20}$ geranylgeranyldiphosphate molecules. This produces the parent $C_{10}$ carbon skeleton from which all the individual variations are derived. They are extremely hydrophobic molecules with little or no solubility in water. The major carotenoids found in nuts include zeaxanthin, β-cryptoxanthin, β-carotene and lutein. Low concentrations in µg/100g are known to be present in nuts however; β-carotene (Figure 5) and lutein are found in pistachios nut at 0.21 and 4.40 mg/100g dry weight respectively (Chen & Blumberg, 2008; Kornsteiner, Wagner & Elmadfa, 2006).

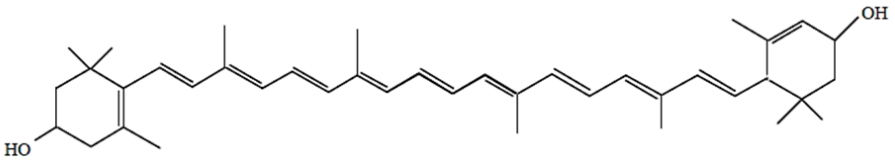



**Figure 4** Structure of lutein

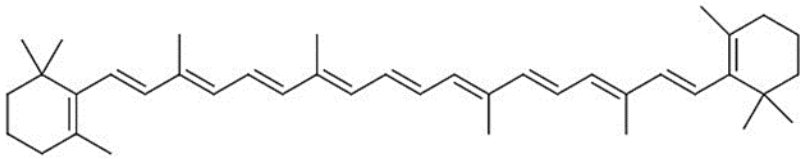


**Figure 5** Chemical structure of β- carotene

### *2.3.3 Phenolics*

These are a class of aromatic organic compounds consisting of one or more hydroxyl groups attached to an aromatic hydrocarbon group. They are often referred to as *Phenolics* (Martins, Mussatto, Martínez-Avila, Montañez-Saenz, Aguilar & Teixeira, 2011). Phenol is a benzene derivative and is the simplest member of the phenolic chemical. Its chemical formula is $C_6H_5OH$ and its structure is that of a hydroxyl group (-OH) bonded to a phenyl ring. Plant phenols, are grouped into three major classes and these include phenolic acids, flavonoids and stilbenes. They all possess hydroxyl groups conjugated to an aromatic hydrocarbon group. Phenolic compounds are ubiquitous in plant foods with total daily intakes estimated at 500-1000 mg (Chen & Blumberg, 2008). The total phenolic content among nuts varies widely. Pecans, pistachios, and English walnuts are so far known to be very rich sources of phenolics, while Brazil nuts, macadamias, and pine nuts containing the lowest concentrations (Kornsteiner, Wagner & Elmadfa, 2006; Yang, Liu & Halim, 2009).

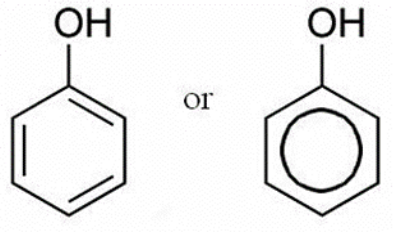



**Figure 6** Structure of phenol

The flavonoids are comprised of six major classes that include anthocyanins, flavanones, flavones, flavanols, flavonols and isoflavones. They are widely distributed throughout the plant kingdom. Flavonoid intake has been associated with a reduced risk of several chronic diseases with their mechanism of action being attributed to their capacity for antioxidation, anti-inflammation, anti-proliferation and modulation of signal transduction pathways. Flavonoids have been identified in nuts with their aglycone profiles included in the United States Department of Agriculture (USDA) database (Chen & Blumberg, 2008). Flavan-3-ols, flavanols and anthocyanins are the major flavonoids in nuts while flavanones and isoflavones are present in lesser amounts. Pecans are known to have the highest concentration of total flavonoids followed by hazelnut and almonds. It is also remarkable to note that so far that flavones have only been found in almonds and pistachios while flavanones are present only in almonds (Bolling, Chen, McKay & Blumberg, 2011). Another important phenolic which has gained great attention in research is resveratrol- a stilbene. It has been found to be present in nuts such as peanuts and pistachios. The level of resveratrol in peanuts ranges from 3-192 µg/100g whereas a pistachio is 9-167µg/100g (Chen & Blumberg, 2008; Bolling, (Bolling, Chen, McKay & Blumberg, 2011). It acts in the plants as a phytoalexin (Tokusoglu, Ünal & Yemis, 2005). In addition to having neuroprotection, immune regulation, antioxidant properties and other bioactivities common in other polyphenols, studies have shown that it might be useful in the chemoprevention of prostate and intestinal epithelial cancer (Aziz, Kumar & Ahmad, 2003; Storniolo & Moreno, 2012). It has been suggested to inhibit proliferation of human prostate cancer cell lines via an inhibition of the production of NO as well as in breast cancer by altering autocrine growth modulator pathways in breast cancer cell lines (Casanova, Quarti, da Costa, Ramos & Da Silva, 2012; Wolfe, 2012). Despite the

scepticisms about the biological availability of resveratrol, there is a growing body of *in vivo* evidence that the compound has protective impacts on several stress and disease models (Nakata, Takahashi & Inoue, 2012). Proanthocyanidines which are basically oligomers of flavan-3-ol linked through carbon-carbon bonds are known to be the most abundant polyphenol in almonds, hazelnuts, peanuts, pecans and pistachios. They consist mainly of (+)-catechin and (-)-epicatechin, afzelechin (found in almonds and peanuts) and epigallocatechin found in hazelnuts, pecans and pistachios (Bolling, (Bolling, Chen, McKay & Blumberg, 2011; Monagas, Garrido, Lebrón-Aguilar, Gómez-Cordovés, Rybarczyk, Amarowicz & Bartolomé, 2009). Although these facts are known about phenols, their bio-accessibility and bioavailability in human are not yet clear as well as the effect of storage and processing on them. These ought to be examined both *in vivo* and *in vitro* for enhanced clarification and conclusions to be made.

### *2.3.4 Phytosterols*

These are a group of over 200 naturally occurring plant sterols with the capacity to inhibit the absorption of dietary cholesterol and lower serum cholesterol as well as antagonize selected inflammatory pathways through competitive uptake mechanism (Brauner, Johannes, Ploessl, Bracher & Lorenz, 2012). Elevated blood cholesterol is well implicated as risk factor for cardiovascular disease. These compounds are structurally similar to cholesterol (Figure 7) and are generally located in the fatty fractions of nuts (Park & Carr, 2012; Vuorio & Gylling, 2012). There are three primary plant sterols that are found in a typical western diet; sitosterol (β-sitosterol, Figure 8), stigmasterol and campesterol. Phytosterol has been implicated in induction of apoptosis in cancer cells (Chong, Yeap, Rahmat, Akim, Alitheen, Othman & Gwendoline-Ee, 2012).

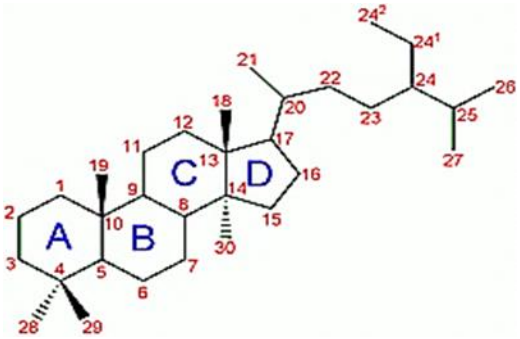



**Figure 7** Basic structure of steroids

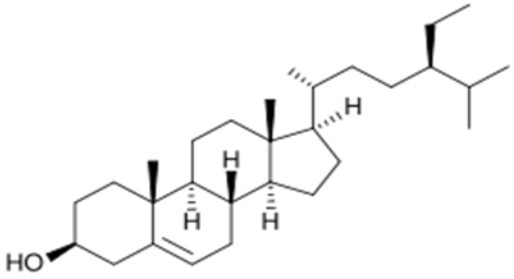



**Figure 8** Structure of β-sitosterol

Nuts are known to be rich sources of phytosterol and the total phytosterol contents of nuts (in mg/100g) as noted by Chen & Blumberg, (2008) are as follows: almonds, 187; Brazil nuts, 95; cashew, 138; hazelnuts, 120; macademias, 198; pecans, 150; pine nuts, 198; pistachios, 280; walnut (English), 113 mg/100g. These concentrations are very comparable to those found in chocolate and flaxseed at 168 and 210 mg/100g however, they differ from that published by the United States department of Agriculture as reported by Chen & Blumberg, (2008).

**Table 1** Average nutrient composition of nuts (per 100 g)

| Nuts | Energy | Total fat | SFA | MUFA | PUFA | Plant sterols | Total protein |
|---|---|---|---|---|---|---|---|
| | (kJ) | (g) | (g) | (g) | (g) | (mg) | (g) |
| Almonds | 2418 | 50.6 | 3.9 | 32.2 | 12.2 | 120 | 21.3 |
| Brazil nuts | 2743 | 66.4 | 15.1 | 24.5 | 20.6 | NR | 14.3 |
| Cashews | 2314 | 46.4 | 9.2 | 27.3 | 7.8 | 158 | 18.2 |
| Hazelnuts | 2629 | 60.8 | 4.5 | 45.7 | 7.9 | 96 | 15.0 |
| Macadamias | 3004 | 75.8 | 12.1 | 58.9 | 1.5 | 116 | 7.9 |
| Peanuts (dry roasted) | 2448 | 49.7 | 6.9 | 24.6 | 15.7 | NR | 23.7 |
| Pecans | 2889 | 72.0 | 6.2 | 40.8 | 21.6 | 102 | 9.2 |
| Pine nuts (dried) | 2816 | 68.4 | 4.9 | 18.8 | 34.1 | 141 | 13.7 |
| Pistachios | 2332 | 44.4 | 5.4 | 23.3 | 13.5 | 214 | 20.6 |
| Walnuts | 2738 | 65.2 | 6.1 | 8.9 | 47.2 | 72 | 15.2 |

Data are from raw nuts, except when specified. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; NR, not reported. Source: Ros & Mataix,, (2006).

## 2.4 African Walnut

### *2.4.1 Botanical description of Tetracarpidium conophorum (African walnut/conophor nut)*

The African walnut plant was originally classified by Hutchinson and Dalziel (1928). It is described to be synonymous with *Pleukenetia conophora*. In the recent times a more comprehensive taxonomic description has been put forward by the United States Department of Agriculture Natural Resources Conservation Service (USDA/NRCS) as presented below (Table 2).

**Table 2** Classification of African Walnut (*Tetracarpidium conophorum*)

| | |
|---|---|
| **Kingdom** | *Plantae*- Plants |
| **Sub-kingdom** | *Tracheobionta* – Vascular plants |
| **Superdivision** | *Spermatophyta* - Seed plants |
| **Division** | *Magnoliophyta* – Flowering plants |
| **Class** | *Magnoliopsida* – Dicotyledons |
| **Subclass** | *Rosidae* |
| **Order** | *Euphobiales* |
| **Family** | *Euphobiaceae* – Spurge family |
| **Genus** | *Tetracarpidium* Pax – Tetracarpidium |
| **Species** | *Tetracarpidium conophorum* (Mull. Arg.) Hutch. & Dalziel |

Source: United States Department of Agriculture Natural Resources and Conservation (USDANRCS) Services database (http://plants.usda.gov/java/ClassificationServlet?source=display&classid=TECO9).

The freshly harvested nuts are greenish in colour and contains between two to four round seeds per pod (Figure 9, B & C). The seed is made up of two cotyledons and enclosed in

hard brown shell-like case within the pods (Nkwonta, Ezeokonkwo, Obidoa & Joshua, 2010); Figure 9D. The seed which is referred to as walnut is hard and the cotyledons are yellowish-white in colour (Aladeokin & Umukoro, 2011); Figure 9E. The leaves are globorous, ovate, long and margin toothed. The bases of the leaves are broad and rounded up to 5 by 3 inches with slender petioles up to 2 inches long (Malu, Obochi, Edem, Nyong, Edem, Obochi & Malu, 2009). A bitter after taste is observed upon drinking water immediately after consumption and this has often been attributed to the presence of alkaloids in the nut although phenols are capable of conferring such characteristic. The seeds take about 14 days to germinate, while the young plant takes 4-6 months to mature. The plant is usually planted near big trees (such as cocoa) which give it strong support while it climbs and covers the crown of the tree (Figure 9A). The nuts are found in local markets between the months of June and September

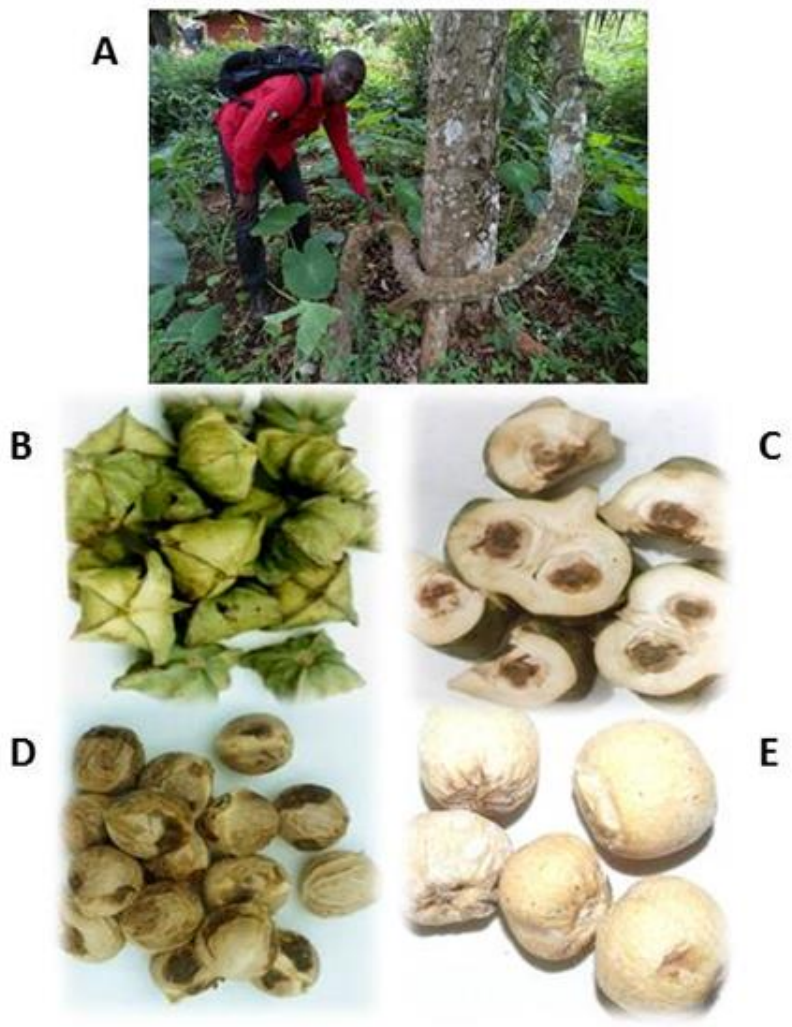



**Figure 9** African walnut (A) climber curling around a supporting tree; (B) harvested mature fruit; (C) cut pods with nuts; (D) nuts released from pods; (E) nuts with shells removed to show kernel.

### *2.4.2 Agro-geography of African walnut*

The nut is indigenous to Nigeria, Benin Republic, Cameroon, Gabon, Equatorial Guinea and Zaire- Democratic Republic of Congo (Fasina & Ajibola, 1989b), Figure 10. It is also found in Sierra Leone; however, it is believed that its naturalization in Sierra Leone may be due to returning slaves because it is known to the *Krio* by the Yoruba (Nigerian) name. It is a perennial climbing shrub found in tropical and coastal regions of Nigeria (West, South, and South-East), Benin Republic and Sierra Leone in West Africa. In West-Central Africa Cameroon, it grows in the west, south and littoral regions (Tchiengang, Blandine

& Kenfack, 2006; Tchiegang, Kapseu & Parmentier, 2001) as well as in Gabon and Equatorial Guinea. In Nigeria, the exotic perennial wild fruit is grown in the traditional farming system of the lowland humid tropics between 4°15′ and 8°N of the equator (Asaolu, 2009). The nut is currently grown in the wild only and has not yet been cultivated.


**Figure 10** Chart showing ecology and distribution of African walnut (*Tetracarpidium conophorum*) in Africa.

Several tribes in the various nations where they are found have baptized the nut with different names. In Nigeria (Figure 11)**,** the Igbo speaking tribes in the south-eastern

region of the country namely, Enugu, Anambra, Ebonyi and Abia states call it *Ukpa*. The Yoruba speaking states in the western region- Ondo, Ekiti, Oyo, Lagos, Osun, Ogun and Kwara states call it *asala or awusa* while the *Efiks* and *Ibibio* – speaking tribes of Cross River and Akwa Ibom states in the south-south region of Nigeria call the nut *Ekporo*. In Cameroon, it is called *Ngak* in the *Bangangte* dialect or *Kaso* in *bush* English.

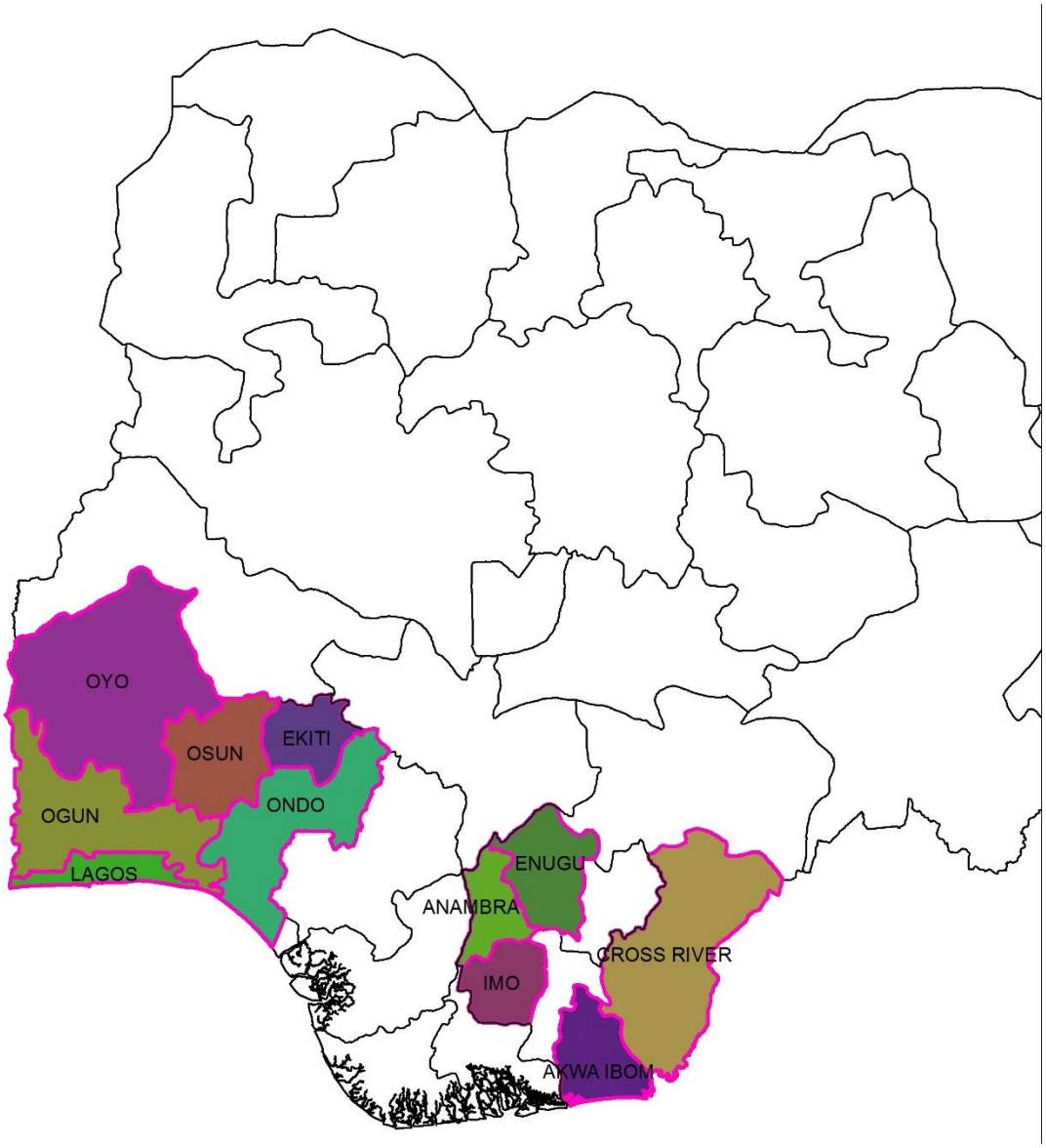



**Figure 11** Chart showing the agro-geography of African walnut in the west and south-eastern Nigeria and major states where the nut is found.

### *2.4.3 Ethno-botanical uses of African walnut*

The nut is basically boiled and consumed as snacks. In the western part of Nigeria, the nuts are roasted or boiled and served to welcome visitors. In Cameroon, adults are known to be the only consumers until recent times (Tchiegang, Kapseu & Parmentier, 2001). The nut is also consumed raw both as food and medicine (Oyekunle, Ogunfowokan, Saheed, Adekunle, Doherty & Abraham, 2013), however, the different parts of the plant – leaves, bark, root and nut has been employed by traditional herbalist in treatment of certain health conditions.

**Table 3** Ethnobotanical uses of African walnut

| **Use** | **Part of plant** |
|---|---|
| As pain relieve for tooth aches, abdominal pains as a beverage and tonic | Decoctions of leaves and nut kernel |
| Treatment of malaria and general fever | Bark of stem, leaves and nut kernel |
| As fertility agent (to increase sperm count) | Root, nut kernel |
| Treatment of constipation and abdominal cramps, diabetes | Nuts kernel, leaves |
| Control of asthma<br>Control chronic cough | Roots, bark |
| Treatment of dysentery, syphilis and thrush | Leaves, roots |
| Reduction of high blood pressure | Roots |
| Diarrhoea treatment | Leaves |
| Cancer | Leaves, nut kernel |

Sources: Onwuli, Brown & Ozoani (2014); Malu, Obochi, Edem, Nyong, Edem, Obochi & Malu (2009); Amaeze, Ayoola, Sofidiya, Adepoju-Bello, Adegoke & Coker (2011); Ajayeoba & Fadare (2006); Akomolafe, Oboh, Akindahunsi & Afolayan (2015)

The scientific bases for the uses (Table 3) are yet to be fully researched and properly documented. It will be quite necessary to identify the key bioactive compounds

responsible for the various pharmacological effects as listed above and their bioavailability as well.

**Table 4** Proposed uses of African walnut (*Tetracarpidium conophorum*) based on research findings

| Industry | Uses | Part of plant |
|---|---|---|
| Food and Nutrition | Conophor butter, chips, dices and grits for incorporation into chocolate products | Nuts |
| | Salad dressings | Nut oil |
| | Biscuits | Nut flour |
| Agriculture | Protein source in livestock feed | Nut cake |
| | Manure in farms | Decaying nut cakes |
| | Forest conservation | Conophor plant |
| Paint | Wood vanish, stand oil, soap and vulcanized oil for rubber and leather substitutes | Nut oil |
| Pharmaceutical | Antimicrobial agent | Leave extracts |

Sources: Aviara & Ajikashile (2011); Nkwonta, Ezeokonkwo, Obidoa & Joshua (2010); Oladiji, Abodunrin & Yakubu (2010); Ehiagbanare & Onyibe (2007); Ajayeoba & Fadare (2006); Adebona, Ogunsua & Ologunde (1988)

### *2.4.4 Survey of previous research works on African walnut*

Over the years, the African walnut has gained popularity and has become a plant of interest to scientists in the field of nutrition, pharmacy/medicine, engineering, and agriculture in Nigeria and parts of west and central Africa.. Following basic research findings, the various components of the plant including the nuts are used in different ways in various industries (Table 4). Although there is continuous increase in the research conducted on African walnut, only very little has been directed towards scaling up the production through mechanise farming as well as postharvest storage and shelf life to maintain its' availability throughout the year.

**Table 5** Nutritional composition of African walnut *(Tetracarpidium conophorum*)

| Proximate analysis (%) | | Mineral (mg/g) | | Phytochemicals (mg/100g) | |
|---|---|---|---|---|---|
| Crude Protein | 21.65-35.22 | Calcium | 1.09-4.31 | Alkaloids | 238 |
| Carbohydrate | 12.58-53.20 | Magnesium | 0.20-1.70 | Tannins | 1.10-8.90 |
| Crude fibre | 3.34-7.34 | Zinc | 0.04-0.10 | Phytate | 3.50 |
| Crude fat & oil | 4.28-8.20/ 37.79-48.90 | Iron | 0.03-0.10 | Gallic acid | 2.40 |
| Moisture | 2.20-4.28 | Copper | 0.01-0.16 | | |
| Ash | 2.03-5.27 | Sodium | 4.83 | | |
| | | Potassium | 6.25 | | |
| | | Manganese | 0.01-0.02 | | |

Sources: Enujiugha, 2003; Edem, Dosunmu & Bassey, 2009; Nkwonta, Ezeokonkwo, Obidoa & Joshua, 2010; Ayoola, Onawumi & Faboya, 2011; Oladiji, Abodunrin & Yakubu, 2010; Ndie, Nnamani & Oselebe, 2010. The values in a range are obtained from different sources.

**Table 6** Vitamin content of African walnut (*Tetracarpidium conophorum*)

| Vitamins | Amount (µg/100g) |
|---|---|
| Ascorbic Acid (C) | 4150 |
| Tocopherol (E) | 122.57 |
| Thiamine (B1) | 0.06 |
| Riboflavin (B2) | 0.02 |
| Niacin (B3) | 0.05 |
| Cyanocobalamine (B12) | 0.12 |

Source: Ayoola, Onawumi & Faboya, 2011

**Table 7** Amino Acid content of African walnut (*Tetracarpidium conophorum*)

| Amino Acid (essential and non-essential) | Concentration (mg/g) |
|---|---|
| Isoleucine | 40.00 |
| Leucine | 70.00 |
| Lysine | 42.2-55.00 |
| Methionine | 1.00-8.00 |
| Cystein | 35.00 |
| Phenylalanine | 23.70 |
| Tyrosine | 21.60-60.00 |
| Threonine | 40.00-52.70 |
| Tryptophane | 10.00 |
| Valine | 50.00-60.00 |
| Alanine | 61.30 |
| Arginine | 63.00 |
| Aspartic acid | 144.00 |
| Glutamic acid | 122.30 |
| Glycine | 138.30 |
| Histidine | 14.40 |
| Proline | 64.30 |
| Serine | 62.00 |

Source: Tchiegang, Kapseu & Parmentier, 2001; Asaolu, 2009. The values in a range are obtained from two sources.

In summary, African walnut contains approximately 21.65-35.22 % protein; 12.58-53.20 % carbohydrate; 3.34-7.34 % crude fibre; 4.28-8.20 %/37.79-48.90 % crude fat and oil; 2.20-4.28 % moisture; 2.03-5.27 % ash content (

**Table 5**). The vitamin contents (Table 6) include ascorbic acid, vitamins E, B1, B2, B12 and Niacin. The ascorbic acid and vitamin E contents are in higher levels compared with other vitamins. It contains such minerals (Table 5) such as magnesium, iron, zinc, calcium, sodium, potassium, nickel, chromium and phosphorus in varying proportions. Heavy metals such as mercury, lead, and cadmium have not been found in it. Phytochemical (antinutrients and bioactives) constituents identified so far although in very minute amounts include tannins, saponins, alkaloids, flavonoids, oxalates and phytates. Cardiac glycosides are noted to be absent. The extracts are known to have antimicrobial activity against *Coliform bacillus*, *Staphylococcus epidermis*, *Streptococcus viridans*, *Pseudomodas aerugenosa*, *Candida albicans* as well as chelate iron (Fe) in the blood. It has been shown to improve sex hormone (testosterone) activity in male guinea pig and lower blood lipid levels in rats thereby reducing risk of cardiovascular disease. Toxicological analyses indicate so far that it causes no damage to organs such as liver, kidney and haematological parameters. The oil yield is dependent on the moisture content of sample after heating, heating temperature and time. The nut contains about nineteen (19) amino acids (Table 7) with nine being essential. The seed is known to germinate best when it is pre-treated with indole acetic acid for twenty-four (24) hours and having been mechanically cracked and the plant as a whole has been recommended as a tool for forest conservation. As can be observed in these findings, there are lots of variations in the nutrient composition of the nut which gave rise to some wide range of values. Apart from minor experimental errors, it is probably an indication that several factors such as differing processing/extraction methods, storage impact, environmental/geographical influence must have played crucial roles. It is pertinent to note that there has been very minimal sophisticated analysis and experiments involving this nut. Nutrient/gene interaction studies, impact of storage on the nutrient composition and bioavailability as well as mycoflora/ mycotoxin contamination of the nut are

important studies that will throw more light in maximizing the potentials of African walnut.

## 2.5 Processing methods and effects on nut nutrients and phytochemicals

Malnutrition results from lack of food especially those with good nutritional quality. The presence of antinutrients such as phytates and oxalates, are factor that limits the quality of plant based diets (Enujiugha 2003). These antinutrients and other toxic substances affect the bioavailability of beneficial nutrients and sometimes cause direct harm to the consumer. This necessitates the need to develop ways of entirely removing them or at least reducing their impact to the barest minimum. Food processing involves subjecting it to controlled conditions which invariably transforms certain properties such as colour and taste/flavour (Ezeokonkwo, 2007). This may also affect the level and quantity of nutrients contained in the plant food. Protein structures often contained in nuts generally undergo both structural and chemical modifications during processing (Clare Mills, Sancho, Rigby, Jenkins & Mackie, 2009). This can lead to epitope destruction or modification which invariably will increase or decrease the allergenicity of the plant food-nut (Sathe, Teuber & Roux, 2005). Some major and common processing methods for nuts which have been developed over the years include thermal processing and mechanical methods.

### *2.5.1 Thermal processing*

This method includes wet and dry heating – boiling, steaming, roasting, blanching, frying, grilling, sun/air-drying, etc. According to Hotz & Gibson (2007), thermal processing is capable of improving the bioavailability of micronutrients such as thiamine and iodine by destroying certain antinutritional factors such as goitrogens and thiaminases. Roasting of peanuts is reported to cause discoloration as a result of Maillard reactions and add flavour to nuts generally (Kita & Figiel, 2007). In peanuts, roasting with microwave was noted to have decreased the moisture content while deep oil frying increased the fat content as observed by Kita & Figiel, (2007). Roasting also increased the allergenicity of peanuts while reducing that of hazelnut (Hansen, Ballmer-Weber, Lüttkopf, Skov, Wüthrich, Bindslev-Jensen & Poulsen 2003; Sathe, Teuber & Roux, 2005). Thermal processing is

known to reduce or totally eliminate phytates and tannins in nuts (Enujiugha, 2003). According to Sze-Tao, Schrimpf, Teuber, Roux & Sathe, (2001), blanching of English walnut was highly effective in producing about 98% decrease in tannin within 2 minutes, while microwaving left tannins undetectable within 1 minute. Roasting, as well, was reported to cause small but significant reduction (about 14%) of tannins in walnuts (Sze-Tao, Schrimpf, Teuber, Roux & Sathe, 2001).

### *2.5.2 Mechanical methods*

Mechanical methods include processes such as pounding, grinding/milling, cutting/chopping, peeling etc. One major effect of these processes is that they increase the surface area of the plant food making volatile substances vulnerable to swift evapouration (Hotz & Gibson, 2007). However, mechanical processing may have negative and/or positive impacts on different nutrient content. Cutting and grinding of nuts may help to improve the bioavailability of carotenoids as noted by Hotz & Gibson (2007) by disrupting the subcellular membranes in which they are bound and making them more accessible for micellarization. The process of shell removal and milling/grinding to a flour state are known to deplete vitamins and minerals; however, the temperature at which this is done may have the capacity to influence loss of these vital nutrients. There are not many research reports known to address the specific impact of mechanical processing on nut nutrients, hence, it remains a potential field for further studies.

## 2.6 Effects of preservation and storage processes on nut nutrients and phytochemicals

It can be inferred from several scientific researches carried out on nuts that two of the most important characteristics of nuts considered during preservation or storage are the fatty acid and moisture contents (Chandrasekara & Shahidi, 2011; Kosoko, Sanni, Adebowale, Daramola & Oyelakin, 2009). Most nuts contain high percentage of fatty acids which when oxidized turn rancid. This chemical reaction tends to affect organoleptic as well as nutritional qualities of nuts over time. Nutritional depletion arises from the reaction of oxidized lipids (peroxides) and other lipid-free radicals with proteins and vitamins (Nejad, Tabil, Mortazavi & Kordi, 2003). The mode of storage of nuts will

help to basically slow down deteriorating process and maintain nutritional quality or encourage the contrary. Unshelled almonds that were stored at ambient temperature for nine months were noted by García-Pascual, Mateos, Carbonell & Salazar, (2003) not to have undergone any changes in their initial fat content, α-tocopherol nor the peroxide values while Buransompob, Tang, Mao & Swanson (2003) showed that heat treated shelled English walnut and almond maintained appearance and nutritional status after 60 days of storage at 25°C which is equivalent to storage at 4°C for 2 years

Drying is another major preservative process employed in nut handling. Whatever means that is employed in drying, all is aimed at reducing the moisture content which also serves as a platform for initiation of several other chemical reactions capable of leading to degradation of nut nutrients. Nejad, Tabil, Mortazavi & Kordi, (2003) reports that controlled drying affected the sensory attributes of pistachio nuts with its roasted flavour increasing with high temperature while cashew nuts steamed for 40 minutes and dried at 70°C best preserved the nutritional quality Kosoko, Sanni, Adebowale, Daramola & Oyelakin, 2009).

## 2.7 Nut fungal invasion and mycotoxin contamination

The discovery of aflatoxins in the 1960s and the subsequent recognition of mycotoxins as issues of significant health concern to humans and animals have led to great interest in the study of moulds and mycotoxin contamination in food substances (Rodrigues, Venâncio & Lima, 2012). These fungi are generally saprophytic and opportunistic. Nuts are known to be susceptible to the growth of these fungi as well as mycotoxin contamination. Nut fungal invasion and mycotoxin contamination usually occur during growth, harvesting and storage periods. Intrinsic and extrinsic factors play crucial roles in the spectrum of fungi that dominate the mycobiota in nuts (Khodavaisy, Maleki, Hossainzade, Rezai, Ahmadi, Validi & Ghahramani,c 2012; (Rodrigues, Venâncio & Lima, 2012). These variable factors include water activity/moisture content, pH, temperature changes, storage conditions, relative humidity, aerobic conditions, and poor harvesting practices which inflict mechanical damages on the nuts. Although these factors favour fungal growth, not all species are found in the mycobiota. Certain characteristics of the invading fungi help to promote its growth in a particular substrate/environment. These important characteristics are those related to the physiology of the invading fungi

and their adaptability to the different nut matrices and environmental conditions (Astoreca, Vaamonde, Dalcero, Ramos & Marín, 2012; Jubeen, Bhatti, Maqbool & Mehboob, 2012). Some of the fungal species known to predominate in nut (almonds, chestnuts, hazelnuts, walnuts, and pistachio) contaminations, most of which are mycotoxigenic include *Aspergillus* section *nigri, parasiticus* and *flavus, Penicillium* spp. *Fusarium* spp. *Rhizopus* spp. *Eurotium* spp. *Botrytis* spp. *Cladosporium* spp. and *Trichoderma* spp. (Jubeen, Bhatti, Maqbool & Mehboob, 2012; Rodrigues, Venâncio & Lima , 2012; Salem & Ahmad, 2010).

Mycotoxins are known to be secondary metabolites. When growth is temporarily restricted in fungi, secondary metabolism is initiated to remove products from intermediate metabolic pathways. Mycotoxins constitute a major part of these products which are removed. They are natural products of low molecular weight produced mainly by filamentous fungi (Rodrigues, Venâncio & Lima, 2012). Its production is not dependent on a specific precursor; rather, different genera, species and strains of fungi employ variable compounds as precursors. Several compounds are being recognised as mycotoxin depending on the definition used since most fungal toxins occur in families of chemically related metabolites; however, the most studied mycotoxins implicated in nut contaminations include aflatoxins (B1, B2, G1, G2), ochratoxin A, trichothecenes, fumonisins, patulin. The role of mycotoxins in nature is not quite clear however, the general idea is that mycotoxin producing fungi are better protected against other organisms that share the same trophic niche.

Although fungal growth on nuts is often associated with mycotoxin contamination, it is not generally synonymous even for mycotoxigenic fungi. This is because of environmental conditions which may allow growth but hinder toxin production. As long as normal growth processes are not interfered with in some species, there may not be need for initiation of secondary metabolism which results in toxin production (Astoreca, Vaamonde, Dalcero, Ramos & Marín, 2012). The economic importance of fungal invasion and mycotoxin contamination cannot be over-emphasized. They cause serious nut-food spoilage which affects the vital sensory qualities. This usually leads to serious economic losses. About 30.97 million tons of pistachio nuts from different Asian countries were recorded to be contaminated by *Aspergillus flavus* and *parasiticus* in 1998

(Khodavaisy, Maleki, Hossainzade, Rezai, Ahmadi, Validi & Ghahramani, 2012). The effects of these toxins in humans when ingested could either be carcinogenic, genotoxic, teratogenic, nephrotoxic, hepatotoxic or immunotoxic (Salem & Ahmad, 2010). There is therefore the need for extensive investigations on how best to minimize fungal invasion and mycotoxin contaminations in nuts.

# 3 Chapter Three: General design of experiment

## 3.1 Introduction

African walnut is usually ripe for harvest between the months of June and September in Nigeria. They are then transported to the open markets and sold either directly to consumers or to retailers who process them by boiling or roasting in hot sand and then sold to consumers through hawking or open-table displays for 1 – 7 days. The nuts are seen in the markets only within this period of harvest and they are often in high demand. During this interval of harvesting and consumption, the nuts are constantly exposed to both high temperatures of 20 - 37°C and relative humidity of 75 – 85 %. This practice predisposes the nuts to fungal infestation and potential mycotoxin contamination hence, raising questions about product quality and safety. Oral interviews with retailers' reveal that processed nuts, not sold out same day are often re-boiled or re-heated in oven for few minutes and re-packaged for sale. So far there is no documented research report on the postharvest storage, shelf-life, fungal and mycotoxin contamination of African walnuts.

Currently, the production of these nuts are still at the subsistent level. Despite the high demand for the nuts, there is still lack of commercialized farming process for the crop which results in low production as well as high scarcity during its harvest season. Due to this scarcity, obtaining experimental samples require long term pre-harvest arrangements involving huge financial commitments as well. Furthermore, the researcher needs to be physicaly present to supervise the harvesting, examine collected fruits and double-check packaging for export. In the light of these sample collection challenges coupled with those posed by Africa to UK cross-border exportation of agricultural crops, this study/experiment was carried out for only one season of the nut.

The experiment was designed to simulate the normal retail practices employed in the postharvest handling of African walnut. The key five factors considered in the treatments were (i) the processing methods (boiling, roasting or left unprocessed), (ii) shelf life storage (3 and 7) days, (iii) retail storage temperatures (25 and 37 °C), (iv) postharvest/cold storage days (0, 10 and 20), and (v) maturity stages (early and late) assessed by visual observation of the pods at harvest (green pods for early maturity and brown coloured pods for late maturity).

## 3.2 Materials and methods

### *3.2.1 Plant materials*

African walnut (*Tetracarpidium conophorum* (Mull Arg.) fruits were harvested in June in Nkalagu Obukpa village, Ibeagwa-ani located in Nsukka Local Government area (Latitude 6°51′21″ N and Longitude 7°23′33″ E), Enugu State, Nigeria. The fruits (i.e. nuts in pods) were packed in net bags to allow adequate ventilation and transported to the United Kingdom within 6 days of harvest. Upon arrival, the fruits were sorted into two categories based on the stage of maturity of the pods (dark brown; late maturity - L and green coloured pods; early maturity – E, respectively) and stored at 5°C.

**Figure 12** African walnut at early/green (left) and late/brown (right) maturity

### *3.2.2 Sampling and treatment of nuts*

A flow chart of experimental procedure from time of sample importation until processed and stored for analysis is shown in Figure 13. Randomized sampling was carried out in three batches; day 0, 10 and 20 respectively. On each sampling day, pods were taken from those at early and late maturity, respectively, and husked to release the nuts. The husked nuts were subsequently treated in triplicates (15 nuts per replicate) by boiling, roasting or left unprocessed. Boiling and roasting were achieved by placing nuts with shells in boiling water (100 °C) on a BioCote Stuart hot plate-stirrer UC152 (Bibby Scientific, Staffordshire, UK) and a Carbolite PF 200 electric oven (Carbolite, Derbyshire, UK) at 200°C, respectively, for 60 min. Treated nuts were then placed in two groups; first group

consisted of those separated for shelf life storage (stored nuts - SN) at both 25 and 37 °C for either 3 or 7 days (on sampling day 0 and 10, and for 3 days only on sampling day 20), while second group were those assigned for immediate cutting and snap-freezing with liquid nitrogen (non-stored nuts – NSN), and kept at – °40 C until analysed. Shelf life storage was carried out using a Sanyo Versatile Environmental Test Chamber unit MLR-250HT (Sanyo, Illinois, United States) with relative humidity set at 78 %. On each sampling day, treated nuts (9 nuts per treatment) were placed in glass jars - 3 nuts per jar - with specially perforated covers for aeration (**Error! Reference source not found.**).

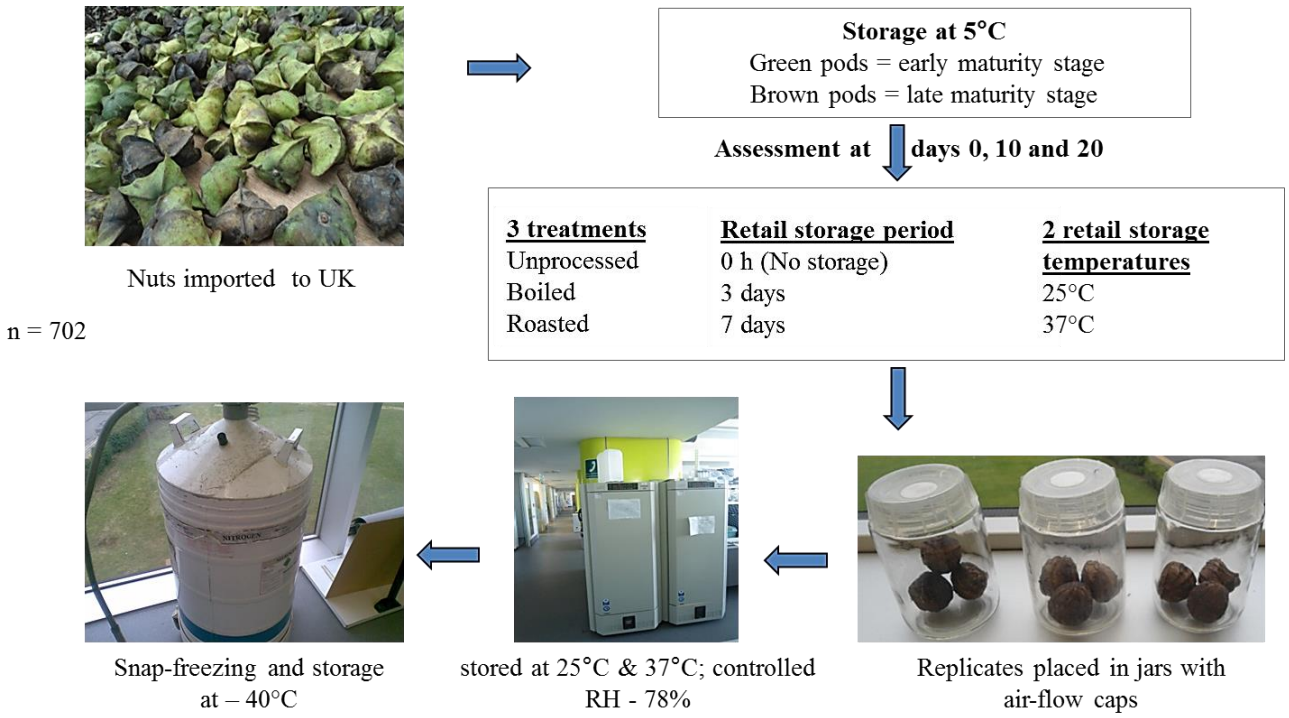



**Figure 13** Flow chart of experiment procedure from importation of nuts to sorting into maturity stages before cold storage. There were 3 assessment days (0, 10 and 20) where nuts from cold storage (45 nuts) were sampled and treated by boiling, or roasting or left unprocessed. Each set of treated nuts were separated either for 'No storage (9 nuts)' or for retail storage. Retail storage of nuts (9 per storage day per storage temperature) was done for either 3 days (at 25 and 37 °C, respectively) or 7 days (at 25 and 37 °C, respectively). Nuts were placed in glass jars which had perforated covers for aeration, before storage in versatile environmental test chamber with controlled temperatures and relative humidity. Samples were cut and snap-frozen and kept at -40 °C at the end of each storage period.

### *3.2.3 Sample preparation*

After 3 or 7 days shelf life storage, nut samples were shelled using a nut cracker and cut to tiny bits. The pieces from the 3 nuts in each replicate were pooled together and snap-frozen with liquid nitrogen. Portions for immediate processing were also shelled, cut and snap-frozen with liquid nitrogen in triplicates (3 nuts per replicate). These cut samples were placed in freezer bags, freeze-dried at –52 °C until stable weight (about 7 days) using ScanVac 55-9 CoolSafe freeze-drier (ScanLaf-Labogene, Denmark), blended using Cuisinart SG20U electric spice and nut grinder (Amazon, UK), and kept at -40°C until needed for further biochemical analysis. These processing and shelf life conditions were adopted in order to simulate normal retail practices. A total of 702 nuts were used.

# 4 Chapter Four: Effects of postharvest storage and processing techniques on the main fatty acids in the profile oil extracted from African walnut (*Tetracarpidium conophorum* Mull. Arg.).

## 4.1 Introduction

Understanding the fatty acid content of any oil enables nutritionists to evaluate the benefits of consuming the food products prepared from the oil source under consideration. So far, this is the first study to evaluate the fatty acid profile of African walnut harvested from South-eastern Nigeria. Again, the modes of storage and processing of nuts and nut products are important postharvest practices that could influence its nutritional quality and shelf life. Inappropriate handling could lead to accelerated degradation through lipid peroxidation pathways, discoloration and off-flavour development (Srichamnong, Wootton & Srzednicki, 2010). Nuts in general are nutrient-dense foods since they contain high total fat content. This is associated with high caloric index (Devitt, Kuevi, Coelho, Lartey, Lokko, Costa & Mattes 2011). They are generally perceived by the public as having fattening potential; however, this perception has changed as research reports show that the fatty acid composition of nuts are more beneficial than harmful (Bes-Rastrollo, Wedick, Martinez-Gonzalez, Li, Sampson & Hu, 2012). The saturated fatty acid contents in most nuts are generally low and most of these nuts contain much of mono- and poly-unsaturated fatty acids such as linoleic acid and α-linolenic acid (Ros & Mataix, 2006). Ros (2009) noted that fatty fraction of nuts also contains sizeable amounts of compounds which are known to have both antioxidant and cholesterol-lowering properties. In general, nuts are suggested to be good sources of protein with high L- arginine content that has been shown to be important in vasodilation processes. Many nuts also contain several other reportedly bioactive compounds such as flavonoids (Huynh & Chin, 2006) and are good sources of dietary fibre; contain sizeable amounts of folate and are rich sources of antioxidant vitamins such as tocopherols (Blomhoff, Carlsen, Frost & Jacobs, 2006; Ros, 2009). Although nuts are composed of healthy nutrients and bioactive compounds as enumerated above, it cannot be conclusively said that their consumption as whole or as part of diet will guarantee healthy state of individuals. Terry & Thompson (2011) stated that various methods of processing and storage, and other postharvest activities before

consumption play significant roles in modifying the quality, bioavailability and interaction between these phytochemicals/nutrients and the consumer. Other research reports showed that two of the most important characteristics of nuts considered during preservation or storage are both the fatty acid and moisture contents (Chandrasekara & Shahidi, 2011). The oxidation of fatty acids in the nuts produces rancid flavours. This chemical reaction tends to affect organoleptic and nutritional properties over time (Nejad Tabil, Mortazavi & Kordi, 2003) and ultimately, lower the market value as well as make them unappealing for consumption. Thus, storage conditions of nuts may help to slow down deteriorating process and maintain nutritional quality, or encourage the contrary.

The current postharvest conditions African walnuts are subjected to, which includes exposure to both high temperature ranges of 20 - 40°C and relative humidity of 75 – 80 % during display (1 – 7 days) sales in the open market, raises concerns about the nutritional quality. Although there have been some studies examining the nutritive value of the whole nut as well as the oil-based diet formulations (Effiong & Udo 2010; Oladiji, Abodunrin & Yakubu, 2010), and the proximate composition of the nut (Oboh & Ekperigin, 2004; Odoemelam, 2003; Enujiugha, 2003), there is none reporting on the nutrient behaviour of stored African walnut harvested in Nigeria, before and after processing, nor on the effect of the common processing methods on the fatty acid profile. This study focuses mainly on profiling the main fatty acid contents and examining the impact these postharvest handling/processing conditions would have on the fatty acid profile.

## 4.2 Materials and methods: analytical method development for fatty acid profiling.

### *4.2.1 Sample Oil Extraction: Cold Hexane Extraction*

Oil extraction was performed on 3.00 ± 0.05 g freeze-dried ground samples, (boiled, roasted and unprocessed, respectively) following the method described by Meyer & Terry (2010) with slight modifications. The extraction method is considered to be less destructive as it does not require heating, thus enabling sequential extraction of other compounds from the same sample which were analyzed in a different study. The process is also less tedious than others (e.g. using Soxhlet apparatus). In brief, hexane (30 mL) was added into the sample beaker and homogenized using an IKA® Ultra-Turrax® T25

homogeniser (IKA®, Staufen, Germany) at 8000 rpm for 30 seconds and allowed to stand for 1 min at room temperature (15 °C). The mixture was filtered under vacuum using a Buchner funnel and flask with a 5.5 cm diameter Fisherbrand QL100 filter paper. The residue was recovered into the beaker and re-extracted twice using 20 and 10 mL hexane, respectively. The lipid-containing hexane extracts were combined and placed in 15 mL centrifuge tubes for each sample and the hexane further evaporated using a miVAC Quattro Genevac centrifuge vacuum evaporator (Genevac, Suffolk, United Kingdom) at 40°C and the [$OH^-$] programme selected. The maximum rotation time was set at 1 h. The clear yellow oil was removed from the tubes and placed in 2 mL screw-cap amber Agilent vials and kept at - 40°C until analysed.

### *4.2.2 Fatty acid methyl ester (FAME) GC- FID Analysis*

The fatty acids were analyzed as fatty acid methyl esters (FAMEs) prepared by trans-esterification of 100 mg ± 0.5mg African walnut oil with a cold methanolic solution of potassium hydroxide (KOH), as recommended by the International Olive Oil Council (IOOC, 2001), with slight modifications. In brief, oil samples were placed in a screw top vial; 2 mL of hexane were added and thoroughly mixed. KOH (2N) prepared by dissolving 2.24 g of potassium hydroxide pellets in 20 mL of methanol was added (200 µL). The mixture was vigorously shaken for 30 seconds and left in a rack at room temperature (15 °C) to stratify. The clear upper layer containing methyl esters was carefully decanted into amber Agilent vials and stored at -40°C until analysed.

#### *4.2.2.1 Stock preparations of FAME standard mix $C_8$ - $C_{22}$ and single standards*

A stock solution of FAME standard mix $C_8$ - $C_{22}$ containing 19 compounds (SUPELCO® Analytical/ Sigma-Aldrich®, Bellefonte, United States) was prepared by weighing 100 mg into a 100 mL volumetric flask and made up to volume with hexane (HPLC grade).

Stock solutions of FAME single standards purchased from Sigma-Aldrich®, Bellefonte, United States were prepared as follows; methyl palmitate standard (15 mg) was dissolved in 1mL of hexane to give a concentration of 15 mg/mL stock. 50 µL of stock was mixed with 950 µL hexane (1:20 dilution) to give a concentration of 0.75 mg/mL. Methyl oleate standard (871.5 mg) was mixed with 5 mL hexane to make the stock solution; 30 µL stock was mixed with 970 µL hexane to give a concentration of 20 mg/mL (1:20 dilution).

Methyl Stearate standard (6 mg) was dissolved in 1 mL hexane to give a concentration of 6mg/mL stock solution. 50 µL stock was mixed with 950 µL hexane to give a final concentration of 0.3mg/mL. Methyl linoleate (837.7 mg) was dissolved in 5 mL hexane to make the stock solution. 15 µL of stock was mixed with 985 µL hexane to give a final concentration of 12.261 mg/mL. Methyl arachidate (10 mg) was dissolved in 5mL hexane to give the stock solution (10 mg/mL). 200 µL stock was mixed with 800 µL hexane to give a final concentration of 2 mg/mL.

Dilutions of African walnut FAMEs and standard mix $C_8$ - $C_{22}$ at 1:100, 1:50 and 1:20 and those of single standards were pre-analysed using an Agilent 6890N Gas Chromatography device equipped with flame-ionization detector (GC-FID) and a multi-chambered autosampler 7683 series (Agilent Technologies, Wokingham, UK), Figure 14. Profiling and quantification was achieved using CP-Sil 88 fused silica capillary column (50m × 250 µm × 0.2 µm nominal; Varian, CP6173- Agilent technologies, Wokingham, UK). A splitless sample injection mode was used. Column temperature was programmed at 80˚C for 3 min, and then raised to 220˚C at 4˚C $min^{-1}$ and then held for 3 minutes. The carrier gas was helium with a column flow rate of 1.0 mL $min^{-1}$ and the detector temperature was 270˚C. The dilution that gave best peak separation was used to analyse the entire African walnut FAME sample set.

#### *4.2.2.2 Identification of African walnut oil sample peaks*

Fatty acids were identified and quantified firstly by comparing sample peak retention times and areas with those of known mixed standards of known composition and concentrations, and secondly by addition of standards (known concentration) addition to samples (spiking). Finally, a gas chromatography coupled mass spectrometry (GC-MS) single-quadruple analysis was carried out as a confirmatory step. The GC-MS analysis was performed using a Perkin Elmer AutoSystemAuto System XL-Turbo Mass machine (Perkin Elmer, Buckinghamshire, UK).

#### *4.2.2.3 Sample spike with standard mix and single standards*

FAME mixed standard ($C_8$ - $C_{22}$) -50 µL was mixed with sample (200 µL) and hexane (750 µL) in amber Agilent vial. Single standards (20 µL of 1:20 dilutions) listed above were mixed with 20 µL of African walnut oil sample and made up to 1 mL with hexane (solvent) in amber Agilent vial. These were placed in the auto sampler chamber of Agilent

6890N gas chromatography machine equipped with flame-ionization detector (GC-FID) and CP-Sil 88 fused silica capillary column (50m × 250 µm × 0.2 µm nominal; model - Varian, CP6173). Analysis was done with settings as earlier described in section 4.2.2.1 above. Calibration curves for FAME single standards and mix were made using dilutions of stock solutions to give concentrations of 0.6, 1.8, 3.0, 4.3, 5.5, and 6.7 mg/mL.

The limits of detection and limits of quantification were determined as 3 and 10 times standard deviations, respectively. (The lowest concentration of each standard in the calibration curve was injected 10 times. The means and standard deviations were calculated for each standard).

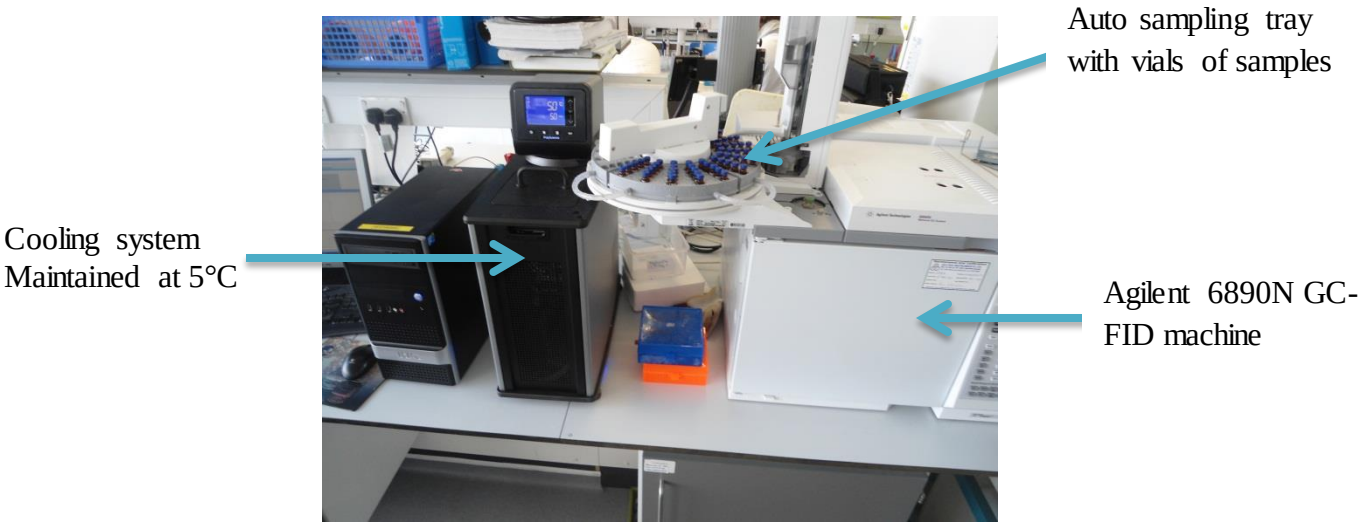



**Figure 14** Gas chromatography-flame ionization device equipped wth a cooling system, used for African walnut oil analysis.

### *4.2.3 GC-MS single-quadruple analysis*

A gas chromatography coupled mass spectrometry (GC-MS) single-quadruple analysis was carried out as a confirmatory step. FAME mix ($C_8$ – $C_{22}$) and African walnut oil sample, 20µL each were placed in amber vials and made up to 1mL with hexane,

respectively. The GC-MS analysis was performed using a Perkin Elmer Auto System XL-Turbo Mass machine (Perkin Elmer, Buckinghamshire, UK). Separation was achieved with a DB-5 polysiloxane polymer capillary column of 15m length, and 0.25mm internal diameter (Agilent Technologies, Wokingham, UK). The method used was as described by De Santana-Filho, Noleto, Gorin, de Souza, Iacomini & Sassaki, (2012) with slight modifications. In brief, initial oven temperature was at 50˚C, held for 2 min and then raised to 90˚C at 20˚C $min^{-1}$ which was held again for 1 min and heated to a final temperature of 280°C at 5˚C $min^{-1}$, and held for 2 minutes. The injector temperature was set at 250˚C and electron ionization obtained at 70eV at 200 °C with a full scan range of 50 – 400 m/z. The carrier flow was at 1 mL $min^{-1}$. A splitless injection of samples was used and selected ion monitoring was performed on the following common ions present in the reference standard mix -74, 87, 55, 43, and 41. The limits of detection (LOD) and quantitation (LOQ) were determined by signal-to-noise ratio of 3 and 10 times standard deviations, respectively (De Santana-Filho, Noleto, Gorin, de Souza, Iacomini & Sassaki, 2012).

## 4.3 Materials and method: analysis of FAMEs of African walnut subjected to experimental conditions.

The plant materials were those processed and kept at - 40°C as described in the general experiment section (section 3.2.3). The samples were removed from storage and dried to constant weight using CoolSafe™ SCANVAC freeze-drier (Figure 15). Freeze-dried samples were blended at room temperature (15 °C) using a nut grinder. The oil samples were extracted following the cold hexane procedure described in the method development section earlier in this chapter (section 4.2.1). However, the oil was recovered from the hexane extract by placing the oil-containing solutions in 15 mL centrifuge tubes and placing them in a miVAC Quattro GeneVac centrifuge evaporator (Suffolk, UK) with temperature at 40°C and the [$OH^{-}$] programme selected (Figure 15). The maximum rotation time was set at 1 h. The solvent was recovered and the clear yellow oil was removed from the tubes and placed in 2 mL screw-cap amber Agilent vials and kept at -40°C until analysed. The results were expressed in relation to freeze dried weight (FDW)

The oil samples from sampling day 0, 10 and 20 were analyzed using the GC-FID equipment (Figure 14), and sample oil extraction and analytical method developed in this

chapter (p. 58). A cooling system was attached to the GC-FID auto sampling tray to maintain a temperature of 5°C throughout the analysis as many samples were being analysed this time.

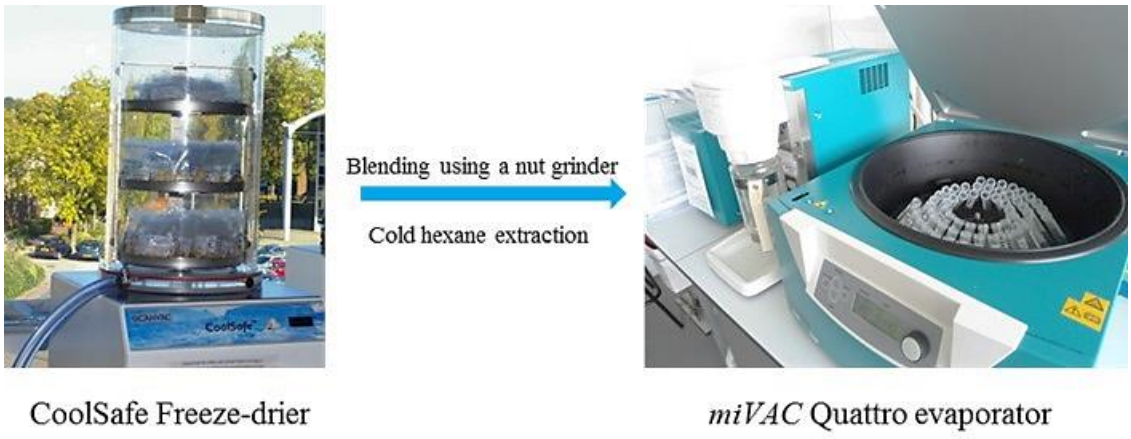



**Figure 15** Sample preparation process and equipment. CoolSafe freeze-drier was used to dry samples at -52 °C until stable weight. Dried samples were blended to powder and oil extracted by 'Cold hexane procedure'. Both extracted oil and solvent were recovered using MiVAC Quattro evaporator.

## 4.4 Statistical analysis

All statistical analysis was performed using GensStat® package for Windows (version 12.0) VSN International Ltd (Hertfordshires. UK). The effects of processing (unprocessed, boiling and roasting), cold storage days (0, 10 and 20), retail storage temperatures (25 and 30 °C) and retail (shelf life) storage (3 and 7 days) and maturity stages (early and late) were considered. The data was analysed first, for all set obtained from 3 days retail storage at both 25 and 37 °C and then set from 7 days storage. Data passed Wilk test for normality and means of variables were separated and their main effects demonstrated using general analysis of variance (ANOVA). Least significant differences of factorial interactions were determined at the 95 % confidence interval ($p < 0.05$).

## 4.5 Results

### *4.5.1 GC-FID and GC-MS single-quadruple analysis of FAMEs and chromatogram peak resolution and identification – method development*

The GC-FID and GC-MS methods used allowed for unambiguous separation, identification and quantification of fatty acids present in the mixed standards and African walnut oil samples. The order of elution of the compounds in the mixed standard is as shown in Table 8. Using GC-FID, six major sample peaks were identified in the African walnut oil sample by comparing the retention times with those of the standards analyzed at the same period. The peaks of sample oil were identified as methyl esters of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1, *cis*-9), linoleic acid (C18:2, *cis*-9, 11), arachidic acid (C20:0) and α-linolenic acid (C18:3, *cis*-9, 12, 15) in order of elution (Table 9). These compounds were confirmed further with the results from GC-MS single-quadruple analysis. Sample spike with standards showed marked increases in the peak areas of eluted compounds (Table 9 and Table 10). The concentrations of the samples peaks were much higher than the limits of detection and limits of quantitation (Table 11).

**Table 8** Order of elution and retention times of compounds in FAME mix ($C_8$ - $C_{22}$) standard.

| Order of elution | FAME $C_8$-$C_{22}$ mix components | Composition (%) | Retention time (min) |
|---|---|---|---|
| 1 | Caprylic Acid (C8:0) | 1.90 | 7.657 |
| 2 | Capric Acid (C10:0) | 3.20 | 10.846 |
| 3 | Lauric Acid (C12:0) | 6.40 | 14.562 |
| 4 | Tridecanoic Acid (C13:0) | 3.20 | 16.444 |
| 5 | Myristic Acid (C14:0) | 3.20 | 18.299 |
| 6 | Myristoleic Acid (C14:1, *cis*-9) | 1.90 | 19.575 |
| 7 | Pentadecanoic Acid (C15:0) | 1.90 | 20.101 |
| 8 | Palmitic acid (C16:0) | 13.00 | 21.869 |
| 9 | Palmitoleic Acid (C16:1, *cis*-9) | 6.40 | 22.823 |
| 10 | Heptadecanoic Acid (C17:0) | 3.20 | 23.531 |
| 11 | Stearic Acid (C18:0) | 6.50 | 25.172 |
| 12 | Elaidic Acid (C18:1, *trans*-9) | 2.60 | 25.707 |
| 13 | Oleic Acid (C18:1, cis-9) | 19.60 | 25.942 |
| 14 | Linoleic Acid (C18:2, *cis*-9,12) | 13.00 | 27.213 |
| 15 | Arachidic Acid (C20:0) | 1.90 | 28.221 |
| 16 | cis-11-Eicosenoic Acid (C20:1) | 1.90 | 28.704 |
| 17 | α-linolenic Acid (C18:3, *cis*-9,12,15) | 6.40 | 28.899 |
| 18 | Behenic Acid (C22:0) | 1.90 | 31.082 |
| 19 | Erucic Acid (C22:1, *cis*-13) | 1.90 | 31.710 |

Arranged from Frau & Frau, (2010).

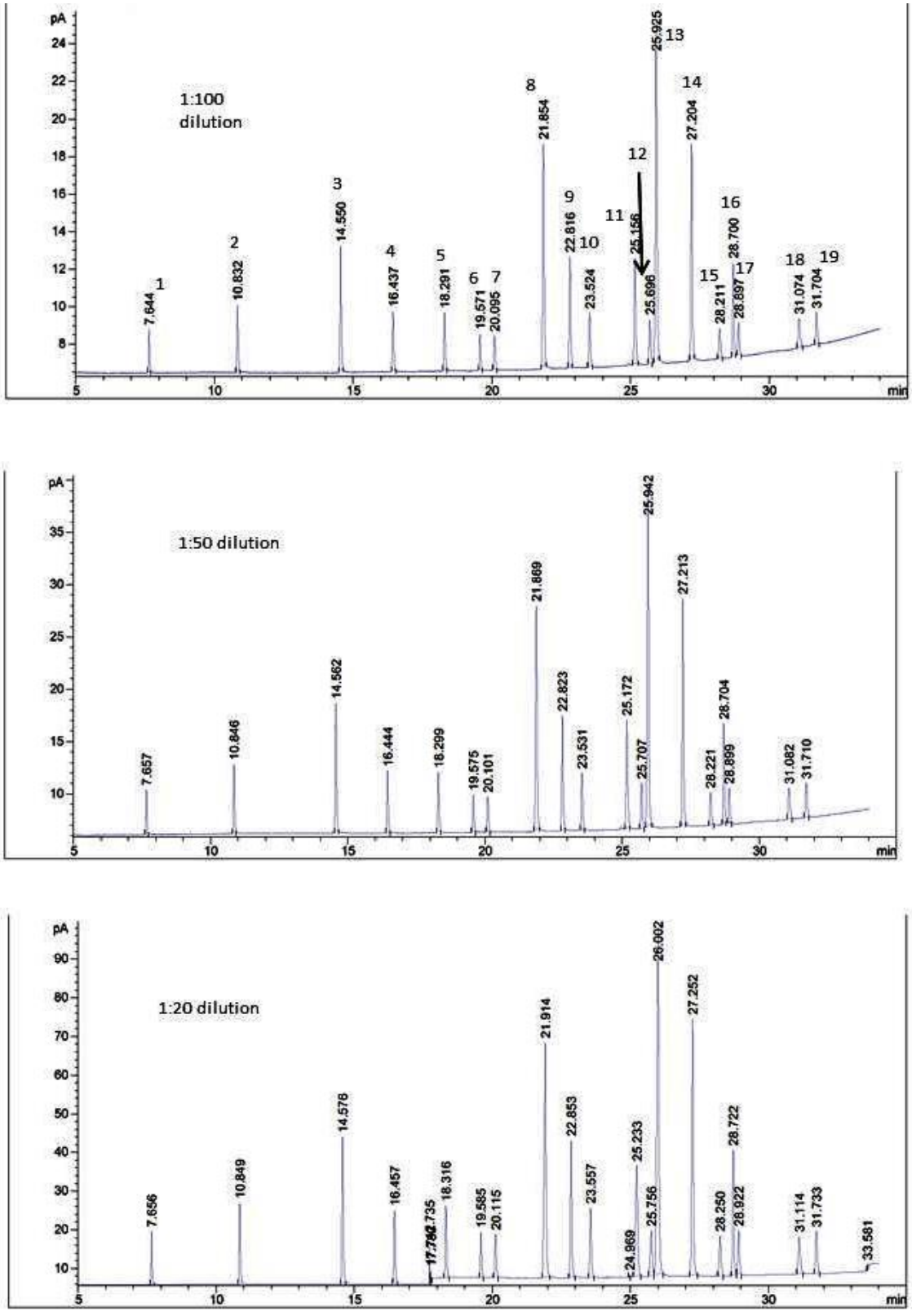



**Figure 16** Peak resolution of of different dilutions (1:100, 1:50 and 1:20) of FAME standard mix ($C_8$ - $C_{22}$).

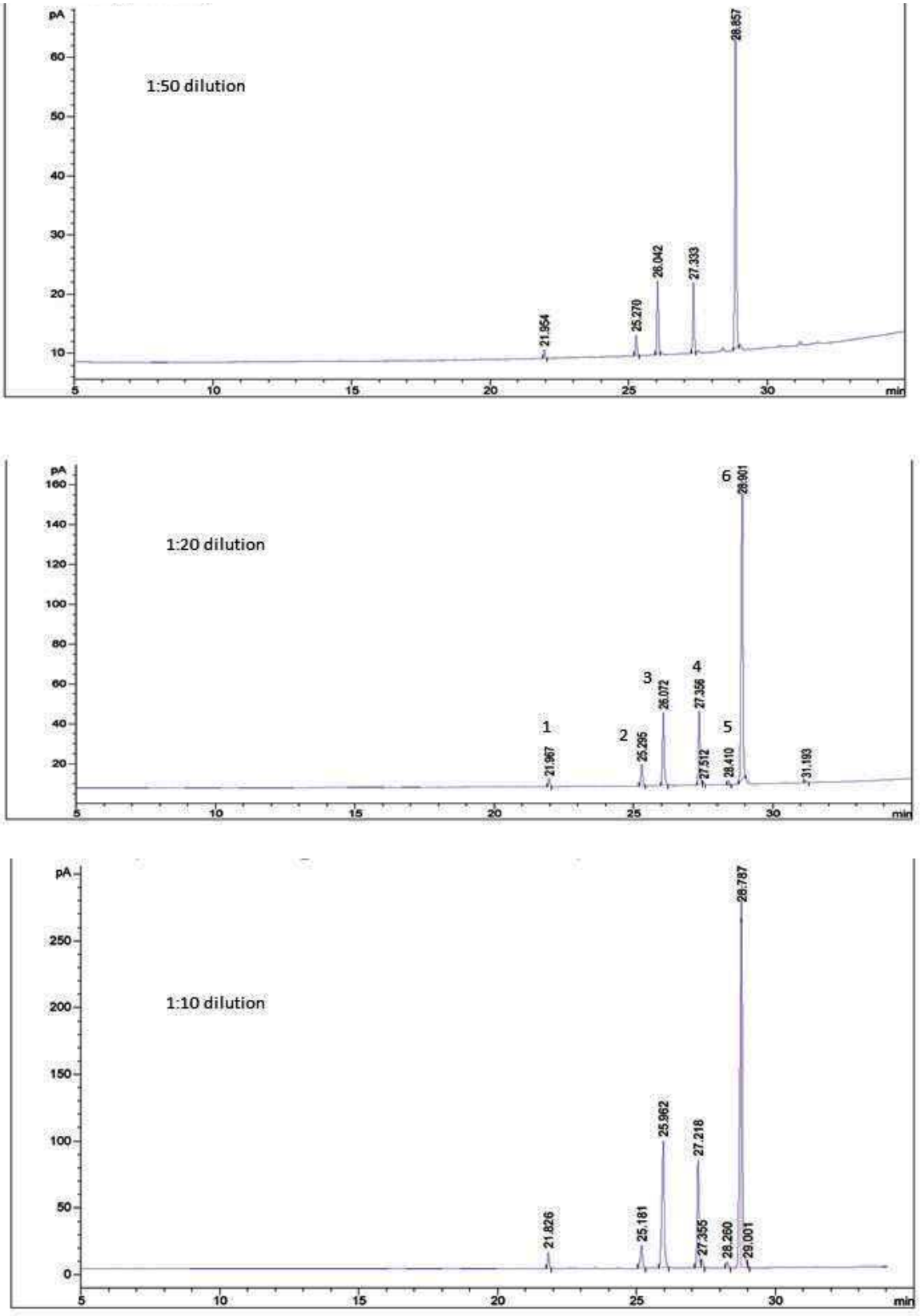



**Figure 17** Peak resolution of different dilutions (1:50, 1:20 and 1:10) of African walnut oil sample.

**Table 9** African walnut oil FAME order of elution and sample spiked with mixed standard. Retention times and peak areas are shown.

| Compound | Retention time (min) | Sample peak area | Mixed standard peak area | Spiked peak area |
|---|---|---|---|---|
| Methyl Palmitate | 21.954 | 45.78 | 267.46 | 410.92 |
| Methyl stearate | 25.181 | 93.88 | 141.44 | 205.76 |
| Methyl oleate | 25.962 | 543.77 | 425.55 | 669.54 |
| Methyl Linoleate | 27.218 | 348.12 | 241.46 | 790.96 |
| Methyl Arachidate | 28.260 | 20.56 | 99.25 | 87.25 |
| Methyl linoleneate | 28.787 | 857.23 | 40.04 | 1697.59 |

Values are means of 3 replicate readings.

**Table 10** African walnut oil sample spike with single standards. Peak areas are shown.

| **Compound** | **Sample peak area** | **Standard peak area** | **Spiked peak area** |
|---|---|---|---|
| Methyl palmitate | 111.52 | 1500.91 | 1854.67 |
| Methyl stearate | 216.56 | 409.55 | 875.17 |
| Methyl oleate | 986.80 | 752.00 | 1857.05 |
| Methyl linoleate | 948.90 | 333.55 | 1196.98 |
| Methyl arachidate | 16.63 | 33.78 | 54.60 |

Values are means of 3 replicate readings.

**Table 11** Limits of detection and quantitation for the single standards

| Compound | LOD (mg/mL) | LOQ (mg/mL) | Sample concentration (mg/mL) |
|---|---|---|---|
| Methyl Palmitate | $4.56\times10^{-5}$ | $9.11\times10^{-5}$ | $7.47\times10^{-4}$ |
| Methyl Oleate | $5.17\times10^{-2}$ | $8.27\times10^{-2}$ | $5.96\times10^{-1}$ |
| Methyl Stearate | $6.86\times10^{-4}$ | $7.43\times10^{-4}$ | $2.17\times10^{-3}$ |
| Methyl Linoleate | $3.10\times10^{-2}$ | $8.74\times10^{-3}$ | $9.06\times10^{-1}$ |
| Methyl Arachidate | $2.59\times10^{-3}$ | $1.55\times10^{-3}$ | $3.04\times10^{-3}$ |

Values are means of 3 replicate readings.

### *4.5.2 Analysis of FAMEs of African walnut subjected to experimental conditions*

Analysis of African walnut oil samples subjected to different postharvest treatments/processing techniques (viz. boiling, roasting, storage days and temperatures ) showed that α-linolenic acid was the most abundant (1.1 – 8.2 mg $g^{-1}$ FDW) followed by, in decreasing order of abundance, linoleic acid (0.05 – 2.00 mg $g^{-1}$ FDW), stearic acid (0.04 – 0.44 mg $g^{-1}$ FDW), oleic acid (0.02 – 0.30 mg $g^{-1}$ FDW) and palmitic acid (0.01 – 0.26 mg $g^{-1}$ FDW) while arachidic acid was the least abundant at (0.01 – 0.07 mg $g^{-1}$ FDW).

When nuts were stored for 3 days at 25 or 37 °C, sampling days (0, 10 and 20 days), storage temperatures (25 and 37 °C) and processing (boiling, roasting and unprocessed) in general, influenced the concentrations of individual fatty acids significantly ($p < 0.05$) while the maturity stage of the nut made no difference. Considering the main effects only (Table 12), cold storage for 10 days, caused the highest increases ($p < 0.05$) in concentration of individual fatty acids across the profile when compared with 0 and 20 days storage. Specifically, linoleate showed the highest increment (46 %) followed by, in decreasing order, palmitate (40 %) arachidate (38 %), stearate (31 %), α-linolineate (21 %) and oleate (13 %) after 10 days cold storage. These increases caused by cold storage, were independent of other experimental factors - maturity stage, processing method or shelf-life temperatures. In relation to values at day 0, extension of storage up to 20 days

caused a significant reduction in the concentration of individual fatty acids except for arachidate. Boiling and roasting caused three-fold increases in the concentrations of, linoleate and two-fold increases in oleate, arachidate and stearate, however, the level of increase observed in α-linolineate was significantly lower, when compared with values from unprocessed samples. In a similar trend across the profile, shelf-life storage at 25 and 37 °C also raised the concentrations of all the fatty acids significantly except in α-linolineate found in nuts stored at 37 °C. Considering the interaction effects (Figure 18) boiling and roasting always raised the concentrations of individual fatty acids compared with unprocessed nuts ($p < 0.05$), whether the nuts were processed immediately after harvest (0 day) or stored (10, and 20 days). There was no statistical difference in the way boiling raised the concentrations compared to roasting. The rate of increase in concentration levels were similar whether the nuts were kept at 25 or 37 °C after cold storage and processing.

The main effects of experimental factors on concentrations of individual fatty acids across the profile after 7 days retail storage simulation was investigated (Table 12). Processing methods (boiling and roasting), cold storage duration and retail storage temperatures all caused a rise in concentrations of individual fatty acids irrespective of the maturity stage of the nut. When boiling was compared with roasting, no difference was found in their effect, however, both caused a significant rise of more than 83 % in concentrations of all the fatty acids except in α-linolineate and palmitate which had about 30 and 50 % rise in both boiled and roasted samples, respectively. The retail storage conditions, with the exception of palmitate, also increased the levels of the fatty acids with arachidate having the highest increment of 25 % while linoleate (2 %) was least. The effect of retail storage was the same whether the nuts were stored at 25 or 37 °C. When values were considered based on interaction of factors (Figure 19) the trends were similar to those of 3 days retail storage. No matter the number of days the nuts were kept in cold storage or immediately processed, boiling and roasting always raised the concentrations ($p < 0.05$) more than those of unprocessed. The rate of increase in concentration levels were also similar whether the nuts were kept at 25 or 37 °C after cold storage and processing. No statistical difference was found between retail storage for 3 and 7 days when both were compared.

**Table 12** Main effects of cold storage (5 °C) durations, processing methods, maturity stages, and retail storage temperatures on concentrations of individual fatty acids after 3 days of retail storage simulation.

| Compound | Cold storage duration (days) | | | | Maturity stage | | | Processing methods | | | | Retail Storage temperatures °C | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 10 | 20 | LSD | Early | Late | LSD | Unprocessed | Boiled | Roasted | LSD | None | 25 °C | 37 °C | LSD |
| Palmitate | 0.025 | 0.034 | 0.020 | 0.002 | 0.027 | 0.027 | 0.002 | 0.016 | 0.032 | 0.032 | 0.002 | 0.024 | 0.028 | 0.08 | 0.002 |
| Oleate | 0.167 | 0.181 | 0.143 | 0.018 | 0.167 | 0.160 | 0.014 | 0.098 | 0.206 | 0.187 | 0.018 | 0.150 | 0.171 | 0.169 | 0.018 |
| Stearate | 0.086 | 0.113 | 0.071 | 0.008 | 0.089 | 0.091 | 0.006 | 0.057 | 0.110 | 0.105 | 0.008 | 0.079 | 0.093 | 0.098 | 0.008 |
| Linoleate | 0.499 | 0.731 | 0.255 | 0.070 | 0.491 | 0.499 | 0.057 | 0.235 | 0.634 | 0.616 | 0.070 | 0.402 | 0.548 | 0.535 | 0.070 |
| Arachidate | 0.042 | 0.058 | 0.045 | 0.004 | 0.047 | 0.049 | 0.003 | 0.026 | 0.059 | 0.059 | 0.004 | 0.042 | 0.050 | 0.02 | 0.004 |
| Linolineate | 2.775 | 3.356 | 2.071 | 0.334 | 2.665 | 2.803 | 0.273 | 2.215 | 3.025 | 2.962 | 0.334 | 2.689 | 2.933 | 2.580 | 0.334 |

Units of values are in mg $g^{-1}$ FDW. Values are means (n = 3) with the LSD shown. The *p* values for individual fatty acids are shown in appendix D, tables D9.4.1.1 - D9.4.1.6. Values were considered significant at $p < 0.05$.

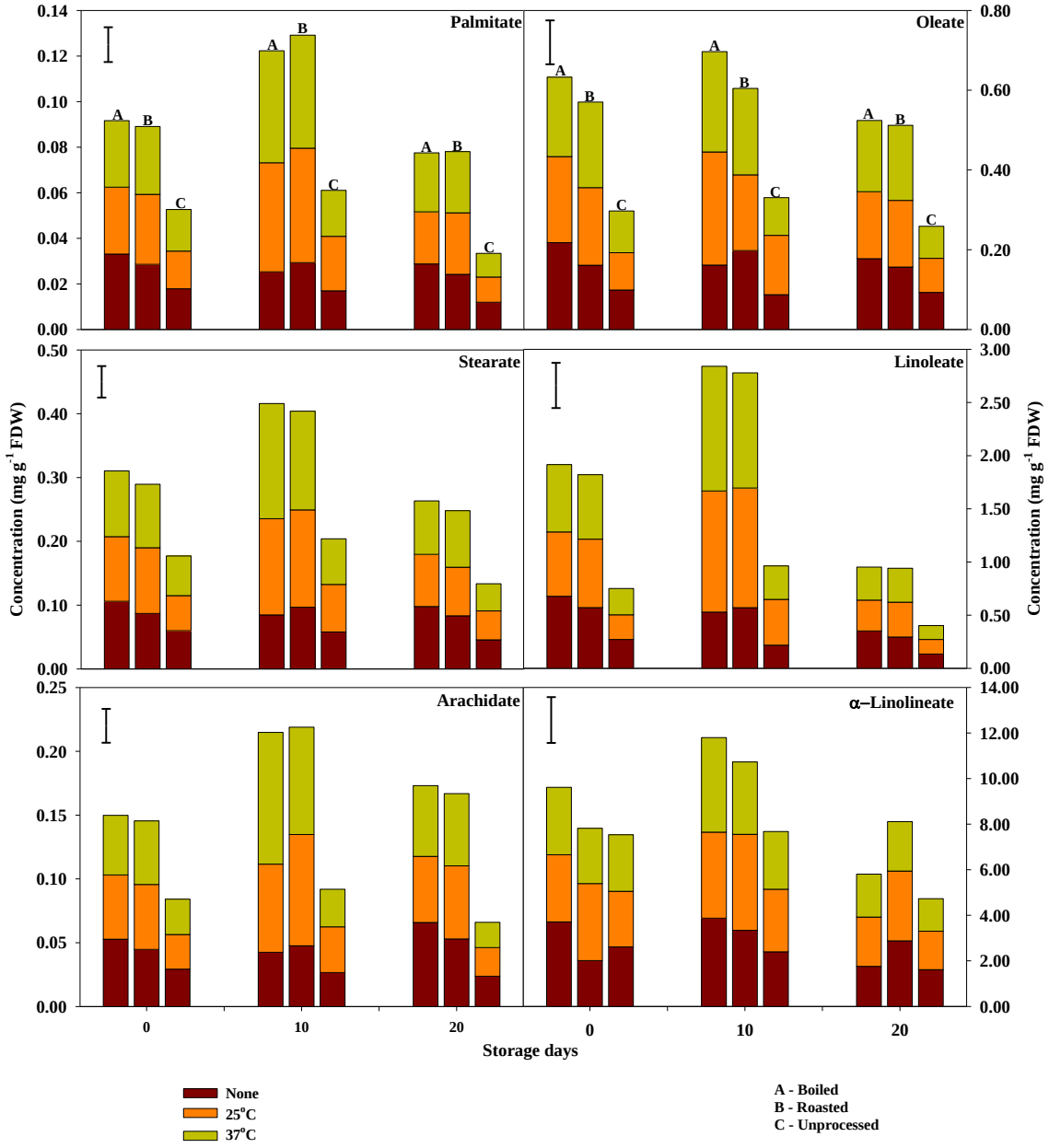



**Figure 18** Interaction effects of cold storage duration, treatment and retail storage temperature on concentrations of individual fatty acids at 95 % confidence interval after 3 days retail storage simulation. The Least Significant Difference (LSD) bars are shown to indicate any differences between factors. The *p* values are also shown in appendices D, tables D9.4.1.1 – D9.4.1.6 Values are considered significant at $p < 0.05$.

**Table 13** Main effects of cold storage (5 °C) durations, processing methods, maturity stages, and retail storage temperatures on concentrations of individual fatty acids after 7 days of retail storage simulation.

| Compound | Cold storage duration (days) | | | Maturity stage | | | Processing methods | | | | Retail storage temperatures °C | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 10 | LSD | Early | Late | LSD | Unprocessed | Boiled | Roasted | LSD | *None | 25 °C | 37 °C | LSD |
| Arachidate | 0.043 | 0.055 | 0.005 | 0.49 | 0.050 | 0.005 | 0.27 | 0.059 | 0.062 | 0.005 | 0.041 | 0.049 | 0.053 | 0.005 |
| Linoleate | 0.411 | 0.547 | 0.052 | 0.471 | 0.487 | 0.052 | 0.234 | 0.610 | 0.593 | 0.063 | 0.473 | 0.483 | 0.476 | 0.071 |
| Oleate | 0.163 | 0.169 | 0.013 | 0.165 | 0.167 | 0.013 | 0.102 | 0.206 | 0.189 | 0.016 | 0.154 | 0.173 | 0.165 | 0.018 |
| Stearate | 0.081 | 0.098 | 0.005 | 0.879 | 0.092 | 0.005 | 0.055 | 0.108 | 0.105 | 0.007 | 0.082 | 0.090 | 0.093 | 0.008 |
| Palmitate | 0.026 | 0.031 | 0.002 | 0.028 | 0.029 | 0.002 | 0.018 | 0.034 | 0.034 | 0.002 | 0.025 | 0.029 | 0.029 | 0.003 |
| Linolineate | 3.123 | 3.481 | 0.371 | 3.310 | 3.294 | 0.371 | 2.752 | 3.549 | 3.605 | 0.455 | 2.991 | 3.479 | 3.280 | 0.508 |

Units of values are in mg $g^{-1}$ FDW. * indicates nuts that were not subjected to retail storage conditions. Values are means (n = 3) with the LSD shown. The *p* values for individual fatty acids are shown in appendix D, tables D9.4.2.1 - D9.4.2.6. Values were considered significant at $p < 0.05$.

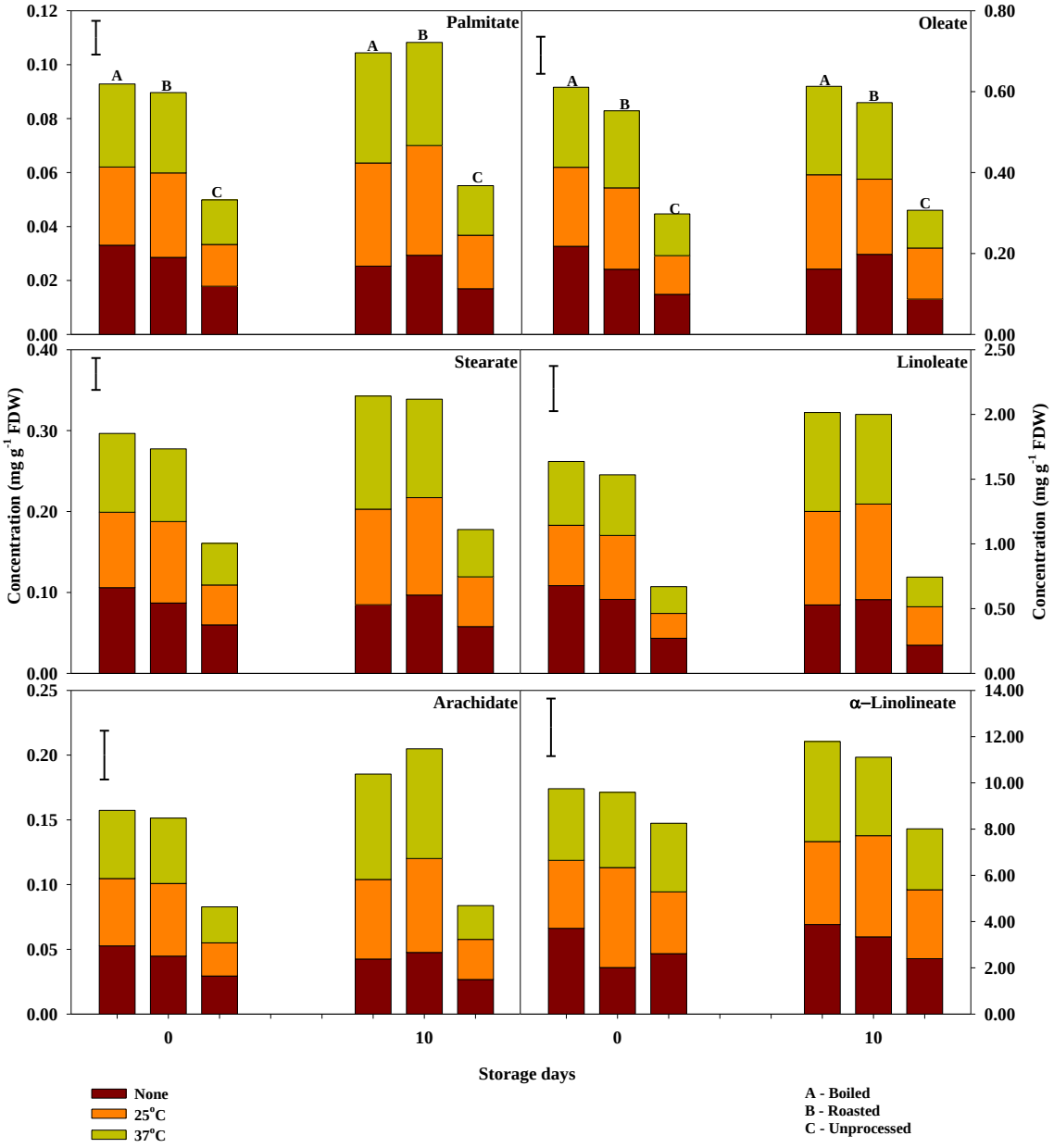



**Figure 19** Interaction effects of cold storage duration, treatment and retail storage temperature on concentrations of individual fatty acids at 95 % confidence interval after 7 days retail storage simulation. The Least Significant Difference (LSD) bars are shown to indicate any differences between factors. The *p* values are also shown in appendix D, tables D9.4.2.1 - D9.4.2.6. Values are considered significant at $p < 0.05$.

## 4.6 Discussion

### *4.6.1 Fatty acid quantification and identification in sample oil*

So far, this is the first study to assess fatty acid composition and the impact of postharvest processing (boiling and roasting) and storage on the fatty acid profile of African walnut harvested in Nigeria. The fatty acid profile of African walnut (Nigeria) contains mainly three saturated and three unsaturated (poly- and mono- unsaturated) fatty acids with α-linolenic acid being the most abundant. These findings were similar to those of Tchiegang, Kapseu & Parmentier (2001) who also reported the presence of the same saturated and unsaturated fatty acids in African walnuts harvested in Cameroon. Although Tchiegang, Kapseu & Parmentier (2001), used a shorter capillary column with different packing, they noted that the most abundant fatty acid was the conjugated C18:3 - α-linolenic acid (70 %) followed by C18:2 (linoleic acid), and C18:1 (oleic acid) fatty acids. These findings suggest a strong similarity between the oil of nuts grown in Nigeria and Cameroon despite environmental possible influences on the plants. The three most abundant fatty acids in the profile belong to the group of polyunsaturated - PUFA (α-linolenic and linoleic acids) and monounsaturated - MUFA (stearic acid) fatty acids. The profile is similar to those of walnuts (*Juglans regia*) which contains PUFA, especially linoleic acid, as the predominant fatty acids (Feldman, 2002; Griel & Kris-Etherton, 2006; Venkatachalam & Sathe, 2006); however, it differs from those of almonds, hazelnuts, macademia, pecan, cashew, and pistachio nuts, which contain mainly monounsaturated fatty acids (Ros & Mataix, 2006) These group of fatty acids (linoleic and α-linolenic) are known as essential fatty acids and have been shown to help reduce the risk factors of type 2 diabetes, cardiovascular and coronary heard diseases by lowering total cholesterol and low density lipoproteins cholesterol (LDL-C) while increasing high density lipoprotein – cholesterol (Hu & Manson, 2012; Shoji et al. 2013; Virtanen, Mursu, Voutilainen, Uusitupa & Tuomainen, 2014). They have also been shown to improve the lipid profile of healthy individuals (Jeppesen, Schiller & Schulze, 2013). It was demonstrated by Melariri, Campbell, Etusim & Smith,(2012) that both α-linolenic and linoleic free fatty acids inhibited growth of *P. berghei* by 70 and 64 %, respectively. They further reported a 96 % *P. berghei* growth inhibition when both compounds were used in a known combination, during an *in vivo* mice-model experiment. This antimalarial activity may justify why the African walnut decoctions are used in ethnobotanical medicine to treat

malaria. The bioavailability and other biological behaviour of these essential fatty acids from rich plant sources such as African walnut, need to be further investigated in order to compare them with those from animal sources. This is important as plant sources may be more readily available and affordable than animal sources.

### *4.6.2 Effects of all experimental factors on the concentrations of the fatty acid profile of African walnut.*

A decline in the concentration of fatty acids, especially α-linolenic acid, was generally observed when nuts were stored for 20 days at 5 °C. This decline may be as a result of degradation following probable hydrolytic, enzymatic, and oxidative reactions on the free fatty acids in the stored nut over time. Similar studies by Ghirardello, Contessa, Valentini, Zeppa, Rolle, Gerbi & Botta, (2013) also reported a decline, although non-significant, in linoleic and α-linolenic fatty acid content of hazelnut after long cold storage (4 °C; 0 – 8 months). However, long cold storage of nuts such as almond was observed to have no negative effect on fatty acid profile (Kazantzis, Nanos & Stavroulakis, 2003). The increases in the concentrations of fatty acids of processed African walnut samples after 10 days cold storage could be attributed to breakdown of conjugated lipids such as glycerophospholipids, phospholipids and glycolipids making them available as free fatty acids. It may also be that the expression and subsequent extraction of these fatty acids were improved as the nuts grew older. Furthermore, it has also been hypothesized that possible structural degradation resulting from cell wall degrading enzyme activities such as cellulases may cause liberation of fatty acids as age sets in (Meyer & Terry, 2008; Platt & Thomson, 1992).

General retail processing methods of boiling and roasting had a main effect of increasing the fatty acid concentrations of African walnut. This finding agrees with the report of Vaidya & Eun. (2013), and Budryn, Nebesny, Żyżelewicz, Oracz, Miśkiewicz & Rosicka-Kaczmarek,(2012). Vaidya and co-workers found increases, though non-significant, in the concentrations of palmitic, linoleic and oleic acid of oil extracted from walnut (*Juglans sinensis* Dode) roasted at 150°C for 15 minutes after 9 days storage in the dark at – 80 °C while Budryn, Budryn, Nebesny, Żyżelewicz, Oracz, Miśkiewicz & Rosicka-Kaczmarek, (2012) observed increases on roasted Robusta seed oil which indicated significant increases on the PUFAs oil when nuts were roasted at 210°C for 60

minutes. This non-significant increase in Vaidya & Eun (2013) study could be as a result of short roasting time employed; unlike in this study where African walnut was roasted for 60 minutes at 200°C. More so, application of dry (roasting) and wet (boiling) heat at the temperatures seen in this study may have caused a breakdown of conjugated fatty acids making them more extractible. According to Blée (2002) and Walley, Kliebenstein, Bostock & Dehesh (2013), environmental stress conditions induce breakdown, alterations or modifications in the fluidity of plant cell membranes, which are lipid-rich structures. Ohlrogge (1995) noted that palmitic, oleic, linoleic and α-linolenic fatty acids are core plant cell membrane lipids, hence, they would be most affected by the processing and storage treatments used in this study. Cold storage (5°C), retail storage at 25°C and 37°C, and postharvest treatments of roasting and boiling at high temperatures (200°C and 100°C , respectively) are stress conditions capable of disrupting the cell membranes of African walnut leading to the breakdown and release of these structural fatty acids. Once released, they became more readily available for extraction.

## 4.7 Conclusions

The findings in this study show that the African walnut oil contains essential fatty acids in the profile which are generally sought for in edible oils. The abundance of mono and poly – unsaturated fatty acids in the profile suggest that the use of the oil in daily cooking may convey potential nutritional benefits on consumers. The study further suggest that the current retail postharvest practice of boiling, roasting and storage between 25 and 37 °C improves the fatty acid profile of African walnut. However, cold storage (up to 10 days) which also had a positive influence on the profile, is not a common practice by retailers and may be challenging to undertake in a large scale giving the instability of electricity and high cost of running fuel generated power in west and central Africa. Consequently, it creates an opportunity for export business. Currently, African walnut is basically produced at subsistence level in Nigeria, however, considering the need for increased affordable plant sources of healthy edible oil in developing nations, industrial scale production of African walnut is herein recommended.

# 5 Chapter five: Impact of postharvest processing on the fungal population contaminating African walnut (*Tetracarpidium conophorum* Mull. Arg) shells at different maturity stages and potential mycotoxigenic implications

## 5.1 Introduction

Nuts (tree nuts) have gained lots of popularity in the recent times following several research reports highlighting the role of nuts and the nutrients they contain in combating chronic diseases such as high blood pressure, coronary heart disorders, diabetes and cancer (Hoevenaar-Blom, Nooyens, Kromhout, Spijkerman, Beulens, Van der Schouw & Verschuren, 2012). However, like most plant food products, they are susceptible to fungal attack and mycotoxin contamination (Molyneux, Mahoney, Kim & Campbell, 2007; Tolosa, Font, Mañes & Ferrer, 2013). The mycotoxins are secondary metabolites that are produced as a result of temporary restriction to normal growth processes resulting in removal of intermediate products through the secondary metabolic pathway which is a very specific biosynthetic pathway (Paterson & Lima, 2010). These fungal and mycotoxin contaminations are generally influenced by variable intrinsic and extrinsic factors such as water activity/moisture content, pH, temperature changes, storage conditions, relative humidity, aerobic conditions, and poor harvesting practices that inflict mechanical damages on the nuts (Khodavaisy, Maleki, Hossainzade, Rezai, Ahmadi, Validi & Ghahramani, 2012; Rodrigues, Venâncio & Lima, 2012). The resultant effect of this biological and environmental interplay on nut food is spoilage which consequently affects the nutritional and sensory qualities through discoloration, rot and off-flavour production (Paterson and Lima, 2011; Rodrigues, Venâncio & Lima, 2012). The health impact in humans when ingested could either be carcinogenic, genotoxic, teratogenic, nephrotoxic, hepatotoxic or immunotoxic (Salem & Ahmad, 2010). The economic impact on export values cannot be over-emphasized especially on farmers who depend on its production and sales for survival. The normal postharvest practices involved in African walnut processing, sales and storage which includes temperature range of 20 - 37°C, relative humidity of 75 - 80 % and open market sales, which take 1 – 7 days, raises strong concerns about the potential fungal infestation and contamination hence necessitating this study.

## 5.2 Materials and methods

### *5.2.1 Nut shells*

Pieces of boiled, roasted and unprocessed nut shells were obtained in replicates of three from both non-stored and retail stored (7 days) groups following the experiment conducted in chapter 3

### *5.2.2 Media preparation, shell culture and incubation*

Two different media purchased from Oxoid limited Basingstoke, England, were used; Malt extract agar (MEA) CM0059, for growth of fungi under high water activity conditions (non-xerophillic) and Dichloran glycerol 18 (DG18) CM0729, media for the growth of xerophilic fungi. These were prepared following the manufacturer's instructions. Both solutions were sterilized by autoclaving with Classic Prestige Medical autoclave (Meadow scientific Ltd. United Kingdom) at 115°C for 10 minutes and then allowed to cool to about 50°C. The cool media were then manually poured in disposable steril plates in a micro flow hood under sterile conditions.

Pieces of nut shells obtained in replicates of three from both non-stored and stored groups were placed in culture plates containing MEA and DG18 agar. Each plate had 4 pieces of shells placed in it. Two out of each 4 shells were placed with the outer side embedded in the media while the other two faced upward. Plates were incubated at 25°C and examined after 7 days. The number of contaminated pieces was recorded such that when 1 out of 24 shell pieces was contaminated it corresponded to 4.17%. The plates were then inspected visually for fungal growth in each shell pieces with the aid of a Motic BA210A trinocular compound microscope (GT Vision, UK). Subsequently, these were sub-cultured on Malt Extract agar (MEA) slants to obtain pure isolates which were kept at 4 °C until identified and tested for toxin production capacity. Pure isolates were prepared on glass slides following staining with lactic acid and lacto-phenol blue and identified to the genus level by observing macroscopic features of cultures such as the colony diameter, colour of conidia on reverse plate, colony texture and exudates (Diba, Rezaie & Mahmoudi, 2007). Microscopic morphological and physiological characteristics such as the conidial head, stipes, colour, conidia size, shape and roughness, vesicles shape and seriation were also assessed using a Motic BA210A trinocular compound microscope

(GT Vision, UK) with magnifications 100× and 400×. These features were compared with those of published standards (Giorni, Magan, Pietri, Bertuzzi & Battilani, 2007; Rodrigues & Menezes, 2005).

### *5.2.3 Mycotoxin determination in randomly selected fungal isolates*

As this was a preliminary investigation, the focus was to simply determine if potential mycotoxigenic isolates from the sample could produce toxins; thus randomly selected Isolates (20), belonging to potentially aflatoxigenic group were sub-cultured in replicates using Oxoid LP0021 Yeast Extract Sucrose (YES) agar prepared by following manufacturer's instructions. Plates were centrally inoculated and incubated at 25°C for 10 days. Agar plugs (8 pieces; 0.5 – 0.7 g each) were taken from the culture plates, processed and analysed for AFG1, AFB1, AFG2, and AFB2 after trifluoroacetic acid pre-column derivatization, using High Performance Liquid Chromatography (HPLC) - Fluorescence detector (FLD), Agilent 1200 series equipped with binary pump system and degasser. Separation of both mixed standard of AFB1, AFG1, AFB2, and AFG2 (50 ng/mL each) obtained from Sigma-Aldrich, UK) and samples were obtained using Agilent Poroshell 120, $C_{18}$ (2.7µm, 100 mm × 4.6 mm) column and following the procedures described by Medina & Magan (2012). In brief, the mobile phase was methanol/water/acetonitrile (30:60:10) with a flow rate of 1 ml/min (isocratic mode). FLD detection was at 360 nm and 440 nm excitation and emission wavelengths, respectively. Sample injection was 20 µl and the total run time was 11 min.

Ochratoxin A (OTA) analysis was carried out by extracting 8 agar plugs (0.5 – 0.7 g each) taken from randomly selected potential OTA-producing isolates (30) and placed in 2.0 mL Eppendorf tubes. Methanol (1mL) HPLC grade was added to the tubes and placed in an electric shaker at room temperature (15 °C) for 1 h. The tubes were then centrifuged at 13, 000 revolutions per minute for 10 minutes and then filtered through 0.22 µm filter disk into Agilent vials (2 mL). HPLC analysis was carried out following the method described by Sultan, Magan and Medina (2014) with slight modifications. In brief, separation was obtained for both OTA standard 250 ng/mL (Sigma-Aldrich, UK) and samples using $C_{18}$ Agilent Zorbax Eclipse Plus (4.6 × 150 mm, 3.5 µm particle size) with parameters set as follows: column temperature 25°C, FLD excitation and emission wavelengths 333nm and 460nm, respectively. Mobile phase consisted of

water/acetonitrile (41:57, v/v) and 2% acetic acid in an isocratic flow rate of 1 mL/min and injection volume of 10 µL with a total runtime of 10 minutes.

The limits of detection and limits of quantification for aflatoxin and ochratoxin A standards were determined as 3 and 10 times standard deviations, respectively (the lowest concentration of each standard in the calibration curve was injected 10 times. The means and standard deviations were calculated for each standard).

Table 14 **Summary of shell culture and incubation plan** performed in replicates (Rep). Pieces of nut shells obtained in replicates of three from both non-stored and stored groups were placed in culture plates containing MEA and DG18 agar. Each replicate culture plate was further duplicated.

| **Nut shell replicates (brown/green)** | **Media** | **Culture duplicates (D)** |
|---|---|---|
| Rep1 | MEA | D1 |
| | | D2 |
| | DG18 | D1 |
| | | D2 |
| Rep2 | MEA | D1 |
| | | D2 |
| | DG18 | D1 |
| | | D2 |
| Rep.3 | MEA | D1 |
| | | D2 |
| | DG18 | D1 |
| | | D2 |

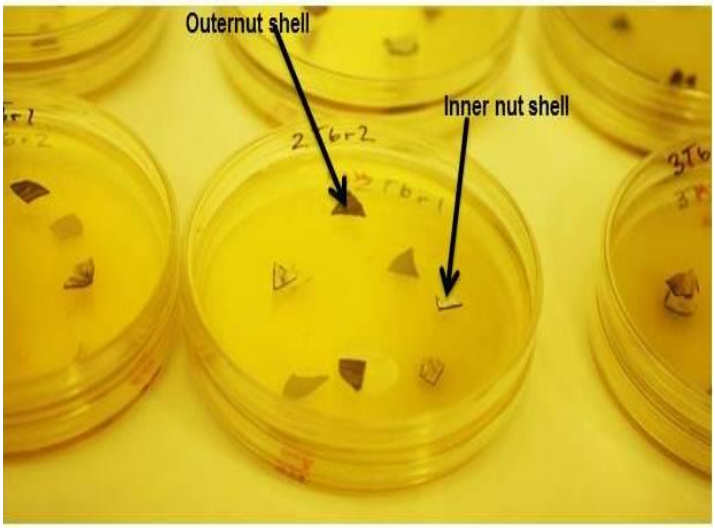



**Figure 20** Nutshell placement in media plates. Each plate had 4 pieces of shells placed in it. Two out of each 4 shells were placed with the outer side embedded in the media while the other two faced upward,

### *5.2.4 Statistical Analysis*

Statistical analysis was performed using the package JMP® 9 (SAS Institute Inc. 2010. Cary, NC, USA). Data sets were tested for normality using the Shapiro-Wilk test. All data failed normality test and variable transformation was performed in order to improve normality or homogenize the variances with no success. Due to non-normality, the data analyses were performed using non-parametric tests (Kruskal-Wallis rank sum test) for testing whether distributions across factor levels were centred at the same location. When only two levels were studied Levene's test was used to investigate homogeneity of variances and the proper *t*-test assuming or without assuming equal variances was performed accordingly.

## 5.3 Results

### *5.3.1 Effect of incubation time, processing and storage temperature on percentage fungal contamination and on potential mycotoxigenic fungal species*

Fungal growth was detected in all shell pieces derived from non-stored nuts (NSN) and cultured in MEA and DG 18 media (Figure 19), except for those nut shells derived from early maturity nuts which had been roasted. The contamination levels of cultured nut shells (Figure 21) in MEA were 100 % in unprocessed nut shells, at both early and late maturity stages. Boiling reduced contamination to58 % in shells from nuts at early maturity. Both boiling and roasting decreased contamination level to 20 % in shells from nuts at late maturity. In DG18 media cultures, unprocessed nut shells (UAW) at early and late maturity stages had contamination levels of 70 and 100 %, respectively. Boiling also reduced contamination in shell pieces from nuts at early maturity to 38 %. However, roasting and boiling gave the highest reduction to 7 % of shell pieces derived from nuts at late maturity. Generally, boiling and roasting significantly reduced contamination levels ($p < 0.05$) of cultured nut shell pieces from African walnuts belonging to NSN group. At storage temperatures of 25°C and 37°C, the level of contamination of nut shell pieces (Figure 21) cultured in MEA and DG 18 media for UAW, BAW at early and late maturity and RAW (early maturity only) were 100 % while those of RAW (late maturity) at 25°C and 37°C showed MEA: 70 % and 40 %, DG 18: 80 % and 55 %, respectively. Incubating both boiled and roasted African walnuts in general, increased contamination levels ($p < 0.05$) of cultured shell pieces irrespective of the stage of maturity.

*Aspergillus* section *Nigri, Aspergillus flavus/parasiticus, Fusarium* spp. and *Penicillium* spp. were the four main potentially mycotoxigenic fungal groups frequently isolated, although *Rhizopus* spp. and *Botyritis* spp. were also isolated. The influence of storage temperature and different processing treatments on these fungal groups is shown in Table 13. Within the non-stored nuts at early and late maturity, *Fusarium* spp. was the main contaminant (21 – 67 %) of shell pieces of unprocessed African walnut in both MEA and DG18 cultures. *Aspergillus* section *Nigri* dominated the contamination of cultured shell pieces of boiled African walnut (29 %) in both MEA and DG18 cultures. Other co-contaminants of BAW were *Penicillium* spp. (16 - 25 %), Fusarium spp. (4 - 8 %) and *Aspergillus flavus/parasiticus* (4 - 25 %).

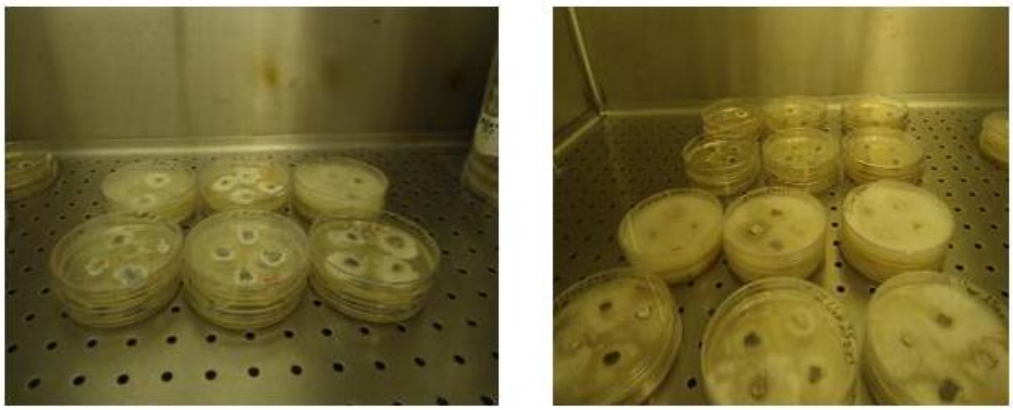


**Figure 21** Fungal contamination of plates containing nut shell samples cultures in DG18 (left) and MEA (right) growth media, respectively. Contamination around one piece of shell is considered as 25 % while that around the four shell pieces is considered as 100 % contamination.

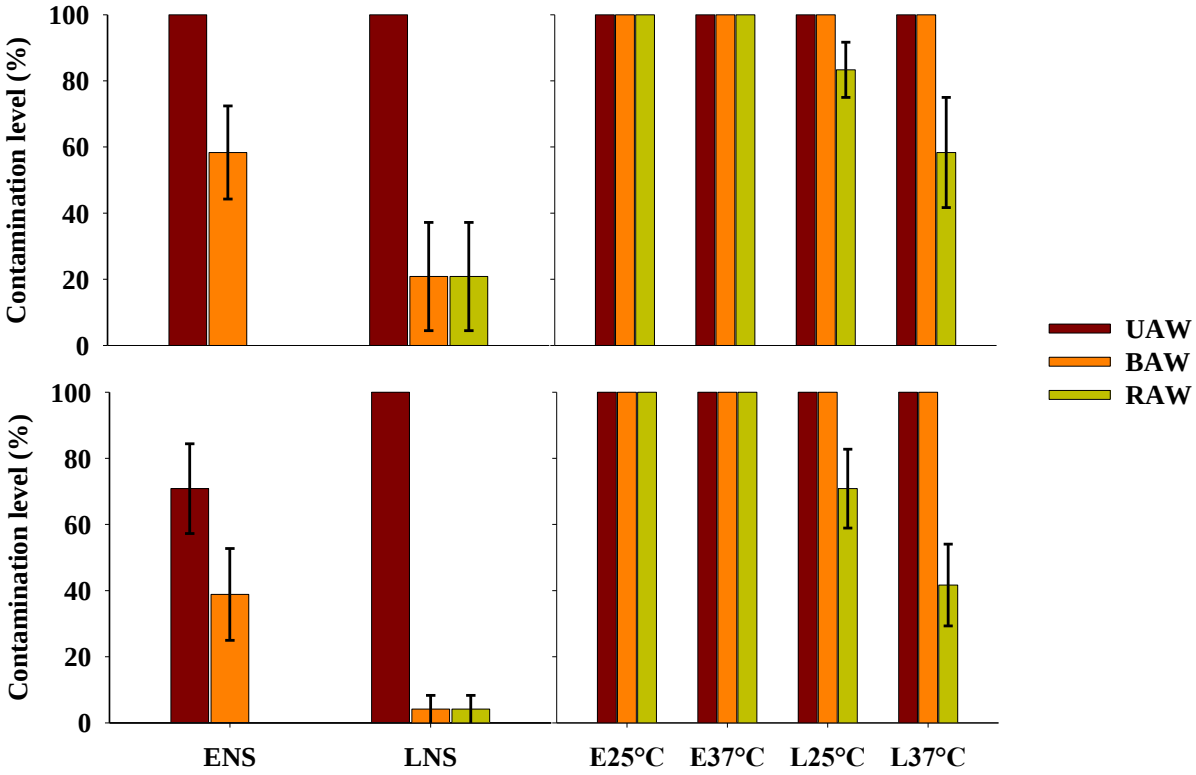



Figure 22 Effect of temperature and incubation time on the percentage of contamination of nut shell pieces in MEA (upper panel) and DG18 (lower panel) culture plates. ENS: Early maturity, non-stored; LNS: Late maturity, non-stored; E: Early maturity; L: Late maturity. UAW, BAW and RAW are unprocessed, boiled and roasted African walnut, respectively. Standard error bars are shown.

**Table 15** Mean percentage contamination of potentially mycotoxigenic species found in MEA and DG 18 agar cultures of pieces of nut shells obtained from treated African walnut at early and late maturity incubated for 7 days at different temperatures under controlled relative humidity of 78 %.

| Storage temperature | Maturity stage | Treatment | Mycotoxigenic fungi present | Mean contamination (%) | |
|---|---|---|---|---|---|
| | | | | MEA | DG 18 |
| NSN | Early | UAW | *Fusarium* spp. | 20 ± 10 | 25 ± 17 |
| | | | *Aspergillus* section *Nigri* | | 4 ± 4 |
| | | | *Penicillum* spp. | | 8 ± 5 |
| | | BAW | *Aspergillus flavus/parasiticus* | 4 ± 4 | 25 ± 17 |
| | | | *Fusarium* spp. | 8 ± 5 | |
| | | | *Aspergillus* section *Nigri* | 29 ± 15 | 29 ± 16 |
| | | | *Penicillum* spp. | 17 ± 5 | 25 ± 17 |
| | | RAW | Nil | | |
| | Late | UAW | *Fusarium* spp. | 67 ± 21 | 50 ± 22 |
| | | BAW | *Fusarium* spp. | Nil | 4 ± 4 |
| | | RAW | *Aspergillus flavus/parasiticus* | 4 ± 4 | 4 ± 4 |
| 25°C | Early | UAW | *Aspergillus* section *Nigri* | Nil | 25 ± 11 |
| | | | *Penicillum* spp. | Nil | 43 ± 15 |

| | | | | | |
|---|---|---|---|---|---|
| | | BAW | *Penicillum* spp. | 12 ± 8 | 17 ± 8 |
| | | RAW | *Fusarium* spp. | 8 ± 5 | Nil |
| | | | *Penicillum* spp. | Nil | 12 ± 5 |
| | Late | UAW | *Fusarium* spp. | 33 ± 21 | 58 ± 20 |
| | | | *Penicillum* spp. | 8 ± 5 | 21 ± 13 |
| | | BAW | *Fusarium* spp. | 33 ± 21 | 33 ± 21 |
| | | | *Penicillum* spp. | 8 ± 5 | 100 ± 0 |
| | | | *Aspergillus flavus/parasiticus* | Nil | 33 ± 21 |
| | | RAW | *Penicillum* spp. | 8 ± 5 | 12 ± 8 |
| | | | *Aspergillus* section *Nigri* | Nil | 42 ± 14 |
| 37°C | Early | UAW | *Aspergillus flavus/parasiticus* | 33 ± 21 | Nil |
| | | | *Aspergillus* section *Nigri* | 100 ± 0 | 62 ± 12 |
| | | BAW | *Aspergillus flavus/parasiticus* | 62 ± 20 | 79 ± 16 |
| | | | *Aspergillus* section *Nigri* | 54 ± 11 | 96 ± 4 |
| | | RAW | *Aspergillus* section *Nigri* | 4 ± 4 | 29 ± 11 |
| | | | *Aspergillus flavus/parasiticus* | Nil | 100 ± 0 |
| | Late | UAW | *Fusarium* spp. | 25 ± 16 | Nil |
| | | | *Aspergillus* section *Nigri* | 25 ± 16 | Nil |
| | | BAW | Nil | | |
| | | RAW | Nil | | |

Values are *mean ± standard error*. NSN: Non stored nuts, UAW: Unprocessed African Walnut, BAW: Boiled African Walnut, RAW: Roasted African Walnut. Nil: absence of mycotoxigenic spp.

### *5.3.2 Mycotoxin analysis of randomly selected fungal isolates*

Results from the HPLC-FLD analysis of 20 isolates from nut shell pieces cultures, randomly picked within each treatment group (unprocessed 2, boiled 10, and roasted 8 isolates) for aflatoxin (AF) analysis, showed chromatogram peaks indicating the presence of aflatoxins G1 (AFG1), B1 (AFB1), G2 (AFG2) and B2 (AFB2) (Figure 23). Aflatoxins B1 and B2 were produced together by 80 % of the isolates while the 4 toxin types (AF - G1, B1, G2, and B2) were produced together by 50 % of the isolates. The concentration levels were as follows: AFG1 10 – 25,225; AFB1 10 – 16, 789; AFG2 5 - 873and AGB2 2 – 1, 947 ng/g plug weights. Although potentially ochratoxin A (OTA) producing fungal groups (*Aspergillus* section *Nigri* and *Penicillium* spp.) were isolated (23 isolates), there was no detection of OTA during HPLC-FLD analysis.

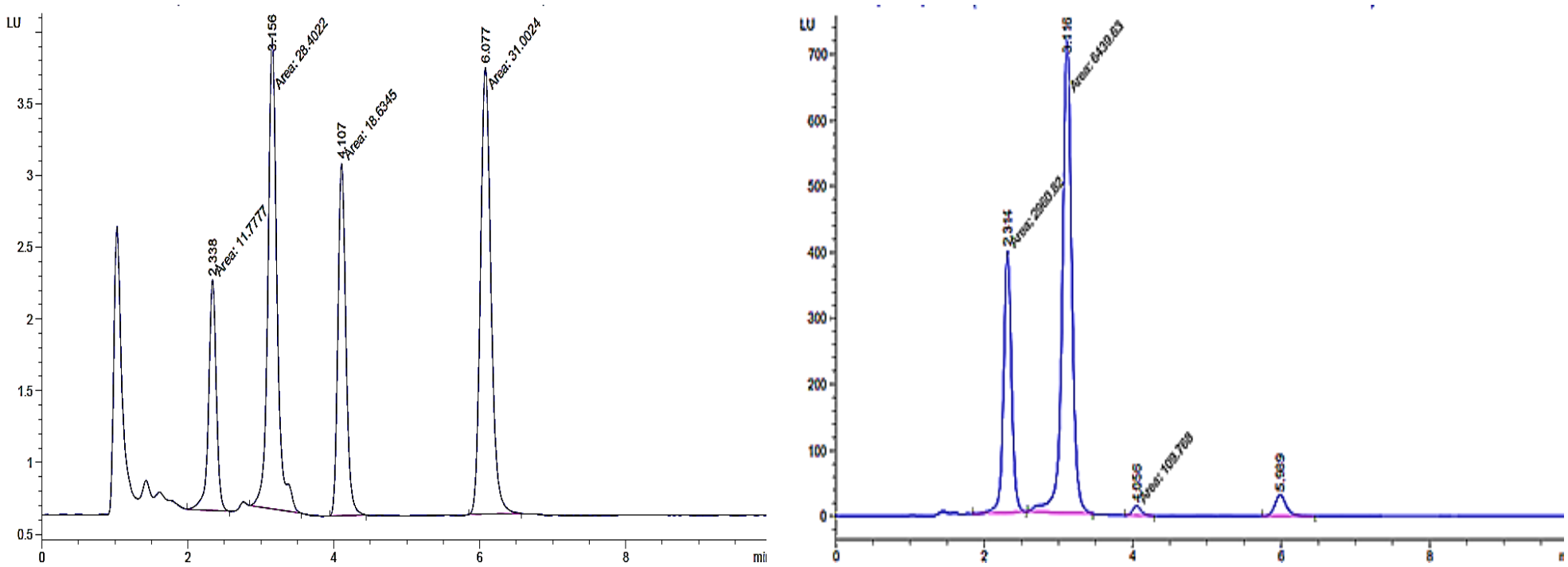



**Figure 23** HPLC-FLD representative chromatograms obtained after the analysis of a mixed standard containing 50 ng/mL each of AFG1, AFB1, AFG2, AFB2 ( Left) and extracts from culture plates of fungi obtained from African walnut shell cultures (Right). Wavelengths: $\lambda_{ex}$ 360 nm and $\lambda_{em}$ 440 nm.

**Table 16** Levels of mycotoxin production by randomly selected fungal isolates from African walnut shell samples cultured in MEA and DG 18 media.

| Sample Treatment | Aflatoxin G1 | | Aflatoxin B1 | | Aflatoxin G2 | | AflatoxinB2 | |
|---|---|---|---|---|---|---|---|---|
| | Peak area | Concentration ng/g plug | Peak area | Concentration ng/g plug | Peak area | Concentration ng/g plug | Peak area | Concentration ng/g plug |
| UAW | 7212 | 20606 | 13417 | 15471 | 373 | 625 | 1522 | 1382 |
| " | 4751 | 19913 | 9141 | 13632 | 204 | 688 | 600 | 1107 |
| BAW | 6399 | 25225 | 11013 | 16280 | 311 | 873 | 1204 | 1949 |
| " | 4328 | 18274 | 7665 | 14118 | 178 | 467 | 534 | 776 |
| " | 5869 | 20945 | 11453 | 16789 | 189 | 525 | 767 | 1379 |
| " | 7192 | 24864 | 13888 | 14075 | 366 | 766 | 1655 | 1369 |
| " | 3260 | 14863 | 9013 | 15109 | 126 | 368 | 511 | 849 |
| " | 0 | 0 | 0 | 0 | 12 | 5 | 0 | 0 |
| " | 7 | 17 | 1822 | 622 | 4 | 7 | 0 | 0 |
| " | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| " | 7 | 26 | 13 | 20 | 4 | 9 | 1 | 2 |
| " | 76 | 143 | 193 | 91 | 0 | 0 | 9 | 5 |
| RAW | 8 | 27 | 21 | 30 | 2 | 6 | 0 | 0 |
| " | 4 | 512 | 9 | 276 | 14 | 43 | 0 | 0 |
| " | 3 | 10 | 6 | 10 | 9 | 24 | 0 | 0 |
| " | 19 | 300 | 65 | 229 | 0 | 0 | 0 | 0 |
| " | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| " | 810 | 1042 | 1288 | 663 | 17 | 12 | 50 | 21 |
| " | 260 | 436 | 17 | 100 | 0 | 0 | 25 | 16 |
| " | 0 | 0 | 18 | 27 | 0 | 0 | 0 | 0 |
| Concentration Range | | 10 – 25,225 | | 10 – 16, 789 | | 5 - 873 | | 2 – 1, 947 |

Values are means of 3 replicate readings. UAW, BAW, and RAW = unprocessed, boiled, and roasted African walnut, respectively. The value '0' signify 'no detection of toxin'.

## 5.4 Discussion

### *5.4.1 Effect of incubation time, processing and temperature on percentage fungal contamination and on potential mycotoxigenic fungal species*

This is the first study quantifying the impact of traditional postharvest practices on the fungal population contaminating African walnut shell and the associated potential mycotoxigenic risk. In order to assess the general and xerophilic fungal contamination of the nuts both MEA and DG18 culture media were used. Cultures (7 days) of unprocessed African walnut shell pieces at early maturity in the "non-stored nuts" group had 100% contamination. Both non-xerophilic and xerophilic fungal species were found to make up the mycobiota of African walnut before any processing was carried out. Results further showed that fungal growth was visible in all cultured nutshell pieces when unprocessed nutshells from both maturity stages, were incubated at 25°C and 37°C. Roasting resulted in a complete reduction of fungal contamination especially in non-stored nuts at early maturity. This can be attributed to exposure to high temperature conditions (200°C, 1 hour). The spore deposits on the nut shells may have been destroyed before they were cultured. According to Tolosa, Font, Mañes & Ferrer (2013), constant heating at high temperatures > 50°C are capable of damaging or killing fungal spores. To a lesser extent, boiling also reduced fungal contamination. This could be as a result of differences in the heating conditions. Boiling involves wet heating at 100°C, while roasting was achieved at 200°C by dry heating. Wet heating increases final moisture content, thus creating a more enabling environment for any surviving spore to grow.

Boiling and roasting were able to modify the mycobiota of the nut shells. The fungi population showed shifts toward more heat resistant genera like *Aspergillus* and *Penicillium* one week after treatment and storage under conditions that mimic that of the normal open market (Table 15). When incubated at 37°C there was an obvious increase in both *Aspergillus* section *Nigri* and the group of *Aspergillus flavus*/*A. parasiticus*. This suggests that storage of both boiled and roasted nuts under open market conditions (37°C and 78 % relative humidity) may potentially favour aflatoxin, and OTA contamination of the nut shells as these fungal species are associated with these toxins.

*Aspergillus* section *Nigri, Aspergillus flavus/parasiticus, Fusarium* spp. and *Penicillium* spp. were frequently isolated from cultures of African walnut shell pieces. These fungi are known to commonly occur in nuts (Baquião, De Oliveira, Reis, Zorzete, Atayde & Corrêa, 2013; Rodrigues, Venâncio & Lima, 2012). Their prevalence have been confirmed in Brazil nuts (Silva, Venâncio, Freitas-Silva & Venâncio, 2011), pistachio nuts (Khodavaisy, Maleki, Hossainzade, Rezai, Ahmadi, Validi & Ghahramani, 2012), chestnut and walnut (Jubeen, Bhatti, Maqbool & Mehboob,2012), peanuts (Adjou, Dahouenon-Ahoussi & Soumanou, 2012), almonds (Riba, Matmoura, Mokrane, Mathieu & Sabaou, 2013) and hazelnuts (Baltaci, İlyasoğlu & Cavrar, 2012). They are also known to have persistent growth under a variety of storage conditions; thus, reports have shown that *Aspergillus* spp. thrives better in lower moisture environments while *Fusarium* spp. is classified as field fungi (Hedayati, Kaboli & Mayahi, 2010; Mylona & Magan, 2011) The predominant genera isolated from nut shell pieces derived from non-stored unprocessed African walnut shell pieces was *Fusarium* spp. Similarly, Baquião, De Oliveira, Reis, Zorzete, Atayde & Corrêa (2012), reported *Fusarium* spp. as the most abundant fungal species colonizing unprocessed Brazil nut shells. Different prevalence of *Fusarium* spp. between early and late maturity stage nuts could be as a result of physiological changes in the composition of the pods. While the pods at early maturity are harder and intact, pods at late maturity were softer and appeared to exhibit tissue degradation which could lead to increased moisture and more neutral pH levels. These conditions may have encouraged rapid growth of *Fusarium* spp. isolates (Marin, Sanchis & Magan, 1995). In same vein, storage of the nuts at 25°C and 37°C with controlled relative humidity of 78 % resulted in a higher isolation frequency of *Aspergillus* spp. and *Penicillium* spp. in nut shell samples from boiled and roasted African walnuts. These genera are relatively resistant to high temperature as shown by Nawar (2008). He demonstrated that *Aspergillus* spp. from Saudi Arabian pistachio nuts had had an optimum growth temperature range between 20°C and 35°C and that similar responses were observed at increased relative humidity range of 70 – 100 %. The potential high growth rate of these mycotoxigenic fungi during postharvest storage under open market conditions (both high temperatures and relative humidity) could result in additional health risk derived from spore inhalation and handling of contaminated nuts. This is because

most farmers, retailers and consumers make direct contacts with these nuts while they remain unshelled.

### *5.4.2 Mycotoxin analysis*

The assessment of the fungal population highlighted the importance of potential mycotoxigenic genera and hence mycotoxigenic isolates. An appropriate media (YES) was then used in order to evaluate the potential risk of toxin contamination. A high proportion of aflatoxigenic isolates were identified. Aflatoxins $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ were detected in *in vitro* cultures of isolates corresponding to potential aflatoxigenic genera of which the majority was seen mainly in shells from stored boiled samples. This suggests that boiling may favour production of aflatoxins over other treatments. Aflatoxins are known to be produced mainly by *Aspergillus* spp. with section *Flavi* and A. *parasiticus* being the most frequent (Yu, Payne, Campbell, Guo, Cleveland, Roberts, Nierman,2008). The production of these toxins has been reported to occur during storage conditions although they could be produced in the field, harvest and processing times (Cotty & Jaime-Garcia, 2007). So far, there are no previous reports on the presence of these mycotoxins in African walnut. The high levels of aflatoxins (ranging 0.11 – 32,200 ng/g of agar) produced by isolates in this study were lower than those produced by aflatoxigenic *Aspergillus* section *Flavi* chemotype isolates (0.50 – 2000 µg/g of agar) from pistachio, hazelnuts, and peanuts in the studies by Riba, Matmoura, Mokrane, Mathieu & Sabaou,(2013). This could result from the differences in the culture media used. This study utilized YES agar while Riba, Matmoura, Mokrane, Mathieu & Sabaou, (2013) used coconut agar media (CAM) which supports better aflatoxin production. Very high concentrations were also observed by Baquião, De Oliveira, Reis, Zorzete, Atayde & Corrêa (2012) for AFs with the highest range in $AFB_1$ (3.5 – 410, 922 ng/g CAM) produced by *Aspergillus flavus* isolates from Brazil nut shells. Apart from the differences in the culture media, Baquião, De Oliveira, Reis, Zorzete, Atayde & Corrêa (2012) incubated their cultures for 14 days before extraction and analysis while our study had only 10 days incubation. This extended incubation time may have allowed for increased toxin production. In addition, more aflatoxigenic isolates (128) were examined by Baquião, De Oliveira, Reis, Zorzete, Atayde & Corrêa (2012) which may have given room for discovery of very high aflatoxin-producing isolates unlike in this study where fewer isolates were examined.

The maximum concentrations observed for $AFB_1$ and $AFG_1$ were 16, 789 ng/g and 25, 225 ng/g of agar, respectively. The high concentration level of toxins produced by isolates in pure cultures suggests that the mycotoxigenic isolates from African walnut shells are strong toxin producers. This further emphasizes the risk that may be associated with the consumption of the nuts without proper postharvest handling as these toxins may potentially be produced *in situ*. However, further studies are required to support this hypothesis. Currently, the concentration limits set by the Commission of the European communities - CEC - are 2 µg/kg or 2 ng/g for $AFB_1$ and 4 µg/kg or 4 ng/g for total aflatoxins (CEC, 2006; Dimanche, 2001;) while legal limits for $AFB_1$ and total Aflatoxins have also been set for specific nuts such as hazelnuts at 5 ng/g ($AFB_1$) and 10 ng/g (total Aflatoxins). Production of toxins by more than 85 % of aflatoxigenic isolates from the nut shell samples compares favourably with those reported on peanuts where 70 - 100 % isolates were seen to be predominantly aflatoxigenic (Nakai, De Oliveira, Gonçalez, Fonseca, Ortega & Corrêa, 2008). Research findings have constantly confirmed that $AFB_1$ and $AFB_2$ are produced together by *Aspergillus flavus* while *Aspergillus parasiticus* produces $AFG_1$ and $AFG_2$ in addition to the former (Baquiao, De Oliveira, Reis, Zorzete, Atayde & Corrêa,2012). This trend was also observed in this study, although $AFB_2$ and $AFB_1$ in few occasions were below detection limits.

Although potentially OTA-producing species were isolated, after growth under conducive conditions in YES medium (10 days incubation at 25°C), no OTA was detected after analysis. Previous research reports have shown that the presence of potentially toxigenic fungi does not guarantee that the substrate will be contaminated with toxins. This result on OTA could also entail that the *Aspergillus* section *Nigri* and *Penicillium* spp. isolates found in this study do not belong to the toxigenic group that produce OTA. Only a few species of *Aspergillus* section *Nigri* (*A carbonarius*, *A. tubingensis* and low percentage of strains of *A. niger*) can produce OTA (Abarca, Bragulat & Cabañes, 2014; Cabañes & Bragulat, 2008; Medina, Mateo, López-Ocaña, Valle-Algarra & Jiménez, 2005) while only two OTA producers are known in the genus *Penicillium* (*P. verrucosum* and *P. nordicum*). Following the results in this study, it can be suggested that aflatoxin is of greater risk in African walnut than OTA although both toxin-producing isolates were present after storage. This is supported by the fact that there was a high proportion of toxigenic isolates among the *Aspergillus flavus*/*parasiticus* group unlike the *Aspergillus*

section *Nigri* and *Penicillium* spp. groups which had no toxigenic OTA-producing isolates.

## 5.5 Conclusion

In the light of the results obtained thus far, it can be concluded that mycotoxigenic fungi were found growing on pieces of nutshells of African walnut upon *in vitro* analysis using the appropriate culture media and incubation conditions. However, it is interesting to note that harvesting nut at early maturity, storing them at 5°C and subsequently processing them by roasting will prevent fungi/mycotoxin contamination while boiling will greatly reduce the fungal load on nut shells for up to seven days. Results have also shown that current postharvest handling of unprocessed African walnuts at both early and late maturity, mainly by exposure to high temperatures (25 – 37 °C) and high relative humidity (78%) during open market sales, may favour growth and development of mycotoxigenic fungi species on the nuts. This poses a great potential health risk that is yet to be considered by policy makers in Nigeria and other African nations where the nut is consumed. This risk is heightened as a result of humans making direct contact with the nut shells during handling right from harvest to consumption. More so, inhalation of the contaminating spores could result in mycotic diseases. Aflatoxins are known to have adverse effects on human health ranging from aflatoxicosis to cancer. Currently, $AFB_1$ is classified as group 1 human carcinogen by the International Agency for Research on Cancer as reported by Shen & Ong, (1996). Furthermore, they lead to very serious economic losses (Baltaci, İlyasoğlu & Cavrar, 2013). Although the results are clearly stated, it cannot be conclusively said that the African walnut kernel is contaminated with mycotoxins as further research needs to be conducted to confirm the hypothesis.

# 6 Chapter six: Phenolic content analysis, and cytotoxicity study of African walnut extracts on lung cancer (A549) cell line

## 6.1 Introduction

The choice to analyse the phenolic components in the African walnut samples, was based on the understanding that many plant materials – fruits, leaves, nuts, roots - contain phenolics compounds. Some of these phenolics have also been reported to have shown antioxidant and anti-carcinogenic activities in biological systems. Dietary flavonoids in combination with other components such as vitamins have been reported to act on reactive oxygen species (ROS), and cell signal transduction pathways related to cellular proliferation, apoptosis and angiogenesis (Yao, Xu, Shi & Zhang, 2011). Tree nuts such as walnut (*Juglans regia* L.) have been reported to contain phenolic acids such as chlorogenic, caffeic, ferulic, sinapic, syringic, ellagic, *p*-coumaric acids and juglone (Colaric, Veberic, Solar, Hudina & Stampar, 2005) while others like Brazil nuts, pistachio and hazel nuts have been shown to contain phenolics from the groups of flavonoids and phenolic acids as well (John & Shahidi, 2010; Schmitzer, Slatnar, Veberic, Stampar & Solar, 2011). As African walnut is well consumed in Nigeria and mixed in different decoctions for management of cancer diseases in ethnobotanical medicine, it is necessary to lay a foundation for the understanding and quantification of the phenolic content of the nut. There is also no research report detailing neither on the type nor amount of phenolic contents of African walnut, thus, the total phenolics of unprocessed, boiled and toasted samples using the Folin-Ciocalteu assay was determined to give understanding of how common processing methods for the nut influence these polyphenols. Also, a qualitative analysis was carried out using HPLC-DAD equipment, in order to separate the potential individual phenolic components and set the platform for their identification.

The extracts of African walnut as mentioned earlier, are often mixed with herbal decoctions and given to patients with varied types of chronic diseases including cancer (Onwuli, Brown & Ozoani, 2014; Akomolafe, Oboh, Akindahunsi & Afolayan, 2015). However, there is neither clear specification on the type of cancer African walnut extracts are applied to, nor information on the stage of the cancer at which the mixture containing

the nut extract should be taken. This raises the question as to whether the extract is used for curative purposes, which implies that the cancer cells become the primary target for the extract bioactives, or simply as a nutritional supplement to boost the immune system of the patient. This is a preliminary investigation to understand the effect of four selected solvent extracts on human lung cancer cells (A549).

## 6.2 Materials and methods

### *6.2.1 Determination of total phenolics in African walnut using Folin-Ciocalteu assay.*

The plant material used were unprocessed, boiled and roasted African walnut samples that were not subjected to retail storage during the general experiment (see chapter 3.2.2). Defatted samples only were used for the estimation of total phenolics while both defatted and non-defatted samples were used in the HPLC analysis. Defatting was done as described in chapter 4.2.1 using the cold hexane extraction method. All chemicals were of analytical grade and purchased from Fisher Scientific, United Kingdom except otherwise stated.

Freeze-dried samples of unprocessed, boiled and toasted African walnut (300 mg) were extracted two times using ethanol : water (80:20, HPLC grade, v/v) solvent (6 mL). The mixtures were placed in water bath with shaker at 70 °C for 2 h with intermittent vortexing every 30 min. Samples were allowed to cool and then centrifuged at 13000 rounds per minute, for 10 min before filtering with a 0.2 µm Milipore filter (StarLab, UK). Total phenolic content was determined following the Folin-Ciocalteu's method which is based on the reduction of a phosphotungstate-phosphomolybdate complex by phenolic compounds to a blue reaction product (Singleton & Rossi, 1965). In brief, 20 µL each of gallic acid calibration standard (Fisher Scientific, UK) or African walnut sample filtrate was mixed with HPLC water (3.2 mL) and placed in 4.5 mL polystryrene cuvettes (10 mm pathlength), respectively. Folin-Ciocalteu's reagent - 200 µL (Sigma-Aldrich, UK) and 600 µL of 0.2 mg/mL sodium carbonate solution (Fisher Scientific, UK) were added thereafter. The mix was vortexed and incubated in the dark at room temperature (15 °C) for 2 h. Absorbance of samples and standards were measured at 765 nm against a blank (contained all other chemicals except sample filtrate and gallic acid standard) using a Camspec M501 UV/Vis spectrophotometer (Camspec Ltd, Cambs. UK). Total phenolic content was estimated against a standard curve of gallic acid. Results were expressed in

milligrams of gallic acid equivalent (GAE) per gram freeze-dried weight of sample (Giné Bordonaba & Terry, 2008).

### *6.2.2 Extraction of phenolic compounds, standard mix and mobile phase solvents for HPLC-DAD analysis*

Different methods of extraction of phenolic compounds from tree nuts have been reported. In order to determine the best extraction solvent for African walnut, dry defatted samples and non-defatted samples (2.00 ± 0.05 mg, respectively) were placed (in triplicates) in 7mL bijou bottles and mixed with 3 mL of extraction solvent. Each extraction solvent contained one organic solvent (ethanol, methanol, ethyl acetate, or acetone) mixed with HPLC water and hydrochloric acid (HCl) in the ratio of 70:29.5:0.5 v/v (organic solvent: HPLC water: HCl). Each extraction mix was vortexed and placed in a shaking water bath set at 35 °C for 30 minutes. The bottles were vortexed intermittently (15 min) during extraction for proper mixing of samples with solvent, and allowed to cool at room temperature (15 °C). Cooled samples were filtered using 0.2 µm Millipore filter (StarLab, UK) into amber HPLC Agilent vials and kept at - 40 °C until analysed.

A mix of the eleven standards (vanillic, caffeic, chlorogenic, syringic, *p*-coumaric, ferulic, sinapic acids; catechin, quercetin-3-glucoside, quercetin-3-rutinoside and hesperidin) obtained from Fisher Scientific, UK, was prepared by pipetting 50 µL of each stock solution (2mg/mL) into Agilent HPLC vial and making it up to 1 mL with 99 % methanol to give a final concentration of 0.1 mg/mL.

Four different mobile phase solvents were used for HPLC analysis of phenolic compounds. Mobile phase solvents (A, B, and C) consisted of methanol, HPLC water and formic acid in varying percentages (A: 95 % HPLC water, 5 % methanol, 5 % formic acid; B: 88 % water, 12 % methanol, 5 % formic acid; C: 20 % HPLC water, 80 % methanol, 5 % formic acid) except D which was made up of 100 % methanol and 5 % formic acid.

### *6.2.3 High performance liquid chromatography – diode array detector analysis and fraction collection of sample peaks*

Extracted samples (defatted and non-defatted), together with the phenolic standard mix, were analysed for presence of phenolic compounds using Agilent 1200 HPLC-DAD

machine equipped with quartenary pump, degasser and multiple wavelength detector. Separation of compounds was achieved by injecting 10 µL of sample extracts at 4 °C, using the autosampler, into Agilent Zorbax Eclipse XBD-C18 column of 250 × 4.6 mm diameter and 5 µm particle size fitted with an Agilent XBD-C18 12.5 × 4.6 mm guard column. Elution and peak identification were according to Alamar & Terry (2013) with slight modifications. In brief, the flow rate for the mobile phase solvents (A, B, C, and D) was 1 mL/min with a maximum column pressure at 400 bar. The elution program timetable started with 100% A and remained isocratic for 7 min followed by a linear gradient from 0 to 50 % B between 7- 9 min, then to 100 % at 20 min. It remained isocratic until 40 min when it changed to 75 % B:25 % C. It it remained linear until 72 % B:28 % C at 50 min and then B:C (50:50) at 52 min. Finally, at 54 min, D was 100 %, isocratic until 58 min stop time. Sample peak signals were detected at 280, 332, 355 and 520 nm (Tomas-Barberan, Gil, Cremin, Waterhouse, Hess-pierce & Kader, 2001).

### *6.2.4 Sequential extraction of African walnut*

Freeze-dried, ground defatted samples of African walnut from assessment day zero (early maturity) were sequentially extracted (Figure 24). Unprocessed, boiled, and roasted samples (3.0 g , respectively) were placed in 15 mL centrifuge tubes respectively, and extracted two times with 100 % methanol (10 mL). The tubes were placed in a water bath (35 °C) for 30 min and then filtered using Buchner funnel and flask. The solvent was evaporated using MiVAC quattro Genevac centrifuge vacuum evaporator system (Suffolk, UK) and the extracts were re-dissolved in HPLC water (5 mL). The water solution was back extracted sequentially using the following solvent in order of increasing polarity; a 50/50 diethyl ether: ethyl acetate mix (diEt:EA, v/v), dichloromethane (DCM, v/v) and n-butanol (n-but, v/v). Each extract was concentrated by vacuum evaporation and kept at – 40 °C until required.

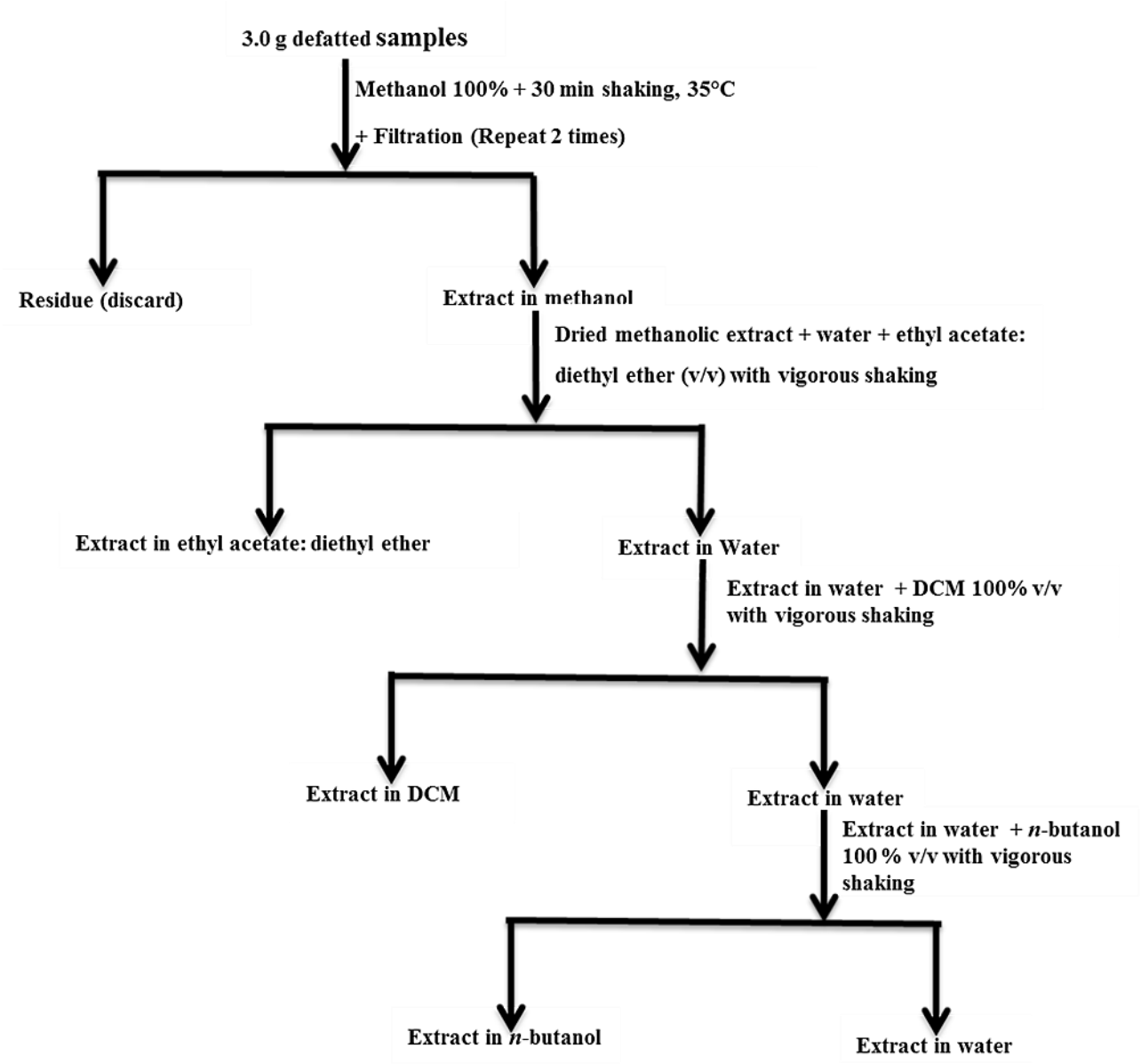



**Figure 24** Schematic representation of sequential extraction of unprocessed, boiled and roasted samples of African walnut. Solvents were used in increasing order of polarity.

### *6.2.5 Cell viability experiment*

All cell culture experiments were carried out under aseptic conditions in a containment level 2 hood.

Considering that this is a preliminary experiment, only A549 cell line was used in the experiment as it was readily available. Frozen A549 cell suspension (Sigma-Aldrich, UK) was thawed in a water bath at 37 °C. The contents were transferred into Nunclon T75 flask containing pre-warmed 12 mL of media made up of Dulbecco modified Eagles media (DMEM/F12) plus foetal calf serum (FCS 10%) and penicillin/streptomycin mix (pen-strep 1%) all supplied by GIBCO Invitrogen – Life Technologies, UK. The flask was placed in humidified (99% relative humidity) cell culture incubator at 37 °C and 5 % $CO_2$ and incubated for 48 h with media change after 24 h. Cell confluence was observed using a light microscope. At 90% confluence, the cells were prepared for counting by removing the media using a sterile disposable pipette and electronic pipettor, washing the cells with pre-warmed PBS (5 mL) and detaching cells by adding trypsin –EDTA mix (3 mL) and incubating (37 °C and 5 % $CO_2$)for 3 min. The trypsin-EDTA was deactivated by adding 5 mL culture media (DMEM/F12 + FCS 10 % + pen-strep 1 %).

The cell mix was transferred into 50 mL falcon tube and 20 µL of the cell was mixed with equal amount of Trypan blue (GIBCO Invitrogen – Life Technologies UK) in an Eppendorf tube and 10 µL of the mixture was placed in the counting slide. The cells were counted using a Countess© counting machine. Live cells were recorded ($a \times 10^b$ cells/mL) and used in calculations to determine seeding density.

#### *6.2.5.1 Seeding cells into 96 well plates*

Two cell seeding densities were used in this study, 5, 000 and 10, 000 cells/well.

Cell-media mix (200 µL) was placed into 60 inner wells in each of 6 nunclon flat bottom 96-well plates assigned for seeding density of 10, 000 cells/well. The outer boundary wells were filled with 200 µL of PBS to help maintain humidity within the closed plates. The seeding density of 5,000 cells/well was achieved by placing 100 µL of culture media (only) first in the wells and then adding 100 µL of the cell-media mix in another set of 6 plates. The plates were incubated (humidified, 37 °C and 5 % $CO_2$) for 48 h.

*6.2.5.2 Extracts and control preparation*

The extracts (diEt: EA, DCM, n-but, and water) of unprocessed (UAW), boiled (BAW) and roasted (RAW) African walnut were dissolved in phosphate buffered saline (PBS) and stock solutions of 10 mg/mL (for diEt:EA and DCM) and 1 mg/mL (for n-butanol and water) obtained. The solutions were filtered using a 0.22 µm pore size sterile filter (Fisher Scientific, UK) into 15 mL centrifuge tubes (Fisher Scientific, UK). The stock solutions were serially diluted with culture media to give concentrations of 1, 10, 100 and 500 µg/mL (media-extract mix).

The negative control was made up of PBS + media (50:50), while positive control was made up of 5-fluorouracil (5-FU) – a common chemotherapeutic drug obtained from Sigma-Aldrich, UK. The drug-media positive control solution was prepared by placing 10 mg of 5-FU in 2 mL Eppendorf tube containing warm media. The mix was warmed in a water bath at 37 °C for 15 - 20 min with intermittent vortexing after every 5 min until a clear solution was formed. The drug-media mix was serially diluted to give concentrations of 1, 10 and 100 µg/mL.

*6.2.5.3 Application of extracts-media and 5-FU drug-media mix to seeded incubated cells*

After 48 h of incubation of cells, the media in the plates were removed by aspiration using a multichannel pipette and washed with pre-warmed PBS. Extract-media and 5-FU-media mix (200 µL) of the different dilutions were added into specified wells in triplicates (3 replicates per concentration) using micro pipettes and tips (Figure 25, Figure 26 and Figure 27). The entire experiment was repeated 4 times. The plates containing cells plus extract at different concentrations were replaced back in the incubator and left for 48 h. The cytotoxicity of the extracts were analysed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay.

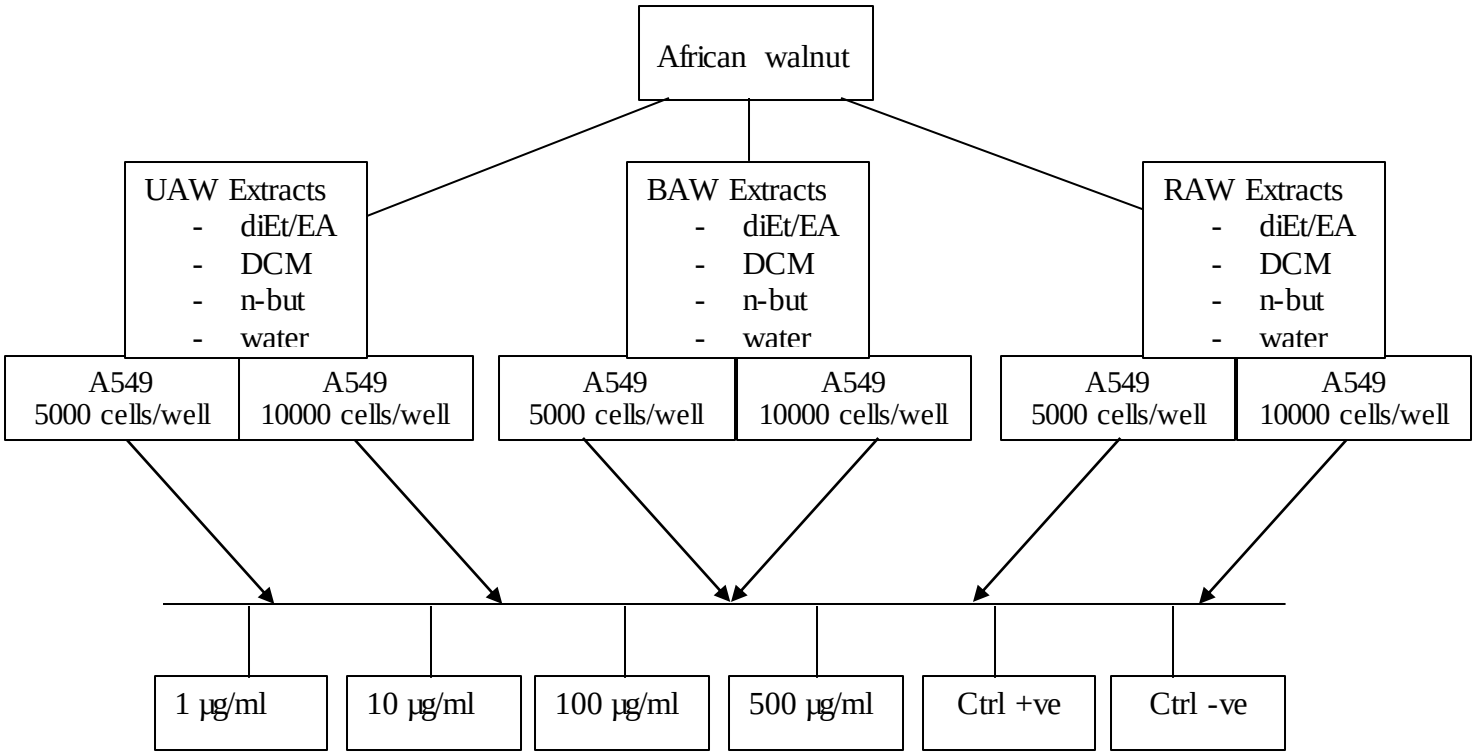



**Figure 25** Summary chart of experimental design for application of extracts to seeded A549 cells at different cell densities.

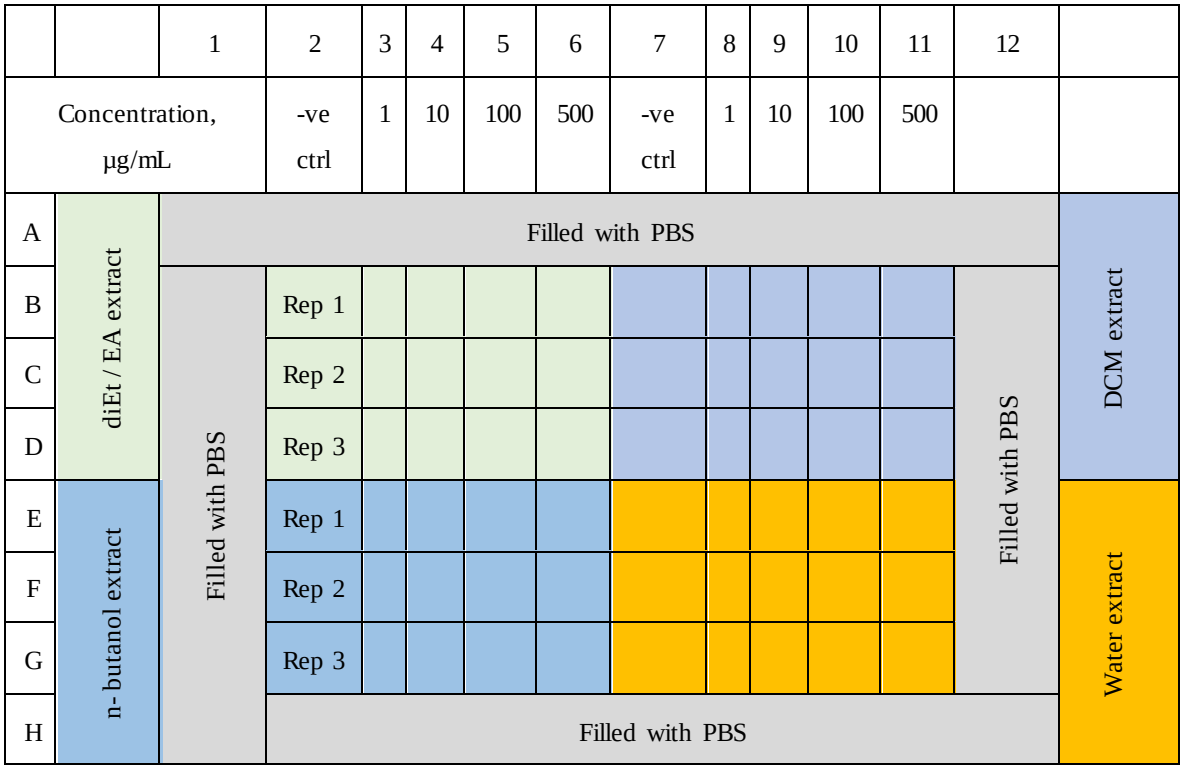



**Figure 26** Outlay of extract application to seeded A549 cells in 96 well plates.

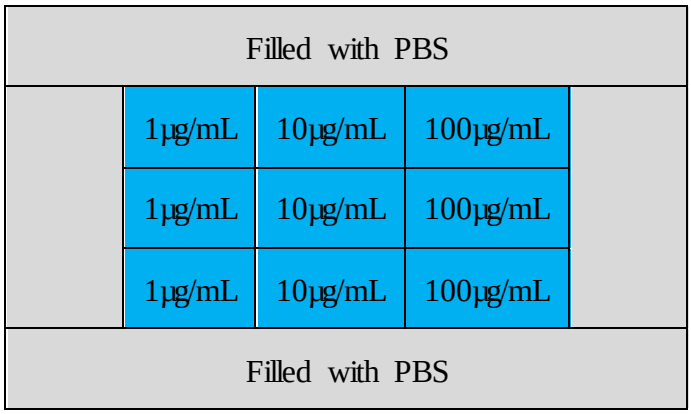



**Figure 27** Outlay for application of positive control (5-fluorouracil) on A549 cells

*6.2.5.4 MTT assay*

MTT is a colorimetric assay and it measures the rate of reduction of the yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide by mitochondrial succinate dehydrogenase. The compound is absorbed by live cells and it is then reduced to an insoluble formazan (dark purple) product in the mitochondria. The product is solubilized by an organic solvent such as isopropanol. Since the compound is only absorbed by metabolically active cells, cell viability is therefore, determined by the level of activity or optical density obtained using appropriate colorimetric machine e.g. Varioscan plate reader.

MTT powder (Sigma-Aldrich, UK), 1 g was dissolved in PBS 40 mL to give a stock solution of 25 mg/mL. The solution was filtered with sterile microfilter (0.22 µm; Fisher Scientific, UK) in falcon tube (50 mL) within a microflow hood. MTT-Media mix was prepared by adding 1.2 mL of stock solution and making it up to 30 mL to give a working solution concentration of 1 mg/mL. Each tube containing MTT was covered with aluminium foil to shield it from light. The MTT-media mix (200 µL) was added into each well in the incubated plates containing cells and test extracts after the extract-media was removed and cells washed with PBS. The plates were then incubated (humidified – 99% RH, 37 °C and 5 % $CO_2$) for 1 h. The MTT-media mix was then removed and isopropanol (100 µL) containing 1 M HCl (8 %, v/v) was added to each well to dissolve the formazan. Each plate was covered with aluminium foil and placed in an orbital shaker (150 revolutions per min) for 5 min at room temperature (15 °C) to ensure adequate dissolution of the formazan. The absorbance/optical density (OD) was read at 540 nm using a Varioscan® micro-well plate reader. Viability (%) was calculated as [OD Sample/OD Control × 100%] (Marks, Belov, Davey, Davey & Kidman, 1992).

*6.2.5.5 Statistical analysis*

All experiments were performed in triplicates and repeated at least four times. Data analysis was performed using Genstat package for Windows (version 12.0) VSN International Ltd (Herts. UK). A one way analysis of variance (ANOVA) non-parametric test was performed after data was confirmed for normality using the Wilk test. The means were separated and the least significant differences of factorial interactions were determined at 95 % confidence interval ($p < 0.05$).

## 6.3 Results

### *6.3.1 Estimation of total phenolic content in African walnut*

The estimation of unprocessed, boiled and roasted defatted samples were standardised for their phenolic content. The calibration curve of gallic acid standard of known concentrations showed linearity in the range of 50 – 500 mg/mL concentration with a coefficient of determination ($R^2$) of 0.99. Unprocessed (20.79 ± 1.00 mg GAE/g FDW) samples showed the highest value for total phenolics while both boiling (9.90 ± 1.80 mg GAE/g FDW) and roasting (9.32 ± 2.70 mg GAE/g FDW) reduced the amount by more than 50 % when compared with unprocessed (Figure 28).

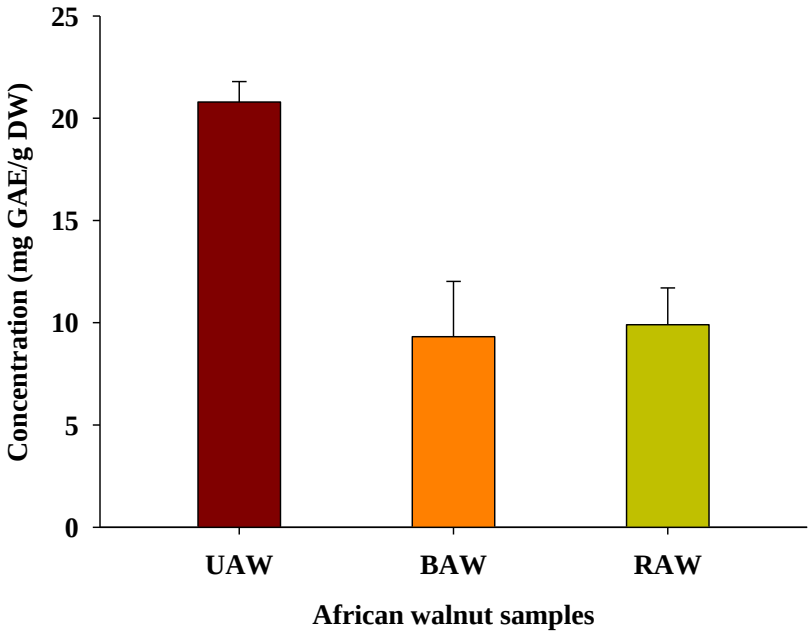



**Figure 28** Total phenolic content of defatted unprocessed (UAW, boiled (BAW) and roasted (RAW) African walnut sample. Values are mean ± SD of triplicate measurements

### *6.3.2 High performance liquid chromatography – diode array detection analysis*

The analysis of defatted and non-defatted samples of unprocessed, boiled and roasted African walnut respectively, using HPLC-DAD showed unambiguous separation of

compounds (Figure 29: Left). The chromatogram from samples extracted with methanol: water: HCl (70:29.5:0.5, v/v) showed better resolution of compounds than those of other solvent combination. The eleven phenolic standards were also separated (Figure 29) with the order of elution of compounds as shown in (Table 17). The chromatograms of defatted African walnut sample showed the elution of compounds with the maximum peak up to 70 mAU at 280 nm, less than 13 and 2 mAU at 332 and 355 nm, respectively (Figure 29). These sample maximum peaks at 280 nm, were lower than those of vanilic, caffeic, syringic, chlorogenic, p-coumaric and ferulic acid mixed standard equivalents but higher than those of catechin, sinapic acid, quercetin-3-rutinoside, quercetin-3-glucoside, and hesperidin, which had peak heights less than 70 mAU (Figure 29). More than 10 major compound peaks were detected at 280 nm in the sample while less than 8 major peaks were seen at 332 nm. Detection was least at 355 nm with maximum of 2 major peaks that were less than 2 mAU. There were no differences between the major compounds eluted in defatted and non defatted samples (Figure 30). Also, boiled, roasted and unprocessed samples, showed similar chromatograms (Figure 31). When eluted peaks were compared with the phenolic standards in terms of retention time, spectrum and area, no match was found for any of the sample peaks.

**Table 17** Order of elution of compounds in mix of phenolic standards detected at 280 nm wavelength.

| S/N | Compound | Retention time (mins) |
|---|---|---|
| 1 | Catechin | 9.339 |
| 2 | Vanillic | 12.888 |
| 3 | Caffeic acid | 13.160 |
| 4 | Chlorogenic | 13.788 |
| 5 | Syringic acid | 17.627 |
| 6 | *p*-Coumaric acid | 20.220 |
| 7 | Ferulic acid | 27.636 |
| 8 | Sinapic acid | 33.181 |
| 9 | Quercetin-3-rutinoside | 40.045 |
| 10 | Quercetin-3-glucoside | 41.075 |
| 11 | Hesperidin | 43.983 |

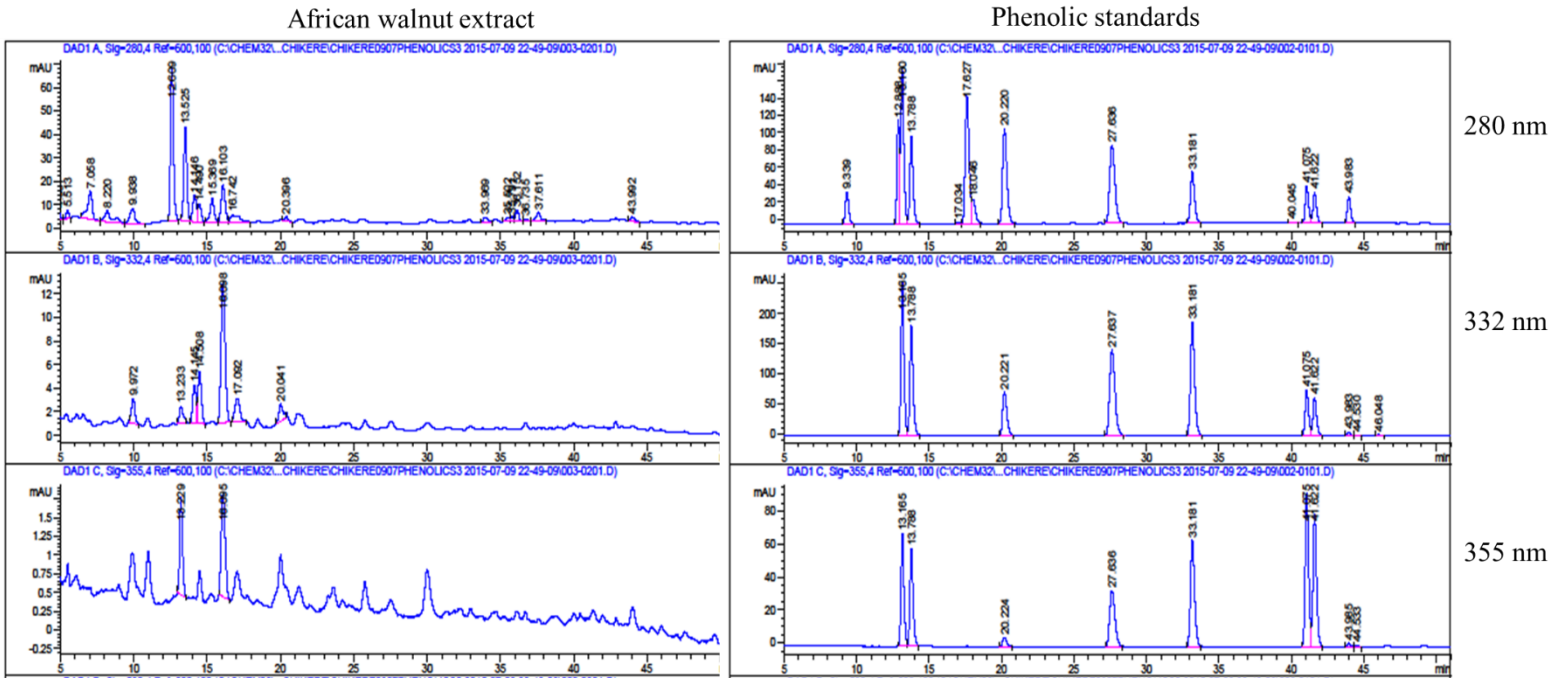
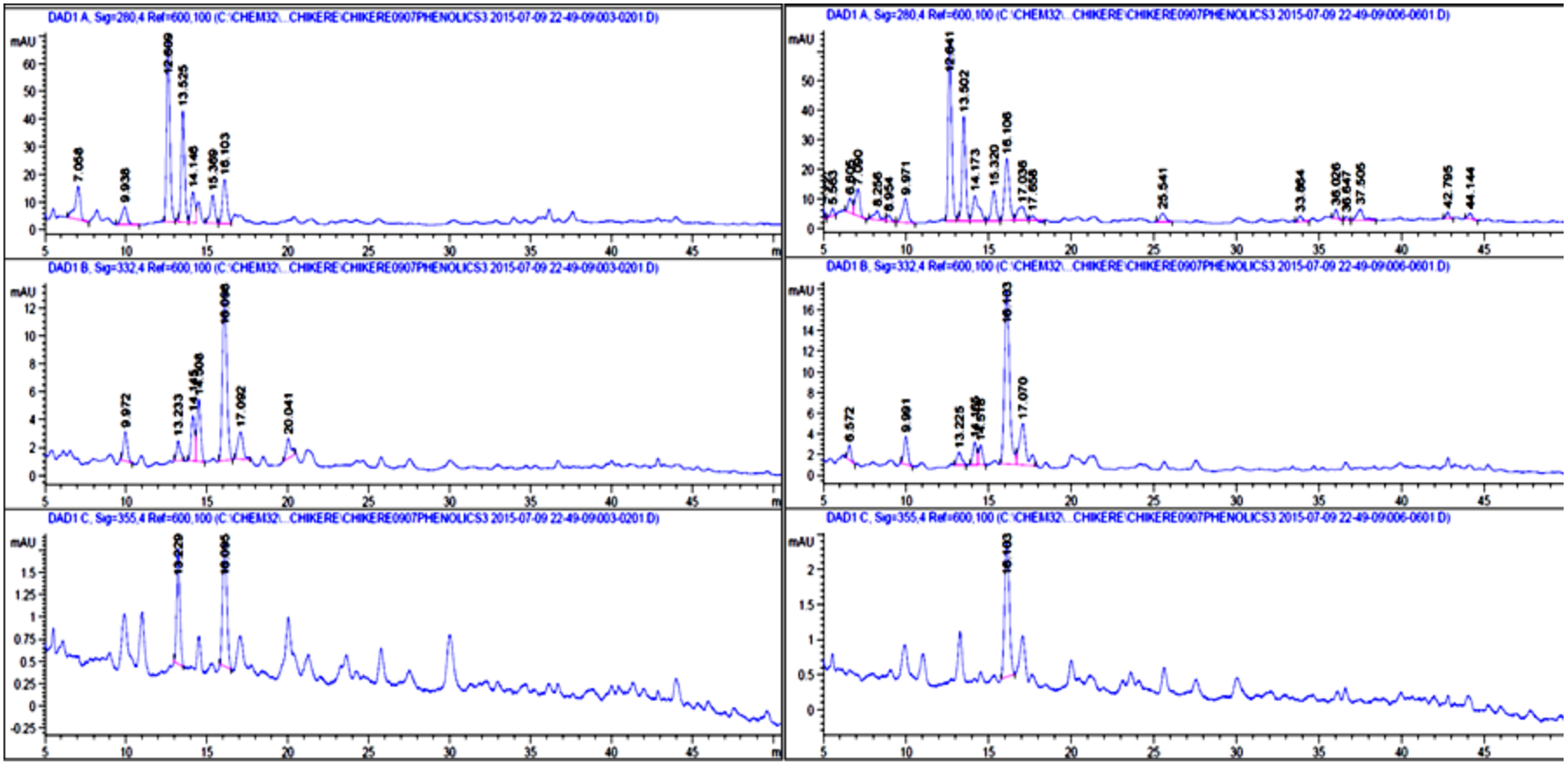



**Figure 29** Separation of potential phenolic compounds contained in unprocessed defatted African walnut extract (left), and mixed phenolic standards (0.1 mg/mL) containing eleven compounds (right). Detection was at 280 nm (upper level), 322 nm (middle), 355 nm (lower level).




**Defatted** **Non-defatted**

**Figure 30** Profile comparison of potential phenolic compounds in defatted and non-defatted African walnut methanolic extracts detected at 280nm (upper level), 332 nm (middle), and 355 nm (lower level).

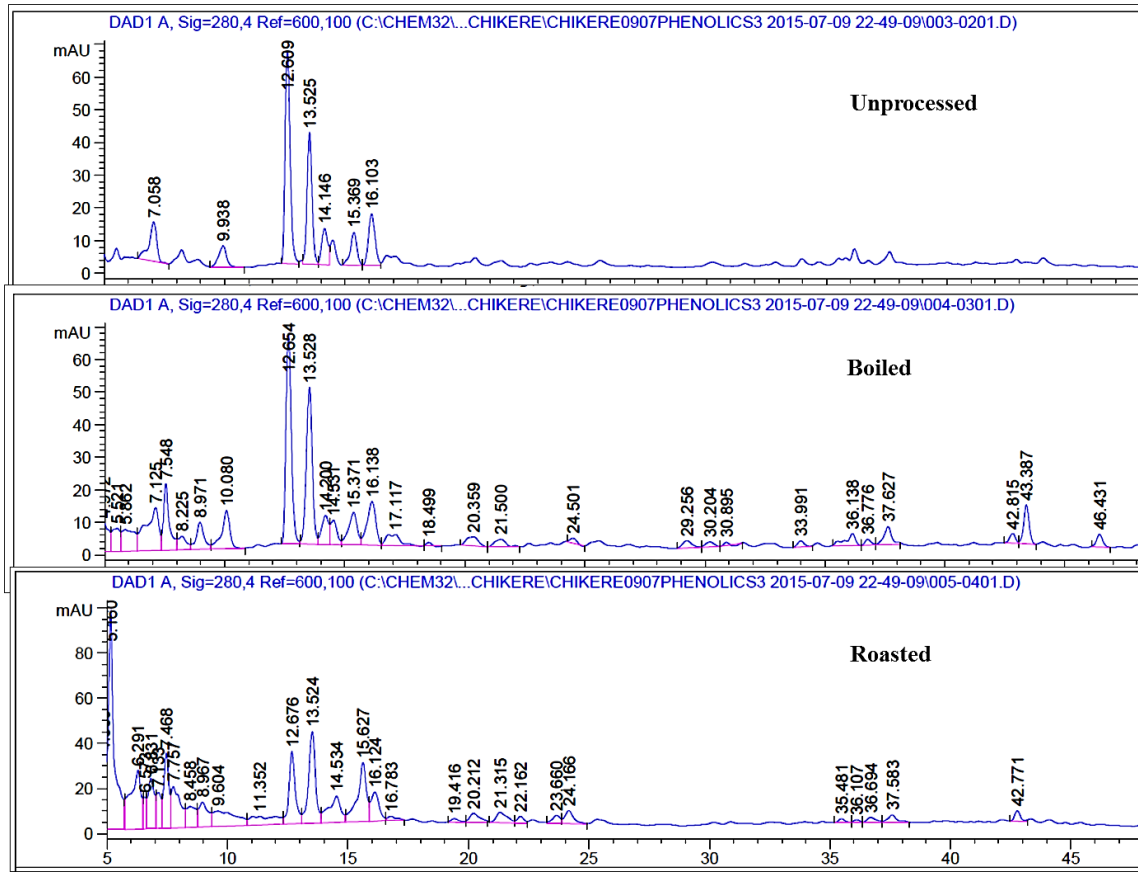



Figure 31 Potential phenolic compounds profile of unprocessed, boiled and roasted African walnut methanolic extract showing similar chromatograms.

### *6.3.3 Cytotoxicity analyses*

The positive control 5-fluorouracil (5-FU) showed a concentration-dependent killing of the cells in both 5,000 and 10,000 cells/well densities. Significant differences were observed when each concentration was compared with the negative control (PBS + media mix). All concentrations of 5-FU reduced the cell viability to at least 40 % (Figure 32).

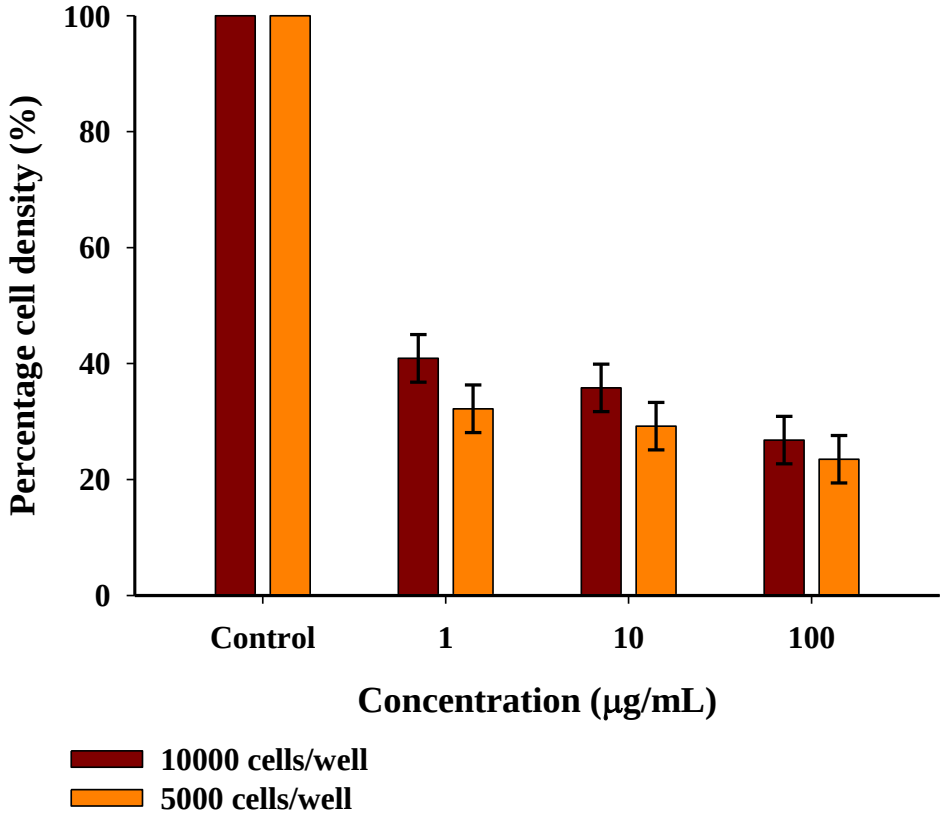



**Figure 32** MTT assay of A549 cells treated with 5-fluorouracil (5FU) at different concentration levels (1, 10 and 100 µg/mL). Values are mean of four replicate readings. LSD bars are shown indicating significant differences ($p < 0.05$) when compared with control.

None of the extracts from unprocessed, boiled and roasted samples altered the viability of the cells in either of the cell densities (Figure 29). Although there was a trend of reduced cell viability at 1 µg/mL water extract of BAW, the effect was not significant ($p > 0.05$). At 1 µg/mL concentration of UAW extracts, there was a trend of increased cell numbers, but again the difference between these and the negative control was not significant ($p > 0.05$).

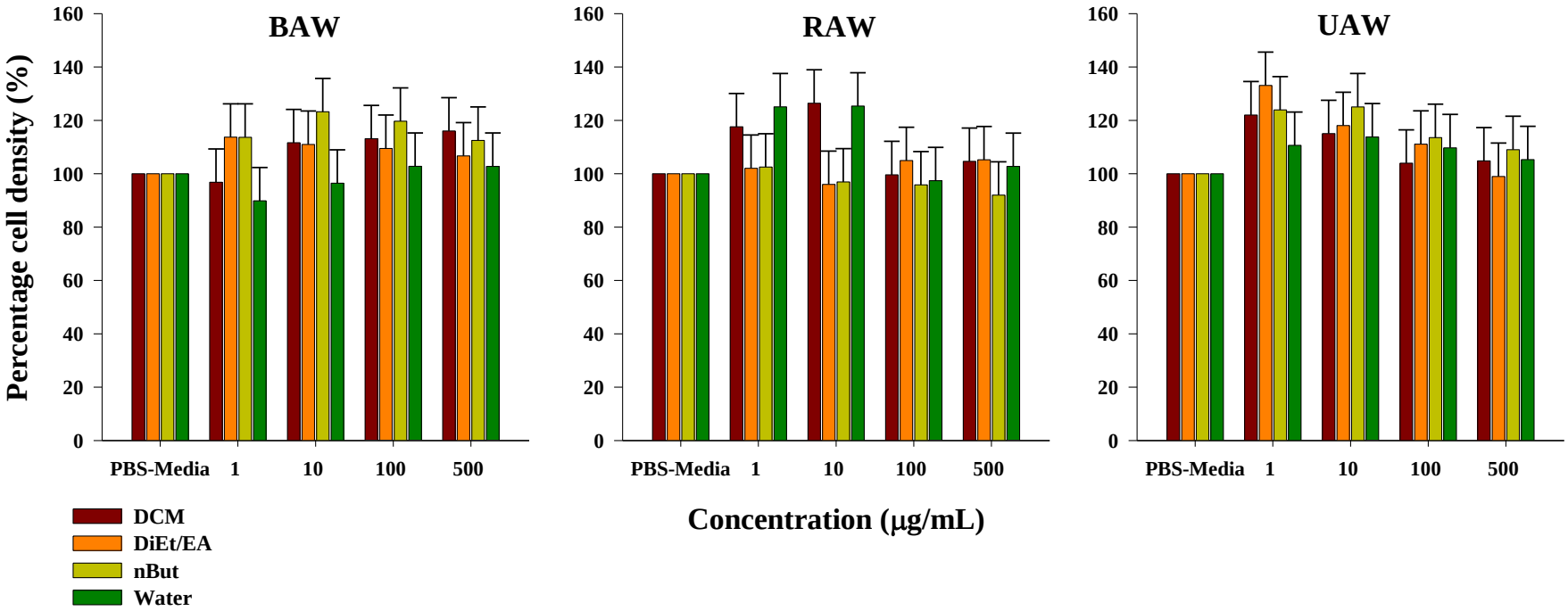



**Figure 33** MTT assay of A549 cell treated with 4 extracts (dichloromethane – DCM; diethyl ether/ethyl acetate – DiEt/EA; n-butanol – nBut and water) of boiled, roasted and unprocessed African walnut – BAW, RAW and UAW, - respectively, at different concentration levels. Values are means ± LSD of four repeat experiments. Control (negative) = PBS + media solution

## 6.4 Discussion

### *6.4.1 Estimation of total phenolic contents in African walnut*

The total phenolic content of African walnut, as estimated, showed the presence of phenolic compounds in detectable amounts. The values of unprocessed African walnut (20.79 ± 1.0 mg GAE/g FDW) were higher than those reported for methanolic extracts of defatted Brazil nuts (kernel 11.99, and skin 17.15 mg GAE/g FDW) (John & Shahidi, 2010); unprocessed whole cashew nuts (7.01 mg GAE/g FDW), and unprocessed hazelnut cultivar (cv. Daviana, 14.77 mg GAE/g FDW) . The presence of these phenolic components in African walnut may be contributing to the astringent taste of the unprocessed nut and the bitter after-taste of boiled and roasted nuts upon drinking of water.

Processing African walnuts by roasting and boiling, reduced the total phenolic content, significantly. Roasting was observed to also reduce total phenolic contents of hazelnuts (Schmitzer, Slatnar, Veberic, Stampar & Solar, 2011). Boiling of the nuts in water may have allowed the leakage of soluble phenolic compounds into the water which was discarded. Heating (wet or dry) plant materials (nuts, seeds and leaves) is generally known to cause both structural and biochemical transformations of compounds which could lead to decrease/loss of secondary metabolites, as well as colour modifications, which are often determined by phenolic constituents (Amaral, Ferreres, Andrade, Valentão, Pinheiro, Santos & Seabra, 2005). As boiling and roasting are the basic methods of processing African walnut, further study on the best procedure to adopt while processing the nuts in order to preserve more of the phenolic contents, may be necessary.

### *6.4.2 High performance liquid chromatography – diode array detection analysis of potential individual phenolic compounds*

This is the first study showing the profile of potential individual phenolic compounds in Nigerian African walnut. As shown in this study, the various compound peaks were detected at wavelengths range of 280 to 355 nm. These are the most common wavelengths extensively used in detection of phenolic compounds in nuts, fruits and vegetables while using HPLC-DAD equipment (Colaric, Veberic, Solar, Hudina & Stampar, 2005; John & Shahidi, 2010; Tomas-Barberan, Gil, Cremin, Waterhouse, Hess-pierce & Kader, 2001).

The resolution of the external standard mix at 280, 332 and 355 nm in the same run with the samples strongly suggests that the compounds eluted from the sample extract are most likely to be phenolic compounds from the family of flavan-3-ols, phenolic acids and Flavonols, respectively.

Again, comparison of the different organic extraction solvent base as reported in this work suggests that methanol:water:HCl (70:29.5:0.5, v/v) combination is the best for phenolic extraction from Nigerian African walnut. This finding supports the general understanding that methanol/water solvent mix is most widely used in extraction of phenolic compounds from plant materials (Escribano-Bailon & Santos-Buelga, 2003). This is probably because the polarity and low boiling point of methanol allows for rapid concentration of the extracted material (Chun, Kim, Smith, Schroeder, Han & Lee,, 2005). The better resolution of potential phenolic compounds extracted with methanol base solvent may also have been positively enhanced as a result of the mobile phase solvent - methanol and water combinations. Since both the extraction solvent and mobile phase solvent contained similar organic base chemicals, molecule interferences would have been reduced to the barest minimum thus, allowing efficient compound detection.

African walnut contains relatively high level of linolenic and linoleic fatty acids as shown in chapter 4 of this thesis. These fatty acids are very prone to oxidation; hence, it is important that antioxidant compounds such as polyphenols are present in the nut to reduce the rate of oxidation of these fatty acids. The findings in this study suggest that majority of these potential individual phenolic compounds may be in very low concentrations. The peak heights/areas of the signal chromatogram gives an indication of the relative abundance of the compound in relation to the weight of sample extracted. In this study, 2 g of the sample was extracted, however, the maximum peak heights were less than 70, 13 and 2 mAU at 280, 332 and 355 nm, respectively. The maximum sample peak height at 280 nm was lower than those of vanilic, caffeic, syringic, chlorogenic, p-coumaric and ferulic acid equivalents. Considering that these phenolic standards had concentrations of only 0.1mg/mL, it can be suggested that the concentration of phenolic compounds in Nigerian African walnut are in very low amounts. In relation to similar nuts, the maximum sample peak height is comparable to those of hazelnut (5g of sample extracted with methanol) with maximum peak height value at about 90 mAU (Jakopic, Petkovsek,

Likozar, Solar, Stampar & Veberic, 2011) but in contrast to walnut (*Juglans regia* L.) which showed maximum peaks height of compounds from 150 mg of sample to be above 70 mAU (Colaric, Veberic, Solar, Hudina & Stampar,2005).

Although there were differences in the total phenolic content of unprocessed, boiled and toasted African walnut, the profile seem not to be altered as the chromatograms of the three groups of samples show similar peaks. This might be an indication that none of the individual phenolic contents was totally lost during processing although their concentrations may have been reduced.

### *6.4.3 Cytotoxicity experiment*

The use of 5-fluorouracil (5-FU) in chemotherapeutic treatment of various types of cancer (especially colorectal and breast cancers) has been an age long practice. It is an analogue of Uracil which rapidly enters the cell through facilitated transport mechanism where it is converted to several forms of active metabolites such as fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate and fluorouridine triphosphate which disrupt ribonucleic acid (RNA) synthesis and the action of thymidylate synthetase (Ghoshal & Jacob, 1997; Kaehler, Isensee, Hucho, Lehrach & Krobitsch, 2014; Longley, Harkin & Johnston, 2003).The drug is classified as an antimetabolite and is cell-cycle specific in its mode of activity, hence, it does not discriminate among actively dividing cells when exerting its effects. This lack of discrimination results in its cytotoxic impact on both normal and cancerous cells. The application of 5-FU at various concentrations to A549 cells grown *in vitro* and incubated for 48 h in this current study demonstrated its cytotoxicity capacity as the cells were still in their actively dividing stages. This observation was similar to those of Huhtala, Tähti, Salminen & Uusitalo, (2002) who reported that application of 5-FU (500 µg/mL) to *in vitro* cultured SV40-immortalized human corneal epithelial cell resulted in significant decrease of cells after 48 h ncubation.

Several phytochemicals (e.g. phytoestrogens, polyphenolics and tannins) present in plant nut extracts such as methanolic, ethanolic, petroleum ether extracts of *Juglans regia* L. have been reported to effect antiproliferative activities or reduce the viability of renal (A-498), breast (MCF-7), liver (HepG-2), and colon (Caco-2) cancer cell lines *in vitro* (Carvalho, Ferreira, Mendes, Silva, Pereira, Jerónimo & Silva,. 2010; Negi, Luqman, Srivastava, Krishna, Gupta & Darokar 2011). These phytomolecules especially the

polyphenolics, have been suggested to inhibit cancer initiation, promotion and progression through various ways; firstly, by suppression of nuclear factor-kB (NF-kB) activation which is a nuclear transcription factor that regulate expression of various genes involved in inflammation and carcinogenesis, whose normal activation induces transcriptional upregulation of genes involved in cell cycle progression; secondly, by induction of apoptosis as well as suppression of growth-factor mediated pathways. Other suggested modes of anti-cancer activities of polyphenolics include suppression of protein kinases (PKC), mitogen activated protein kinases and through antioxidant activities which protects pre-malignant cells from oxidative breakage of cellular DNA (Bonfili, Cecarini, V. Amici, M. Cuccioloni, M. Angeletti, M. Keller, J. N. & Eleuteri,2008; Carvalho, Ferreira, Mendes, Silva, Pereira, Jerónimo & Silva, 2010; Russo, 2007). Although the sequential extraction applied in this study involved the use of solvents that should target extraction of both polar and non polar bioactive compounds such as polyphenolics and phytoestrogens, respectively, extracts from African walnut samples processed by boiling, roasting or left unprocessed did not affect the viability of the cells negatively nor reduce proliferation of cells. As seen in the total phenolic content assay and HPLC-DAD qualitative analysis of individual phenolic components, African walnut contain low amounts of these compounds which may not have been enough to have measurable effect on the cells. Previous studies evaluating the phytochemical composition of African walnut have also often reported very low presence of polyphenols in both processed and unprocessed samples (Ekwe & Ihemeje, 2013; Nwaoguikpe, Ujowundu & Wesley, 2012). According to Malu, Obochi, Edem, Nyong, Edem, Obochi & Malu, (2009), the amount of phytochemicals (polyphenols) in African walnut are often reduced by the general processing and extraction method of boiling and roasting. The report herein, suggests that the potential bioactive phytochemicals (such as polyphenolics) in processed African walnut extracts did not exhibit antiproliferative activities on A549 cell line as they may have been present in very low concentrations following the sequential extraction and processing methods (boiling and roasting) employed. On the other hand, no study has reported on the specific types of phenolic compounds present in African walnut, thus, it is unclear whether the class of phenolics normally detected in total phenolic assays performed in African walnut are among those involved in anti-cancer activities or not. Further research will be required for verification.

## 6.5 Conclusion

It has been established in this study that potential phenolic compounds are present in African walnut. This was based on the conditions of analyses adopted in this study, which were specific for determination of phenolic compounds. However, the concentration of individual phenolic compounds may be in very low amounts. The concentrations were however comparable to commonly consumed nuts such as Brazil nut, hazel nut, and English walnut. The concentration of these compounds may be greatly reduced by the basic processing methods commonly employed by retailers. Hence, further studies on better processing method that will minimise loss of the phenolic components may be necessary in future. As phenolic compounds are important biomolecules with some of them known to have antioxidant effects that influence several other molecules in biological pathways when consumed, it will be necessary to determine the daily serving or recommended daily allowance for African walnut that will achieve the required concentration of the phenolic compounds necessary for maximum function.

Although the extracts of the sample in this current report suggests that they had no cytotoxic or antiproliferative effects on A549 cells, it is not possible to say conclusively that the extracts of African walnut have no anti-cancer activity as the study is limited by the use of only one cell line. The activity of the extracts on other cancer cell lines need to be investigated in order to ascertain the scope of influence of the nut extracts. On the other hand, the study suggests that the nut extracts had no lethal effect on the A549 cells, regardless of the processing method applied (unprocessed, boiling or roasting). More so, the results suggest that the use of the nut extract in ethnobotanical medicine for management of chronic diseases such as cancer may be for the purpose of general body nourishment as against the notion of direct chemotherapeutic effect on the cancer cells.

# 7 General discussion

## 7.1 Introduction

African walnut is currently not a staple crop in Africa as it still grows in the wild. Consequently, it can be classified as one of the neglected and underutilised species (NUS) that is beginning to gain attention among scientists in Africa. This growing interest in African walnut is necessitated by the persistent menace of malnutrition which has been earmarked as one of the major challenges facing developing nations (Bhat & Karim, 2009). As of the year 2000, it was estimated that over 800 million malnourished people live in the world with majority in developing countries, including Nigeria (Bhat & Karim, 2009; IFPRI, 2002; Müller & Krawinkel, 2005). The Nutrition Society of Nigeria noted Nigeria to be among the top 20 countries with the highest burden of malnutrition (Ogbebo, 2014). They further identified that most people are malnourished not because of lack of food but as a result of ignorance on how to utilise the available local food resources such as nut, fruits and vegetables, and maximise their nutritional components. Thus, in collaboration with multi-national companies like Unilever as well as the Ministry of Health and Agriculture, the Nutrition Society of Nigeria launched a sensitization campaign on nutritional education in schools (Ogbebo, 2014). Creating nutritional awareness among school children and farmers has remained a major driving factor in increasing understanding about alternative sources of nutrients available in most of the neglected and under-utilised local species such as African walnut.

The nutritional potentials of African walnut as reported by previous researches have shown that it has high protein content which can serve as alternative source in the wake of animal protein scarcity and high cost in developing nations. It is also reported to have good amino acid profile with essential amino acids present in appreciable amounts. These nutritional properties are essential in the fight against protein-energy malnutrition.

Another factor that has helped to draw attention to African walnut is the continued quest for plants with medicinal properties. The discovery that some secondary metabolites (phytochemicals) produced by plants may possess beneficial characteristics, which includes those with reported antioxidant activities, has brought in to the lime light, several local herbs used in ethnobotanical medicine. African walnut – kernel, leaves, root and

bark - is constantly mixed in decoctions used in treatment or management of different ailments including chronic diseases such as diabetes and cancer. This existing practice of treatment of diseases, using African walnut extracts in local herbal medications, underscores the need for adequate research to validate the claims and much more, re-package the possible products from African walnut for global commercial acceptability. Also, as African walnut continues to be in high demand for consumption as food product or use in different industrial sectors, there is need to increase research data on the adaptability of the nut for industrial scale farming, and describe the possible cultivars in existence at genetic level, so as to promote informed breeding.

This current study was undertaken to understand further the fatty acid profile, biochemical and biomedical properties of African walnut from a postharvest perspective. One major large scale experiment was designed to simulate the normal retail practices employed in the postharvest handling of African walnut. The impact of processing methods (boiling, roasting or unprocessed), storage time (cold storage - 0, 10 and 20 days; retail storage - 3 and 7 days) and temperatures (25 and 37 °C), and maturity stages (early and late) on the major fatty acid profile, and total and potential individual phenolic content of African walnut were assessed. Two other sub-experiments were also performed, firstly, for mycological determination of possible fungal infestation and potential mycotoxin contamination of the nut; and secondly, biomedical analysis to assess the cytotoxicity effect of four different extracts of the samples on cancer cell line (A549), so as to understand the reason for its use in herbal decoction for cancer management. The key findings in this current study are herein discussed in relation to their relevance and economic importance in food/nutrition, health/medical, and agricultural industries.

## 7.2 Food and Nutrition

### *7.2.1 Major fatty acid composition*

The burden of malnutrition in Africa and the world at large, as briefly mentioned in the introductory section of this chapter, is of great concern. The impact on vulnerable groups such as neonatal, children, pregnant women and the elderly cannot be over-emphasized. Results from this study showed that African walnut contains both essential and non-essential fatty acids with predominant ones being α-linolenic and linoleic acids. These are essential fatty acids necessary for several biological activities including cell membrane

functions, in humans and animals. Thus, the consumption of the nut kernel, oil or its products may contribute in reducing nutritional deficiencies resulting from lack of these essential fatty acids. The fatty acid profile of African walnut was shown in this study, to be comparable to some popular and well consumed nuts such as European walnut (*Juglans regia*). Hence, it adds to the diversity of nuts sources for edible oils with desirable fatty acids. Although the oil content may not be as high as those of groundnuts, or some other popular vegetable oil sources, increased industrial scale extraction and consumption/use of African walnut oil, as recommended in this study, presents an extra or alternative choice to consumers. The habit of diversifying sources of similar nutrients is highly encouraged by health personnel and seen as good dietary practice that helps to reduce risk of unhealthy chemical accumulation. It is also significant in terms of food security as the plant is a perennial climber, producing fruits/nuts for oil extraction annually, without having to be replanted.

### *7.2.2 Processing method*

The processing methods adopted in this study were applied in order to simulate the normal retail practice in preparing the nut for consumption. Boiling and roasting are the most common processing methods for the nuts although boiled African walnut seem to be more popular in the streets. This could be because of differential processing costs between both methods. Little effort is needed to prepare boiled nuts when compared to roasting procedures; the nuts are simply placed in a pot of boiling water, covered and left to boil for an hour while roasting will require regular stirring and monitoring in the hot sand for the entire duration of one hour. However, findings in this research suggest that roasting of the nuts may be more beneficial to both retailers and consumers in terms of food safety. Roasting totally prevented growth of both xerophilic and non-xerophilic spoilage fungi in non-stored harvested nuts at early maturity. In terms of packaging of finished products for export, roasted African walnuts may also be more desirable than boiled nuts. This is because, roasting has been reported to help decrease moisture content (Alamprese, Ratti & Rossi, 2009) which is a necessary factor for growth of spoilage organisms. Thus, shelf life of roasted packaged African walnut may be better prolonged under appropriate storage temperature conditions than those of boiled packaged nuts. In addition, other microstructural changes such as flavour and colour enhancement have been reported to be achieved through roasting, making it more appealing to consumers (Alamprese, Ratti

& Rossi, 2009; Alasalvar & Shahidi, 2008; Schlörmann, Birringer, Böhm, Löber, Jahreis, Lorkowski, 2015).

### *7.2.3 Maturity stage and storage*

Two maturity stages (early maturity – green pods; late maturity – brown pods) of African walnut were identified for the first time in this study. Analysis of the major fatty acid composition of the nut showed that neither of these maturity stages made any significant impact over the other. Thus, it implies that consumption of the nut processed by boiling or roasting supplies similar fatty acid requirements. This finding may enable dieticians, while making recommendations on African walnut consumption to clients, not to discriminate between the stages of maturity of the nut. Furthermore, it increases the accessibility of the nuts to consumers as farmers may not concentrate on harvesting only at a particular stage of maturity. In contrast, the results of the experiment conducted for mycological analysis showed that nuts harvested at early maturity and roasted immediately, had no fungal growth while those at late maturity were contaminated with fungi. Thus, the proper timing of harvest of African walnut at early maturity may play a pivotal role in ensuring the safety of the final product when roasting is considered as the preferred processing method. Most nuts that have gained global recognition (pistachio, hazel, Brazil, peanut, almonds, macadamia, walnut, etc.) are usually roasted and packaged for export; hence, for the purpose of commercialization/export of roasted African walnut, use of nuts at early maturity is herein recommended for better safety of finished product.

Another factor necessary for preservation of the nutrients, and avoidance of early spoilage in export food products, is cold storage. Simulating retail storage temperatures of 25 and 37 °C showed contamination of African walnut with fungi, irrespective of the processing method. Hence, it increased the risk of mycotoxin contamination in the nut. Cold storage of African walnut (processed and unprocessed) is not a common practice in Nigeria and other parts of Africa where it is cultivated and consumed. This is as a result of irregular supply of electricity, and the high cost of maintaining alternative sources of electricity in these developing nations. The global demand for vegetarian options in food delicacies necessitates that such nuts as African walnut optimum cold storage conditions (for unprocessed and processed) be further explored by multinational food based companies

in developed nations that have constant electricity, with the view to create international export business opportunities.

## 7.3 Medical/health implications

### *7.3.1 Fatty acid and Total phenolic component*

Several nuts (walnut, hazelnut, almonds) are generally considered to have health-promoting properties. The key components of these nuts often referenced when considering their effect on human health have been the type of the fatty acids as well as the phenolic compounds present. The presence of poly and mono – unsaturated fatty acids have been confirmed to be contained in African walnut. The significant amount of α-linolenic acid found (1.1 – 8.2 mg $g^{-1}$ FDW) in African walnut harvested from Nigeria distinguishes the fatty acid profile from those of the most consumed nuts such as hazel, macadamia, almonds, cashew, peanuts, Brazil nut, and pistachio nuts but puts it in the same category as the common walnut, which also contains α-linolenic acid in an appreciable amount. This essential fatty acid together with linoleic acid also present in African walnut, have been shown to help reduce the risk factors of type 2 diabetes, cardiovascular and coronary heard diseases by lowering total cholesterol and low density lipoproteins cholesterol (LDL-C) while increasing high density lipoprotein – cholesterol (Hu & Manson, 2012; Shoji, Kakiya, Hayashi, Tsujimoto, Sonoda, Shima, Shioi, 2013; Virtanen, Mursu, Voutilainen, Uusitupa & Tuomainen, 2014). They have also been shown to improve the lipid profile of healthy individuals (Jeppesen, Schiller & Schulze, 2013). It was demonstrated by Melariri, Campbell, Etusim & Smith, (2012), in an antimalarial experiment, that both α-linolenic and linoleic free fatty acids inhibited growth of *Plasmodium berghei* (protozoan test model) by 70 and 64 %, respectively. They further reported a 96 % *P. berghei* growth inhibition when both compounds were used in a known combination, during an *in vivo* mice-model experiment. The continued search for antimalarial drugs remains a huge area of research in the developing nations. Hence, the confirmation of the presence of chemical components in African walnut (α-linolenic and linoleic fatty acids) known to have antimalarial activity may help to justify why the decoctions of the nut are used in ethnobotanical medicine to treat malaria.

Although this study was limited in terms of full identification of individual phenolic compounds contained in African walnut, the results of the Folin-Ciocalteu assay for Total

phenolic content as well as HPLC-DAD qualitative analysis of potential individual phenolic compounds indicated that the nut contains phenolic compounds which may have health-promoting effects. The study further suggests that the potential individual phenolic compounds are most likely to be from the family of flavonoids (flavan-3-ols, and flavonols) and phenolic acids. Flavonoid intake has been associated with a reduced risk of several chronic diseases with their mechanism of action being attributed to their capacity for anti-oxidation, anti-inflammation, anti-proliferation and modulation of signal transduction pathways (Chen & Blumberg, 2008). As the nut decoctions are already being used in treatment/management of different ailments including chronic diseases such as diabetes (Type 2) and cancer, the results from this study provides another platform for further guided investigation on bioavailability of phenolic components contained in African walnut and their roles in disease management.

### *7.3.2 Cytotoxicity experiment*

The cytotoxicity experiment was carried out on lung cancer cell line (A549) as a preliminary study to help understand the use in ethnobotanical medicine with respect to cancer treatment. The results of the experiment showed that the nut had no direct cytotoxic effect on the A549 cell lines. Thus necessitating alternative hypothesis that the use in cancer treatment may actually be for boosting the immune system of the patient rather than being therapeutic. Although the limitations of this cytotoxicity analyses are obvious, in that only one cell line was used, the resulting new hypothesis is another line of research worth investigating in order to increase the knowledge on the health-promoting properties of African walnut.

### *7.3.3 Fungal invasion and potential mycotoxin contamination*

The burden of mycotoxins in Africa has been on the increase since the last two decades. In East and central provinces of Kenya in particular, about 317 persons were diagnosed with aflatoxicosis as a result of an outbreak through aflatoxin contamination of stored maize in 2004. This resulted in eventual death of 125 patients as reported by Centre for Disease Control (CDC, 2004). The re-occurrence of this outbreak was recorded by World Health Organization in 2005 and 2006 (WHO, 2006). Research reports have constantly mentioned occurrence of mycotoxins, especially aflatoxins, in high concentrations in staples such as rice, maize, and groundnuts grown in Nigeria and Africa at large (Makun,

Dutton, Njobeh, Mwanza & Kabiru, 2011; WHO, 2006). *Aspergillus* spp. (*A. flavus and A. parasiticus*) are the main producers of aflatoxins and these fungi were frequently isolated from African walnut sample shell cultures in the study herein. The concerns over aflatoxin contamination of food and feed consumed in Nigeria is further heightened by the results of this current study which showed that all four aflatoxins (AFB1, AFG1, AFB2, and AFG2) were detected in the African walnut sample shells cultured under appropriate conditions. The health implications cannot be over-emphasized as aflatoxins, especially AFB1, are known as potent carcinogens involved in liver cancer. More so, the chronic incidences of aflatoxins has been evident from the presence of Aflatoxin M1 in human breast milk and umbilical cord blood samples in Ghana, Nigeria, Kenya, Sierra Leone and Sudan (Bhat, R.V. and Vasanthi, 2003; WHO, 2006). The consumption of contaminated African walnut by adult pregnant women, therefore, may be a potential health hazard for foetus, infants and children. Stunted growth and neurological impairments in children are already associated with early exposure of infants to aflatoxins in Benin and Togo (Gong, Egal, Hounsa, Turner, Hall, Cardwell & Wild, 2003).

Since mycotoxin contamination was noted as a public health issue, efforts have and are still being made by both national and international organizations such as World Health Organization, in Africa to reduce the incidence of mycotoxin health effects. The basic recommendations, following study reports, suggest that multidisciplinary approaches and good practices including manual/mechanical sorting, winnowing, washing combined with deshelling, are effective in reducing overall mycotoxin contamination (WHO, 2006). Creating awareness among key actors for a concerted action has been identified as a major step. Other suggested measures are increase in consumer awareness through the education systems and the media, strengthening surveillance and laboratory capacities as well as establishing early warning systems (WHO, 2006). These good postharvest practices could be applicable to African walnut in order to reduce the burden of aflatoxin contamination.

## 7.4 Agricultural and Economic importance

The findings in this study provide relevant information for farmers who look to starting a large scale farming of African walnut. The mycological analysis in this study reveals that both field and storage fungi such as *Fusarium* spp. and *Aspergillus* spp. respectively, infect the nut. This is necessary when choice of fungicides for treatment of the crop is to

be advised. Although the effect of boiling and roasting on the fatty acid profile are comparable to each other, their impact on the growth of potential mycotoxigenic fungal species are significantly different on nuts harvested at early maturity. Thus, the suggested better agricultural practice will be to harvest the nuts at early maturity. The colour and texture appearance of the fruit at this stage of early maturity is usually more attractive and would appeal more to both local and international retailers, hence, increasing the market value. In the recent past, there have been rejections of export crop products from African countries with Nigeria having the highest reject. For instance, the number of rejects in major foreign markets between 2012 and 2013 revealed that Benin republic had 2 rejects; Egypt had 95, Ethiopia 3, Zambia 5, and South Africa 56 while Nigeria recorded 102. One of the major factors among other, noted to be responsible for the rejection of exported food item include non-compliance with regulatory requirements (Adekoya, 2015) such as allowable limits for mycotoxin contamination in cereals, nuts and other food stuff. So far, these rejections are projected to cause potential economic loss of about $ 6.9 billion. This rejection incidence underscores the importance of the mycotoxin analysis performed in this research work. As an emerging potential export crop, this study gives a preliminary assessment of possible mycotoxin contamination levels that could arise if good agricultural postharvest practices (storage, processing and packaging in particular) are not applied in the large scale production of African walnut. Although there are government institutions responsible for assessment and maintenance of standards in export food produce in Nigeria, there are no national or regional legislation regarding tolerable aflatoxin contamination levels in nuts and cereals. This research report, therefore, highlights the need for establishment of such a policy as African walnut has shown potential for high level aflatoxin contamination.

## 7.5 General Conclusion

Many popular tree nuts and their products, were once known only to the local residents/ farmers where they were indigenous. However, the act of continuous rigorous research helped to increase awareness regarding their nutritional, medicinal, pharmaceutical and economic importance. It further brought them to global prominence. Thus, this research report in its entirety, has aimed to throw light on some interesting, expressed and inherent characteristics of African walnut, in both processed and unprocessed form with the view

to establish strong basis for future research with more sophisticated facilities. The major findings in this study indicate that;

- The profile contains essential and non-essential fatty acids with abundance of α-linolenic and linoleic fatty acids. Their concentrations were enhanced by current retail processing methods of boiling and toasting.
- Mycotoxigenic fungi among others were isolated from shell of harvested African walnut. However, roasting prevented completely, the growth of these fungi in shells of nuts harvested and processed without storage. Aflatoxins (AFB1, AFG1, AFB2, and AFG2) were produced by some isolated fungi when cultured in appropriate media.
- The nut contains phenolic compounds although they may be in low concentrations. Processing by boiling and toasting decreases further the concentration of these phenolic components. The potential individual phenolic components may include those in the class of flavonoids.
- The extracts of the nut showed no cytotoxic effect on A549 cancer cell lines;

In line with the relevance of these major findings as discussed in this chapter, as well as the attractive physical appearance of African walnut, it could be suggested that the prospects for the nut in the global market would be high in the near future.

## 7.6 Suggestion for future work

As most studies, including this current research, are limited by time, resources and several other factors, further work will be necessary in order to better explain some of the findings, and confirm certain hypotheses made, in the course of this study. A few suggestions for further work are herein listed although not exhaustively.

- To carry out a more comprehensive long term cold storage experiment with unprocessed nuts in pods and without pod as to determine the best temperature and form in which to store after harvest.
- Assess the shelf life of processed nuts (boiled and roasted samples) under cold storage scenarios and packaging conditions (e.g. controlled atmosphere environment)

- Determine the possible growth of fungi as well as potential mycotoxin contamination on the nut kernel.
- Conclusively identify and quantify the individual phenolic compounds and other bioactives such as alkaloids, contained in the nut and test their bioavailability in animal and human studies
- Assess the cytotoxicity of the extracts on other types of cancer cell lines such as prostate and colon cancer cell lines.

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# 9 Appendices

## 9.1 Appendix A

### *9.1.1 Peer reviewed journal publications*

- Impact of postharvest processing on the fungal population contaminating African walnut shells (*Tetracarpidium conophorum Mull. Arg*.) at different maturity stages and potential mycotoxigenic implications.

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## Impact of postharvest processing on the fungal population contaminating African walnut shells (*Tetracarpidium conophorum* Mull. Arg) at different maturity stages and potential mycotoxigenic implications

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ABSTRACT

African walnut (*Tetracarpidium conophorum* Mull. Arg) is commonly processed by boiling or toasting and consumed as a snack or used as a thickener in many West African soup preparations. The nuts are usually exposed to both high temperatures and high relative humidity in open markets which predisposes them to fungal growth. Hence, the dangers of spore inhalation and resultant mycosis cannot be over-emphasized as retailers and consumers are always in direct contact with these nuts during harvest, processing and consumption. So far, there is no reported research on potential mycotoxin contamination of African walnut and whether this risk might be accentuated by processing. African walnut, at early and late maturity stages, were processed by toasting, boiling or left unprocessed before being stored at 25 °C and 37 °C, respectively under controlled relative humidity for 7 days. Nuts were cracked and shell pieces cultured in malt extract agar (MEA) and Dichloran Glycerol 18 (DG18) media and incubated at 25 °C for 7 days. Results revealed that potential mycotoxigenic species – *Aspergillus* section *Nigri*, *Aspergillus flavus/parasiticus*, *Fusarium* spp. and *Penicillium* spp. – were frequently isolated. When compared with unprocessed nuts, toasting completely prevented fungal contamination in shell pieces from nuts in the non-stored (NSN) group at the early maturity stage, while boiling significantly reduced the level of contamination to about 58% ($p < 0.05$). In general, simulating open market conditions caused 100% fungal contamination in all boiled samples and toasted samples at early maturity. However, contamination in toasted samples at late maturity was increased to 90 and 70% at 25 °C in DG18 and MEA, respectively, while at 37 °C

- Effects of postharvest storage and processing techniques on the fatty acid profile of African Walnut (*Tetracarpidium conophorum* Mull. Arg.). *Journal of Food Composition and Analysis.*

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Original Research Article

# Effects of postharvest storage and processing techniques on the main fatty acids in the profile of oil extracted from African Walnut (*Tetracarpidium conophorum* Mull. Arg.)

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Nut processing

ABSTRACT

African Walnut (*Tetracarpidium conophorum* Mull. Arg.) is a perennial climber which grows western and central regions of Africa. The nuts are processed by boiling and roasting and are sold 1–5 days to consumers through the open market system. During processing, storage and distributi nuts are typically exposed to high temperatures raising concerns over nutrient quality and Although African walnut, like several other nuts, contains high amount of oil, there is no study rep on how the common processing methods (boiling and roasting) affect the fatty acid profile. Nut sa ($n$ = 702) at both early and late maturity were harvested and stored at 5 °C. Randomized samplir done (0, 10 and 20 days) and nuts grouped according to treatments (boiling, roasting, unprocessed were then held for 3 and 7 days at either 25 °C or 37 °C to simulate normal retail practices. O extracted and analysed as fatty acid methyl esters using gas chromatography flame ionization det and gas chromatography coupled mass spectrometry. Retention times were compared with standards. Results indicated the presence of C16:0, C18:0, C18:1 *cis*-9, C18:2, *cis*-9, 12, C20:0, with C18:3 being the most abundant (1.1–8.2 mg $g^{-1}$ dry matter). In general, postharvest stor 25 °C or 37 °C for 3 or 7 days after boiling and roasting significantly increased concentrations fatty acids (>50%) in nuts stored for 10 days compared to unprocessed. Current processing m and retail storage practices improved concentrations of the fatty acids in African walnut stored 10 days at 5 °C.

### *9.1.2 Conference Presentations*

- Presenter at Gordon Research Seminar (GRS) - speaker; and Conferences (GRC) – poster - 2013 on Mycotoxins & Phycotoxins held at Stonehill College in Easton MA, United States from June 15 – 21, 2013.

**Nkwonta, Chikere**

**From:** Holly Tobin <HTobin@grc.org>
**Sent:** 17 June 2013 06:00
**To:** Nkwonta, Chikere
**Subject:** Gordon Research Conferences: Participation Certificate

Gordon Research Seminars

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**Certificate of Participation**

06/17/2013

CHIKERE NKWONTA
CRANFIELD UNIVERSITY
PLANT SCIENCE LABORATORY
VINCENT BUILDING 52A
CRANFIELD
BEDFORD, MK430TD
UNITED KINGDOM

***This is a certificate of participation for:***
Seminar: Mycotoxins & Phycotoxins (GRS)
Dates: 06/15/2013 - 06/16/2013
Location: Stonehill College in Easton MA United States

This letter certifies your participation as a Speaker at the Gordon Research Seminar on Mycotoxins & Phycotoxins (GRS) held 06/15/2013 - 06/16/2013 at Stonehill College in Easton MA United States.

Presented poster titled: Impact of postharvest processing on the fungal population and potential mycotioxin risk of African walnut - Tetracarpidium conophorum (Mull. Arg) - at different maturity stages

Nancy Ryan Gray, PhD, Director and President
Gordon Research Conferences

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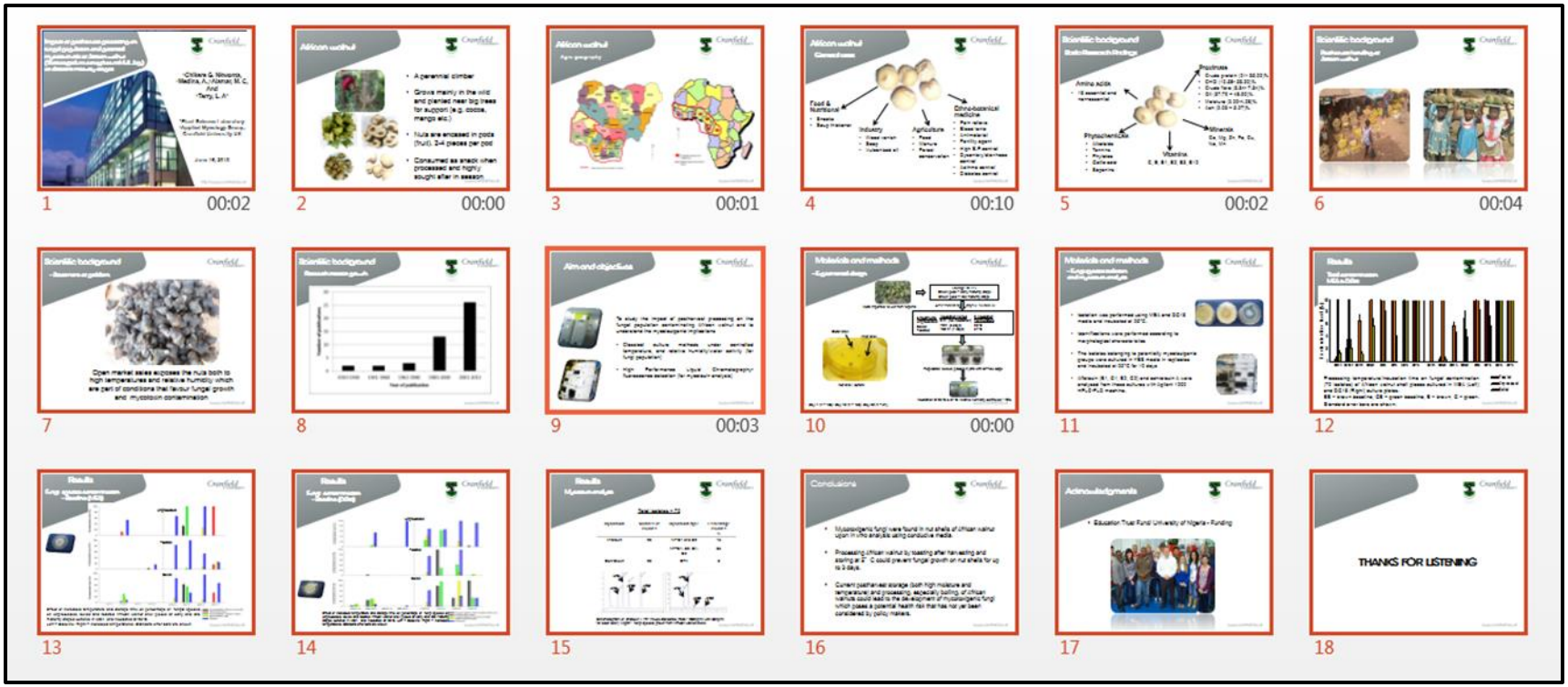



**Title**: Impact of postharvest processing on fungal population and potential mycotoxin risk of African walnut (*Tetracarpidium conophorum* Mull. Arg.) at different maturity stages

# Cranfield Health

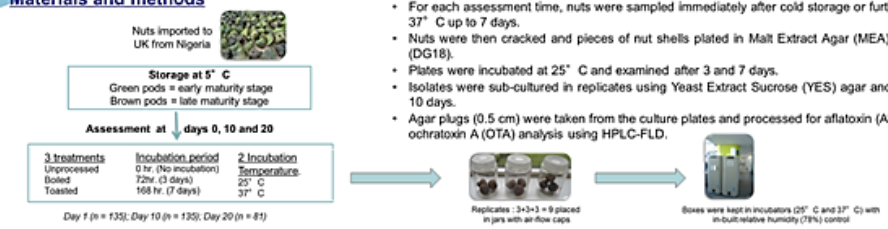
## Impact of postharvest processing on fungal population and potential mycotoxin risk of African walnut (*Tetracarpidium conophorum* - Mull. Arg.) at different maturity stages

**[1]Nkwonta, Chikere G., [2]Medina, Angel, [1]Alamar, M. Carmen, [1]Terry, Leon A.***
[1]Plant Science Laboratory, [2]Applied Mycology Group, Cranfield University, Bedfordshire, MK43 0AL, UK
* Corresponding author. Tel.: +44 7500 766490; Fax: +44 1234 758380; Email: l.a.terry@cranfield.ac.uk

### Background

African walnut (*Tetracarpidium conophorum* Mull. Arg.) is a tropical climbing plant that grows in the western regions of Africa and is usually processed by boiling or toasting before consumption as a snack. The ethno-botanical uses are widespread with respect to treatment of disease conditions and biological disorders. The nuts are usually exposed to both high temperatures and relative humidity while being sold or hawked in the open markets; these conditions favour fungal growth. So far, there is no reported research on fungi/mycotoxin contamination in African walnut and how this risk might be accentuated by processing.
**Aim:** To study the impact of postharvest processing on the fungal population contaminating African walnut and to understand potential mycotoxigenic implications.

### Materials and methods




- For each assessment time, nuts were sampled immediately after cold storage or further stored at 25° C and 37° C up to 7 days.
- Nuts were then cracked and pieces of nut shells plated in Malt Extract Agar (MEA) and Dichloran Glycerol (DG18).
- Plates were incubated at 25° C and examined after 3 and 7 days.
- Isolates were sub-cultured in replicates using Yeast Extract Sucrose (YES) agar and incubated at 25° C for 10 days.
- Agar plugs (0.5 cm) were taken from the culture plates and processed for aflatoxin (AF) – G1, B1, G2, B2 and ochratoxin A (OTA) analysis using HPLC-FLD.

### Results

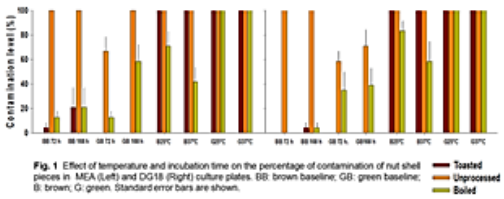



**Fig. 1** Effect of temperature and incubation time on the percentage of contamination of nut shell pieces in MEA (Left) and DG18 (Right) culture plates. BB: brown baseline; GB: green baseline; B: brown; G: green. Standard error bars are shown.

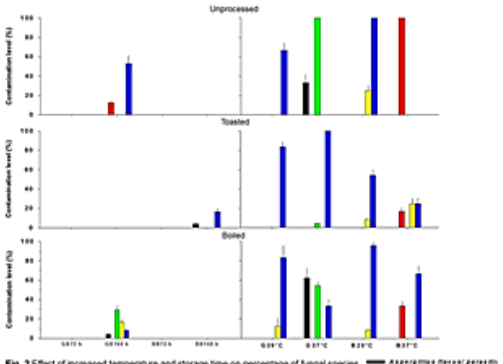



**Fig. 2** Effect of increased temperature and storage time on percentage of fungal species isolated from unprocessed, boiled and toasted African walnut shell pieces at early and late maturity stages cultured in MEA and incubated at 25° C. Left: baseline; Right: increased temperatures. BB: brown baseline; GB: green baseline; B: brown; G: green. Standard error bars are shown.

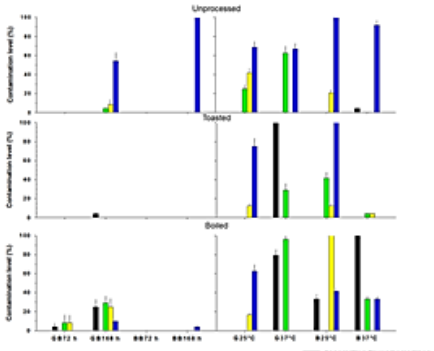



**Fig. 3.** Effect of increased temperature and storage time on percentage of fungal species isolated from unprocessed, boiled and toasted African walnut shell pieces at early and late maturity stages cultured in DG18 and incubated at 25° C. Left: baseline; Right: increased temperatures. BB: brown baseline; GB: green baseline; B: brown; G: green. Standard error bars are shown.

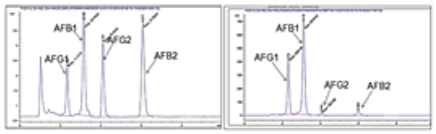



**Fig. 4.** HPLC-FLD chromatograms obtained for aflatoxins standard mix (Total - 200ng/ml with 50ng/ml for each toxin) (Left) and a 10 days *Aspergillus flavus/parasiticus* isolate culture on YES medium (Right)

- Toasting and boiling reduced the contamination level significantly ($p < 0.05$)
- *Aspergillus* section *Nigri, Aspergillus flavus/parasiticus, Penicillium* spp. and *Fusarium* spp. were the most frequently isolated fungi.
- AFB1 and AFB2 were produced by 15% of 20 isolates while AFB1, AFG1, AFB2, and AFG2 were produced by 85% of 20 isolates.
- No OTA production was observed for any of the black *Aspergilli* or *Penicillium* spp.

### Conclusions

- Mycotoxigenic fungi were found in nut shells of African walnut upon *in vitro* analysis using conducive media.
- Processing African walnut by toasting after harvesting and storing at 5° C could prevent fungal growth on nut shells for up to 3 days.
- Our results have shown that current postharvest storage (both high moisture and temperature) and processing, especially boiling, of African walnuts could lead to the development of mycotoxigenic fungi which poses a potential health risk that has not yet been considered by policy makers.

### Acknowledgement

Education Trust Fund/ University of Nigeria - Funding



### References

A. Medina and N. Magan (2012). Comparison of $C_{18}$ HPLC columns with different particle sizes for the optimization of aflatoxins analysis. *Journal of Chromatography B* 889 -890, 138 -143.

- Oral presenter at International Society of Horticultural Sciences (ISHS) conference on Management of Quality in Chains (MQUIC) September 2 – 5, 2013 at Cranfield Univrsity.


ISHS

Cranfield UNIVERSITY

VI International Conference on

Managing Quality in Chains (MQUIC 2013)

2nd – 5th September

Certificate of participation

This is to certify that

Mr Chikere G. Nkwonta

participated in the conference as a Speaker

Professor Leon A. Terry
Convener, MQUIC 2013

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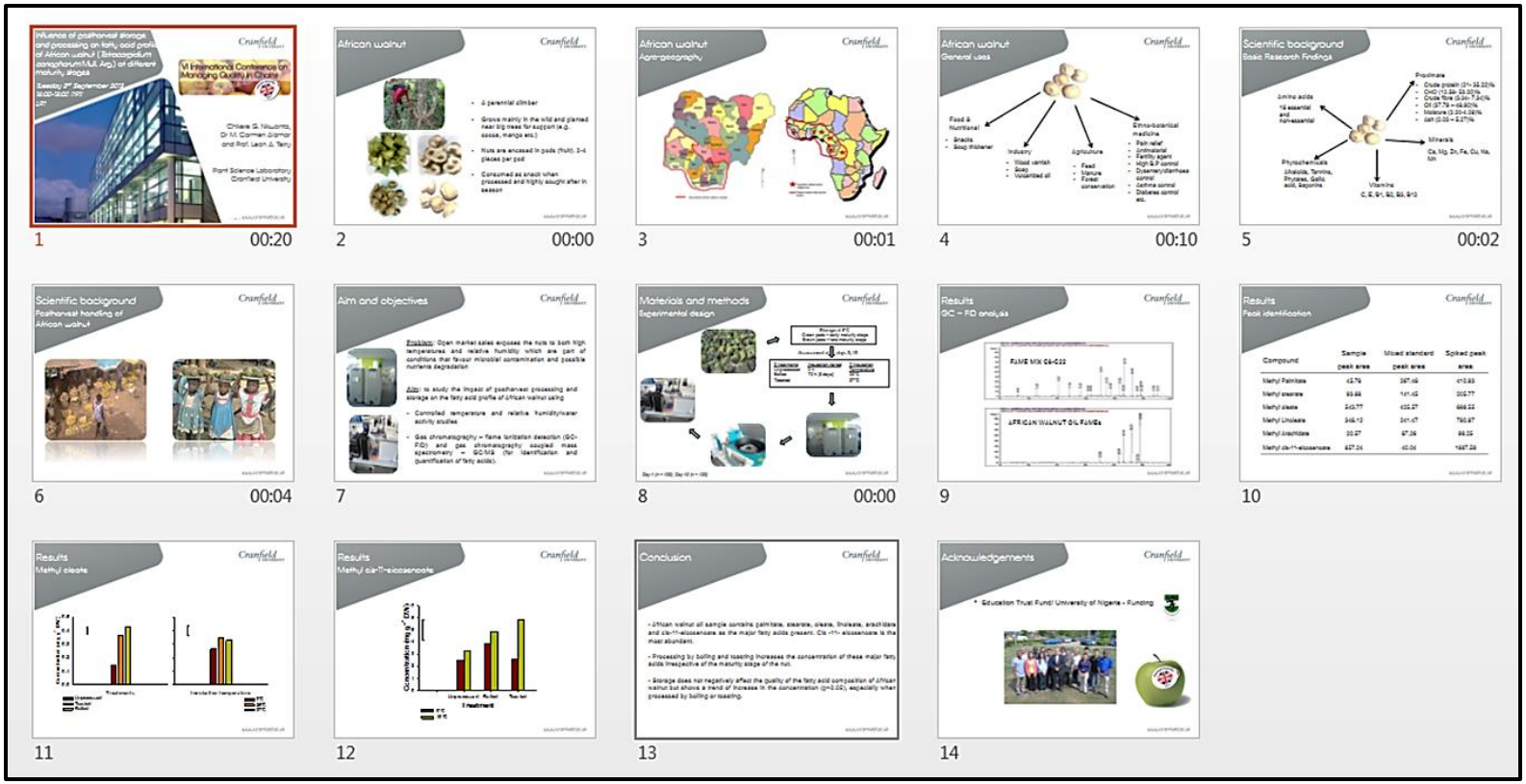



**Title**: Influence of postharvest storage and processing on fatty acid profile of African walnut (*Tetracarpidium conophorum* Mull. Arg.) at different maturity stages

- Oral presentation at the International Society of Horticultural Sciences (ISHS) 2nd international symposium on Mycotoxins in nuts and dried fruits , 8th – 12th September, 2015 in Abuja, Nigeria.

**Fungal contamination and aflatoxin risk in African Walnut – a postharvest perspective.**

Chikere G. Nkwonta[a], Angel Medina[b], M. Carmen Alamar[a], and Leon A. Terry[a]

[a]Plant Science Laboratory

[b]Applied Mycology Group,

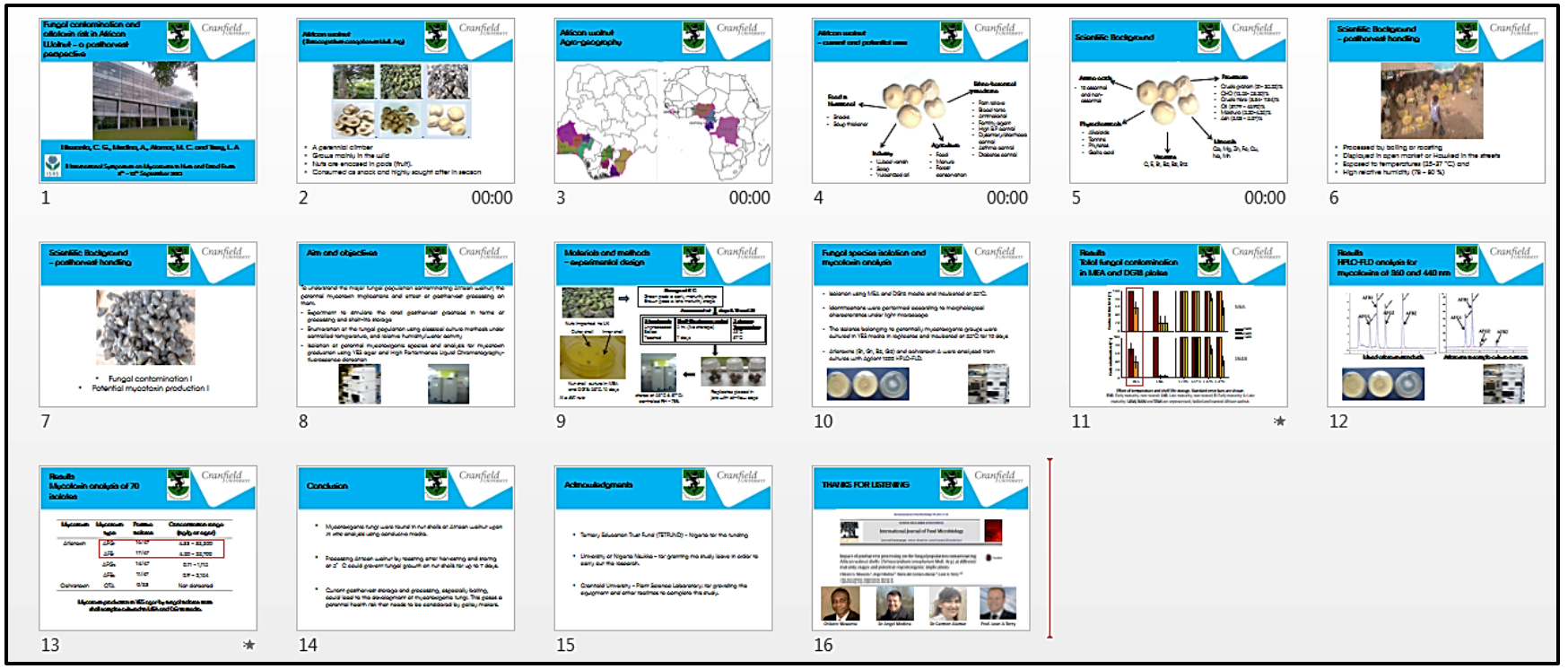
**Address**: Cranfield University, United Kingdom MK43 0AL.

**Presenting Author:** Email- c.g.nkwonta@cranfield.ac.uk

**Abstract**

African walnut (*Tetracarpidium conophorum* Mull. Arg) also known as *Ukpa* and *Asala* among the Igbo and Yoruba tribes in Nigeria, respectively, is processed by boiling or toasting and consumed as a snack or used as a thickener in many West African soup preparations. The nuts are usually exposed to both high temperatures and high relative humidity in open markets predisposing them to fungal infestation and possible mycotoxin contamination. So far, there is no reported research on potential mycotoxin contamination of African walnut and whether this risk might be accentuated by processing. African walnut, at early and late maturity stages, were processed by toasting, boiling or left unprocessed before being stored at 25°C and 37°C, respectively, under controlled relative humidity (78 %) for 7 days. Nuts were cracked and shell pieces cultured in Malt Extract Agar and Dichloran Glycerol 18 media and incubated at 25°C for 7 days. Results revealed potential mycotoxigenic species - *Aspergillus section Nigri, Aspergillus flavus/parasiticus, Fusarium* spp. and *Penicillium* spp. - were frequently isolated. Comparing with unprocessed nuts, toasting completely prevented fungal contamination in shell pieces from nuts in the non-stored group at early maturity stage, while boiling significantly reduced the level of contamination to about 58 % ($p < 0.05$). In general, simulating open market conditions caused 100 % fungal contamination in all boiled and toasted samples at early maturity. Capability for mycotoxin production on potentially toxigenic isolates was tested in Yeast Extract Sucrose (YES) agar. Mycotoxin analysis using High Performance Liquid Chromatography - Fluorescence detector showed that Aflatoxins - $G_1$ ($AFG_1$), $B_1$ ($AFB_1$), $G_2$ ($AFG_2$), and $B_2$ ($AFB_2$) were produced by 20 isolates with both $AFG_1$ and $AFB_1$ being predominant at concentration ranges of 4 – 32,200 and 4 – 22,700 ng/g plug weight, respectively. The study suggests that toasting of nuts, preferably at early maturity, is a safer processing technique than boiling in terms of prevention of fungal growth on nut shells and possible risk of mycotoxin contamination.

**Key words**: *Tetracarpidium conophorum*, Mycotoxins, Aflatoxins, African Walnut.




**Title:** Fungal contamination and aflatoxin risk in African Walnut – a postharvest perspective

## 9.2 Appendix B: Relevant trainings and short courses

- Cranfield doctoral training centre (DTC) core research skill training

4/30/2015 Training Diary

Cranfield UNIVERSITY

# My History (Courses & Events Attended)

We have on record that you have attendance the following events...

| Course | Date | Time | Duration |
|---|---|---|---|
| Communicating Your Progress at the 9 month review - part1 | 27 Feb 2012 | 14:00 | 3.5 hours |
| Communicating Your Progress at the 9 month review - part2 | 29 Feb 2012 | 14:00 | 2 hours |
| Working Safely With Chemicals | 05 Mar 2012 | 10:00 | 2 hours |
| Choosing The Right Statistics | 09 Mar 2012 | 11:00 | 1.5 hours |
| Research Integrity and Being An Ethical Researcher | 12 Mar 2012 | 14:00 | 2 hours |
| Communicating Your Science To The Public and Your Peers | 08 May 2012 | 09:00 | 4 hours |
| An Introduction To The Statistical Treatment of Experimental Data | 25 Nov 2013 | 14:45 | 2.5 hours |
| Linear Regression | 17 Mar 2015 | 09:00 | 3 hours |
| Visualising & Presenting Data | 19 Mar 2015 | 11:00 | 1.5 hours |
| Design of Experiments | 20 Mar 2015 | 09:30 | 3.5 hours |
| Getting Started With LaTex | 26 Mar 2015 | 10:00 | 2 hours |
| Assisting In The Supervision of MSc Students | 29 Apr 2015 | 10:00 | 3 hours |

- Information technology (IT) training

4/30/2015 Training Diary

Cranfield UNIVERSITY

# My History (Courses & Events Attended)

We have on record that you have attendance the following events...

| Course | Date | Time | Duration |
|---|---|---|---|
| PowerPoint: Creating Cranfield Presentations | 08 Feb 2012 | 14:00 | 2 hours |
| Word: Using the Thesis Template | 14 Feb 2012 | 10:00 | 2 hours |
| Excel: Creating Charts | 07 Mar 2012 | 14:00 | 2 hours |
| Outlook: Email Techniques (Staff) | 08 Mar 2012 | 10:00 | 2 hours |
| Word: Essentials | 05 Apr 2012 | 09:30 | 3.5 hours |
| Word: Using the Thesis Template | 11 Apr 2012 | 10:00 | 2 hours |
| Excel: Formulas and Functions | 26 Apr 2012 | 10:00 | 2 hours |
| Excel: Analysing Data | 09 May 2012 | 10:00 | 2 hours |
| PowerPoint: Creating Cranfield Presentations | 10 May 2012 | 10:00 | 2 hours |
| Project: Creating a simple project plan | 15 Oct 2014 | 14:30 | 2 hours |

- Safety awareness training on Liquid Nitrogen decanting

Certificate

*If you can't identify the hazards you can't assess the risks*

This is to certify that

Chikere Nkwonta
of
Cranfield University

has attended a

Safety Awareness Training Workshop

Liquid Nitrogen
Practical Decanting

covering:

Suitability of equipment & location of supply vessels
Pre-fill checks
Fill procedure
Filling of liquid nitrogen into dewars
Regulatory & maintenance requirements of dewar filling
Actions in the event of spillage

Dated
12th January 2015

Signed on behalf of Gas Safe Consultants Limited

Cert. No.
5092418

T.J. Broughton
Managing Director

*This certificate remains valid for a period of 2 years from the date of certificatio*

Gas Safe Consultants Ltd
Waterloo House, 2 Station Road, Elworth, Sandbach, Cheshire, CW11 3JG.
Tel 01270 758890 Fax 01270 758891 E-mail admin@gassafeconsultants.co.uk
www.gassafegear.com www.gassafeconsultants.co.uk www.gas-safe-interactive.co.uk

- 7th European short course on quality and safety of fresh-cut produce

- 12th Annual Nutrition and Health Conference/Course at Phoenix, Arizona, USA

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CMEsolutions PO Box 68680 Tucson, Arizona 85737

*This certificate is presented to*

Chikere Nkwonta

For successful participation in

12th Annual Nutrition & Health Conference

Phoenix, AZ on May 4-6, 2015

Shirlene DeHart
Signature of Contact Person, CMEsolutions

16.50 Hours

## 9.3 Appendix C : Experimental design summary figure

Batch 1

| Brown pod nuts in replicates | | | | | | Green pod nuts in replicates | | | |
|---|---|---|---|---|---|---|---|---|---|
| Incubation Temperature | Out turns\Treatments | Boiled | Roasted | Unprocessed | | Boiled | Roasted | Unprocessed | Out turn Dates |
| | No incubation | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 11 |
| 25°C | Day 3 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 14 |
| | Day 7 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 18 |
| 37°C | Day 3 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 14 |
| | Day 7 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 18 |

Batch 2

| Brown pod nuts in replicates | | | | | | Green pod nuts in replicates | | | |
|---|---|---|---|---|---|---|---|---|---|
| Incubation Temperature | Out turns\Treatments | Boiled | Roasted | Unprocessed | | Boiled | Roasted | Unprocessed | Out turn Dates |
| | No incubation | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 20 |
| 25°C | Day 3 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 23 |
| | Day 7 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 27 |
| 37°C | Day 3 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 23 |
| | Day 7 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 27 |

Batch 3

| Brown pod nuts in replicates | | | | | | Green pod nuts in replicates | | | |
|---|---|---|---|---|---|---|---|---|---|
| Incubation Temperature | Out turns\Treatments | Boiled | Roasted | Unprocessed | | Boiled | Roasted | Unprocessed | Out turn Dates |
| | No incubation | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | July 30 |
| 25°C | Day 3 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | August 2 |
| 37°C | Day 3 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | | 3 +3 +3 = 9 | 3 +3 +3 = 9 | 3 +3 +3 = 9 | August 2 |

## 9.4 Appendix D: Statistics Tables

### *9.4.1 ANOVA tables for the general effects of cold storage (5 °C) durations, processing methods, maturity stages, and retail storage temperatures on concentrations of individual fatty acids after 3 days of retail storage simulation (Table 12 and Figure 18).*

**Table D 9.4.1 1 Arachidate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 2 | 0.0080853 | 0.0040427 | 30.13 | <.001 |
| Maturity stage | 1 | 0.0003107 | 0.0003107 | 2.32 | 0.131 |
| Treatment | 2 | 0.0380213 | 0.0190107 | 141.68 | <.001 |
| Retail storage temperature | 2 | 0.0027364 | 0.0013682 | 10.2 | <.001 |
| Cold storage*Maturity stages*Retail storage temperature | 4 | 0.0101855 | 0.0025464 | 18.98 | <.001 |
| Residual | 104 | 0.0139547 | 0.0001342 | | |
| Total | 159 | 0.1079442 | | | |

**Table D 9.4.1 2 Linoleate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 2 | 6.12716 | 3.06358 | 89.16 | <.001 |
| Maturity stage | 1 | 0.00233 | 0.00233 | 0.07 | 0.795 |
| Treatment | 2 | 5.48008 | 2.74004 | 79.74 | <.001 |
| Retail storage temperature | 2 | 0.70418 | 0.35209 | 10.25 | <.001 |
| Cold storage*Treatment*Retail storage temperature | 8 | 0.38165 | 0.04771 | 1.39 | 0.21 |
| Residual | 104 | 3.57347 | 0.03436 | | |

| | | | | | |
|---|---|---|---|---|---|
| Total | 159 | 23.79498 | | | |

**Table D 9.4.1 3 Oleate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 2 | 0.038394 | 0.019197 | 8.33 | <.001 |
| Maturity stage | 1 | 0.002194 | 0.002194 | 0.95 | 0.332 |
| Treatment | 2 | 0.356199 | 0.178099 | 77.27 | <.001 |
| Retail storage temperature | 2 | 0.015385 | 0.007693 | 3.34 | 0.039 |
| Cold storage*Treatment*Retail storage temperature | 8 | 0.032639 | 0.00408 | 1.77 | 0.091 |
| Residual | 104 | 0.2397 | 0.002305 | | |
| Total | 159 | 0.797922 | | | |

**Table D 9.4.1 4 Stearate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 2 | 0.0492793 | 0.0246397 | 53.27 | <.001 |
| Maturity stage | 1 | 0.0001706 | 0.0001706 | 0.37 | 0.545 |
| Treatment | 2 | 0.091073 | 0.0455365 | 98.46 | <.001 |
| Retail storage temperature | 2 | 0.0098912 | 0.0049456 | 10.69 | <.001 |
| Cold storage*Treatment*Retail storage temperature | 8 | 0.0093066 | 0.0011633 | 2.52 | 0.015 |
| Residual | 104 | 0.0481011 | 0.0004625 | | |
| Total | 159 | 0.294419 | | | |

**Table D 9.4.1 5 Palmitate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 2 | 0.00522645 | 0.00261322 | 58.74 | <.001 |
| Maturity stage | 1 | 0.00000074 | 0.00000074 | 0.02 | 0.897 |
| Treatment | 2 | 0.00957808 | 0.00478904 | 107.66 | <.001 |
| Retail storage temperature | 2 | 0.00083588 | 0.00041794 | 9.4 | <.001 |
| Cold storage*Treatment*Retail storage temperature | 8 | 0.00066511 | 0.00008314 | 1.87 | 0.073 |
| Residual | 104 | 0.0046264 | 0.00004448 | | |
| Total | 159 | 0.0303485 | | | |

**Table D 9.4.1 6 α-Linolineate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 2 | 44.6941 | 22.347 | 29.04 | <.001 |
| Maturity stage | 1 | 0.7713 | 0.7713 | 1 | 0.319 |
| Treatment | 2 | 21.9155 | 10.9578 | 14.24 | <.001 |
| Retail storage temperature | 2 | 3.5464 | 1.7732 | 2.3 | 0.105 |
| Cold storage*Treatment*Retail storage temperature | 8 | 5.5611 | 0.6951 | 0.9 | 0.517 |
| Residual | 104 | 80.0222 | 0.7694 | | |
| Total | 159 | 253.4058 | | | |

### *9.4.2 ANOVA tables for the main effects of cold storage (5 °C) duration, processing method, maturity stage, and retail storage temperature on concentrations of individual fatty acids after 7 days of retail storage simulation (Table 13 and Figure 19)*

**Table D 9.4.2 1Arachidate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 1 | 0.0061507 | 0.0061507 | 16.98 | <.001 |
| Maturity stage | 1 | 0.0000858 | 0.0000858 | 0.24 | 0.627 |
| Treatment | 2 | 0.0433569 | 0.0216784 | 59.83 | <.001 |
| Retail storage temperature | 2 | 0.0042268 | 0.0021134 | 5.83 | 0.004 |
| Cold storage*Treatment*Retail storage temperature | 4 | 0.0021156 | 0.0005289 | 1.46 | 0.219 |
| Residual | 116 | 0.0420302 | 0.0003623 | | |
| Total | 177 | 0.1501449 | | | |

**Table D 9.4.2 2 Linoleate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr(p value). |
|---|---|---|---|---|---|
| Cold storage | 1 | 0.83138 | 0.83138 | 26.88 | <.001 |
| Maturity stage | 1 | 0.0124 | 0.0124 | 0.4 | 0.528 |
| Treatment | 2 | 5.39765 | 2.69883 | 87.25 | <.001 |
| Retail storage temperature | 2 | 0.00449 | 0.00224 | 0.07 | 0.93 |
| Cold storage*Treatment*Retail storage temperature | 4 | 0.12129 | 0.03032 | 0.98 | 0.421 |
| Residual | 116 | 3.58824 | 0.03093 | | |
| Total | 177 | 24.24501 | | | |

**Table D 9.4.2 3 Oleate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 1 | 0.001779 | 0.001779 | 0.83 | 0.364 |
| Maturity stage | 1 | 0.000327 | 0.000327 | 0.15 | 0.697 |
| Treatment | 2 | 0.375271 | 0.187636 | 87.66 | <.001 |
| Retail storage temperature | 2 | 0.00849 | 0.004245 | 1.98 | 0.142 |
| Cold storage*Treatment*Retail storage temperature | 4 | 0.024955 | 0.006239 | 2.91 | 0.024 |
| Residual | 116 | 0.248299 | 0.002141 | | |
| Total | 177 | 0.814398 | | | |

**Table D 9.4.2 4 Stearate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 1 | 0.0138345 | 0.0138345 | 35.03 | <.001 |
| Maturity stage | 1 | 0.0011216 | 0.0011216 | 2.84 | 0.095 |
| Treatment | 2 | 0.1041758 | 0.0520879 | 131.89 | <.001 |
| Retail storage temperature | 2 | 0.0029607 | 0.0014804 | 3.75 | 0.026 |
| Cold storage*Treatment*Retail storage temperature | 4 | 0.0035508 | 0.0008877 | 2.25 | 0.068 |
| Residual | 116 | 0.0458125 | 0.0003949 | | |
| Total | 177 | 0.3013259 | | | |

**Table D 9.4.2 5 Palmitate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 1 | 0.001237 | 0.001237 | 31.08 | <.001 |
| Maturity stage | 1 | 4.841E-05 | 0.00004841 | 1.22 | 0.272 |
| Treatment | 2 | 0.0104757 | 0.00523787 | 131.58 | <.001 |
| Retail storage temperature | 2 | 0.0004382 | 0.00021909 | 5.5 | 0.005 |
| Cold storage*Treatment*Retail storage temperature | 4 | 0.0002429 | 0.00006073 | 1.53 | 0.199 |
| Residual | 116 | 0.0046176 | 0.00003981 | | |
| Total | 177 | 0.029139 | | | |

**Table D 9.4.2 6 α-Linolineate**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cold storage | 1 | 5.742 | 5.742 | 3.63 | 0.059 |
| Maturity stage | 1 | 0.012 | 0.012 | 0.01 | 0.932 |
| Treatment | 2 | 27.307 | 13.654 | 8.64 | <.001 |
| Retail storage temperature | 2 | 5.773 | 2.886 | 1.83 | 0.166 |
| Cold storage*Treatment*Retail storage temperature | 4 | 7.398 | 1.85 | 1.17 | 0.327 |
| Residual | 116 | 183.3 | 1.58 | | |
| Total | 177 | 399.56 | | | |

### *9.4.3 ANOVA table for MTT assay of A549 cells*

**Table D 9.4.3 1 Optical densities (%) for cells treated with 5 fluorouracil (5FU) at different concentration levels**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cell Density | 1 | 172.12 | 172.12 | 5.03 | 0.034 |
| %5FU | 3 | 28752.78 | 9584.26 | 279.95 | <.001 |
| Cell Density*%5FU | 3 | 86.92 | 28.97 | 0.85 | 0.482 |
| Residual | 24 | 821.65 | 34.24 | | |
| Total | 31 | 29833.47 | | | |

**Table D 9.4.3 2 Optical densities (%) for A549 cell treated with 4 extracts**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr.(p value) |
|---|---|---|---|---|---|
| Cell density | 1 | 66.7 | 66.7 | 0.21 | 0.645 |
| Treatment | 2 | 3298.6 | 1649.3 | 5.25 | 0.006 |
| Extract | 3 | 1587.3 | 529.1 | 1.68 | 0.17 |
| Dilution | 4 | 12664.9 | 3166.2 | 10.07 | <.001 |
| Cell density*Treatment | 2 | 5539.3 | 2769.6 | 8.81 | <.001 |
| Cell density*Extract | 3 | 444.9 | 148.3 | 0.47 | 0.702 |
| Treatment*Extract | 6 | 11305.6 | 1884.3 | 5.99 | <.001 |

| | | | | | |
|---|---|---|---|---|---|
| Cell density*Dilution | 4 | 207.4 | 51.9 | 0.16 | 0.956 |
| Treatment*Dilution | 8 | 6446.8 | 805.9 | 2.56 | 0.01 |
| Extract*Dilution | 12 | 6637.6 | 553.1 | 1.76 | 0.053 |
| Cell density*Treatment*Extract | 6 | 5464.2 | 910.7 | 2.9 | 0.009 |
| Cell_density*Treatment*Dilution | 8 | 4131.3 | 516.4 | 1.64 | 0.111 |
| Cell density*Extract*Dilution | 12 | 5599.1 | 466.6 | 1.48 | 0.128 |
| Treatment*Extract*Dilution | 24 | 10772.6 | 448.9 | 1.43 | 0.09 |
| Cell*density*Treatment*Extract*Dilution | 24 | 6484.3 | 270.2 | 0.86 | 0.659 |
| Residual | 360 | 113195 | 314.4 | | |
| Total | 479 | 193845 | | | |