INVESTIGATION OF *TYLOSEMA FASSOGLENSE* SEED AS AN ALTERNATIVE SOURCE OF PROTEIN IN ANIMAL FEED

BY

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DECLARATION

This thesis is my original work and has not been presented to any institution for award of degree or certificate whatsoever.

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DEDICATION

This work is dedicated to my family, friends and colleagues whose support and encouragement pushed me all the way to its successful conclusion

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ABBREVIATIONS

AAS:	Amino acid score
AES:	Atomic emission spectroscopy
AFS:	Atomic fluorescence spectroscopy
AR:	Analytical reagent
BSE:	Bovine spongiform encephalopathy
CF:	Crude fibre
CO:	Crude oil
CP:	Crude protein
CRM	Certified reference material
CV:	Calorific value
EAA:	Essential amino acids
FA:	Fatty acids
FAO:	Food and Agriculture Organization
GC:	Gas chromatography
GC-FID:	Gas chromatography-flame ionization detector
GR:	General purpose reagent

ICMG:	Inorganic Chemical Metrology Group
ICP:	Inductively coupled plasma
ICP-MS:	Inductively coupled plasma-mass spectrometry
ICP-OES:	Inductively coupled plasma-optical emission spectrometry
IEC:	Ion exchange chromatography
KEBS:	Kenya Bureau of Standards
LDPE:	Low-density polyethylene
MUFA:	Monounsaturated fatty acids
NEAA:	Non Essential amino acids
NIST:	National Institute for Standards and Technology
nM	nano mole
NS:	Not specified
OPA:	Orthophthaldehyde
PMT:	Photomultiplier tube
PUFA:	Polyunsaturated fatty acids
RF:	Radio frequency
SFA:	Saturated fatty acids

SRM:	Standard reference material
TS:	*Tylosema fassoglense*
U:	Uncertainty of measurement
U_c:	Combined uncertainty of measurement
UFA:	Unsaturated fatty acids
USDA:	United States Department of Agriculture
UV:	Ultra violet
WHO:	World Health Organization

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ABSTRACT

Formulation of animal feeds entails use of various raw materials in order to come up with a final product capable of meeting dietary needs of the animal under consideration. To achieve this goal, animal and plant based raw materials are investigated for various nutritional components such as amounts of proteins, carbohydrates, fats, fibre and minerals present in order to facilitate formulation of the optimal nutritional feed. Plants of the leguminosae family are known to be rich in the essential nutrients and are therefore used extensively in the animal feed industry. *Tylosema fassoglense* is a member of the leguminosae family growing wildly in Western, Rift valley and sections of North Eastern parts of Kenya. Various parts of the plant have several uses among different communities; the plant seed is consumed, tuber used as medicine by some communities in Western Kenya, while the leaves are used as pasture for livestock by some other communities in Rift Valley and North Eastern parts of Kenya.

Local regulations specify recommended levels of the feed components for crude protein, crude fat, total ash and mineral contents for dairy meal, pig feeds, poultry feeds and also raw materials such as maize bran, cotton seedcake, sunflower seedcake and wheat bran. Ban on animal based protein sources in ruminant feed by the European Union following the outbreak of mad cow disease has pushed the local industry towards looking for additional plant based protein sources for use in the feed formulation process. In the feed industry, crude protein sources are still classified as the most expensive feed formulation component and therefore identification of additional sources may lead to reduction in costs. Protein malnutrition is still a problem to poor households in the developing world hence need to identify additional sources of protein.

In this project *Tylosema fassoglense* seed was analysed for various nutritional components such as crude protein, crude oil, crude fibre, total ash, mineral components (copper, iron and zinc). Crude protein was determined using kjeltec analyzer and found to be 24.2±0.4%. Crude oil was determined using sohxlet solvent extraction technique and found to be 22±0.3%. Crude fibre was determined using acid/alkali digestion followed by ignition in muffle furnace and found to be 13.4±0.1%. Mineral composition was determined using Inductively Coupled Plasma-Optical Emission Spectrometry and found to be 16.9±0.6mg/kg, 57.4±1.5mg/kg, and 29.3±1.9mg/kg for copper, iron and zinc respectively. Total ash was determined by igniting the seed sample in a muffle furnace and found to be 3.3±0.05%. Amino acid profile was determined using Ion Exclusion Chromatography and found to contain essential amino acids. The essential amino acids was made up of 0.760% methionine, 6.007% Lysine, 3.046% threonine, 5.216% valine, 4.688% isoleucine, 6.176% leucine, 4.844% phenylalanine and 13.746% tyrosine of the total amino acids respectively.

Fatty acid profile was determined using gas chromatography with Flame Ionization detection and found to contain 24% saturated fatty acids (SFA), 24.7% monounsaturated fatty acids (MUFA) and 48.7% polyunsaturated fatty acids (PUFA). The dominant saturated fatty acid was found to be palmitic acid at 15.18% followed by docosanoic acid at 5.08% and stearic acid at 3.77% respectively. MUFA comprised of oleic acid at 23.50% and docosenoic acid at 1.44% as the major components. PUFA was predominantly linoleic acid at 46.64% and linolenic acid at 2.75%. The carbohydrate content was determined by difference and found to be 31.2%.

The results of the study show that *Tylosema fassoglense* seed is a good source of protein, fat and minerals that may be useful in animal feed formulation as well as human food.

CHAPTER ONE

1.0 INTRODUCTION

Formulation of animal feeds entails combination of various raw materials in order to come up with a final product capable of meeting dietary needs of the animal under consideration. To achieve this goal, animal and plant based raw materials are investigated for various nutritional components such as proteins, carbohydrates, fats, fibre and minerals present for consideration in formulation of animal feed. This calls for use of different analytical techniques such as gravimetry, atomic spectroscopy, gas chromatography, ion exclusion chromatography to quantify the proximate components (proteins, fats, carbohydrates, and moisture), mineral elements, fatty acids and amino acids.

Plants of the leguminosae family are known to be rich in the essential nutrients and are therefore used extensively in the animal feed industry. Proteins are necessary for provision of energy and maintaining normal metabolic demands of the body; Oils also supply energy and essential polyunsaturated fatty acids like linoleic acid which cannot be synthesized by animals and therefore must be obtained from dietary sources. Mineral elements for example; iron; zinc and copper play various roles in biochemical processes such as support of plant growth, formation of reactive oxygen species, protein metabolism, nucleic acid synthesis or stabilization of DNA binding proteins. Fibre ensures maintenance of healthy condition of the intestines by holding water and reducing stool transit time in the large intestine (Potter *et al, 2007*).

In Kenya as well as in other developing countries, crop produce constitute a major part of diet both for human and animal consumption. Cereals and pulses contribute about 50 % of protein needs for human consumption while for domestic animals; compounded feeds are also used to

complement these cereals and pulses sources especially in dairy farming or commercial poultry farming (Belitz and Grostch, 2009). The composition of these foods/feeds is poorly documented in the Kenyan market especially in the informal sector where raw materials are rarely monitored for the essential nutrients like protein, fat, fibre and mineral composition. Formulation of Kenya Standards through Kenya Bureau of Standards (KEBS) for animal feeds and raw materials for the same takes into consideration the stated essential nutrients levels as well as setting limits for toxic materials in the final products (KEBS, 1989, KEBS, 2009, KEBS, 1990). Kenya Standard Specifications for dairy meal, poultry feeds, pig feeds and dog feeds indicate the accepted levels of moisture, protein, oils, fibre and mineral elements such as iron copper and zinc among other requirements (KEBS, 1989, KEBS, 2009, KEBS, 1990). Protein component is still classified as the most expensive ingredient in the whole feed manufacturing process owing to scarcity of the raw materials locally. At the moment, maize bran, wheat bran, cotton seedcake, sunflower seedcake, fish meal and bone meal are the most commonly used sources of protein in feed formulation (KEBS 2009, KEBS, 2008).

Legumes are a well known source of protein in local diet and members of the leguminosae family such as beans (*phaseola vulgaris*) are widely cultivated in the country; however, crude protein and mineral composition of plants tend to be affected by phenotype and location or soil factors (Potter *et al*, 2007). The plant *Tylosema fassoglense* belongs to the genus Tylosema (synonym bauhinia) and is a member of the Leguminosae/Fabaceae family and sub-family Caesalopinioideae (Turner, 1971). It is widely distributed in Nyanza, Western, Rift valley and Eastern provinces of Kenya and parts of North Eastern province of Kenya, (Maundu *et al*, 1999). The plant is a tuber, grows as a climber with yellow/purple flowers and is known to be hardy (Herklots, 1976, Maundu *et al*, 1999). The seed pods are used locally by potters to smoothen the

surfaces of their pottery works while the seeds are occasionally consumed after roasting; the dried root tuber is used as a beverage and medicine by the Luo community; the leaves are used as fodder for domestic animals; the stem is used in some communities as a makeshift rope (Kokwaro , 1976, Maundu *et al*, 1999).

CHAPTER TWO

2.0 Literature Review

Mature seeds of plant family leguminosae commonly known as legumes are an important source of proteins for much of the world population. They contain relatively high amounts of proteins which explain their use as an indispensable supply of protein within the economically emerging countries (Mathews, 1989, Mark J.M, 1999). The extent of world production of legumes is illustrated in Table 2.1 below (Belitz *et al*, 2009).

Table.2.1: World production of seed legumes in 2006 (1000t) [Belitz and Grotsch,2009]

CONTINENT	Legumes Total[a]	Beans[b]	Broad beans	Peas	Chick peas	Lentils	Soybeans	Peanuts[c]
World	60194	19559	4577	10563	8241	3455	221501	47768
Africa	11111	2856	1321	382	324	105	1417	8967
America (central)	2185	1853	37	8	163	7	124	175
America(north)	6025	1430	18	3405	231	928	91203	1479
America(south and caribbean)	6865	6153	158	94	170	16759	98885	1026
Asia	28505	8701	2256	2392	7365	2316	26334	36258
Europe	6841	404	719	3898	43	48	3607	9
Oceania	846	15	104	392	108	41	55	29

[a]without soybeans and peanuts

[b]without broad beans

[c]with hull included

Cases of protein malnutrition still persist inspite of the production levels illustrated (Saskia and Bloem, 2009, Iqbal *et al*, 2006). Previous work on plant seeds as source of protein for food or feed has shown that members of the leguminosae family are rich in essential amino acids recommended for daily nutritional requirements (VanEtten *et al*, 1967; .Olafoe, 1994; Viano *et*

al, 1995; Maestri *et al*, 2002;Vadivel and Janardhan, 2005). Legume seeds such as soybeans and peanuts are used in the industrialized world as sources of raw protein as well as oil (Karr-Lilienthal *et al*, 2004). *Tylosema fassoglense* is a member of leguminosae family (Maundu *et al*, 1999). It is widely distributed in western parts of Kenya where it grows in the wild and as such there is no record of it being cultivated for either domestic or commercial use. Among the *Luo* ethnic community, the plant is known as *'ombasa'*. Over the years, potters in this community have used the seed pod for smoothening pot surfaces during molding (Maundu *et al*, 1999) while the tubers are dried and used as medicine (Kokwaro J.O, 1976). The mature seed is consumed occasionally after roasting though this tends to be done mainly by children (Maundu *et al*, 1999). The picture of the plant and its various parts (leaves, flower, seed pod and seed) is shown in Figures 2.1 and 2.2 below; the geographical distribution of the plant in Kenya is shown in the Figure 2.3 below (Maundu *et al*, 1999)

Figure 2.1: *Tylosema fassoglense* plant

Figure 2.2: *Tylosema fassoglense* seed and pod

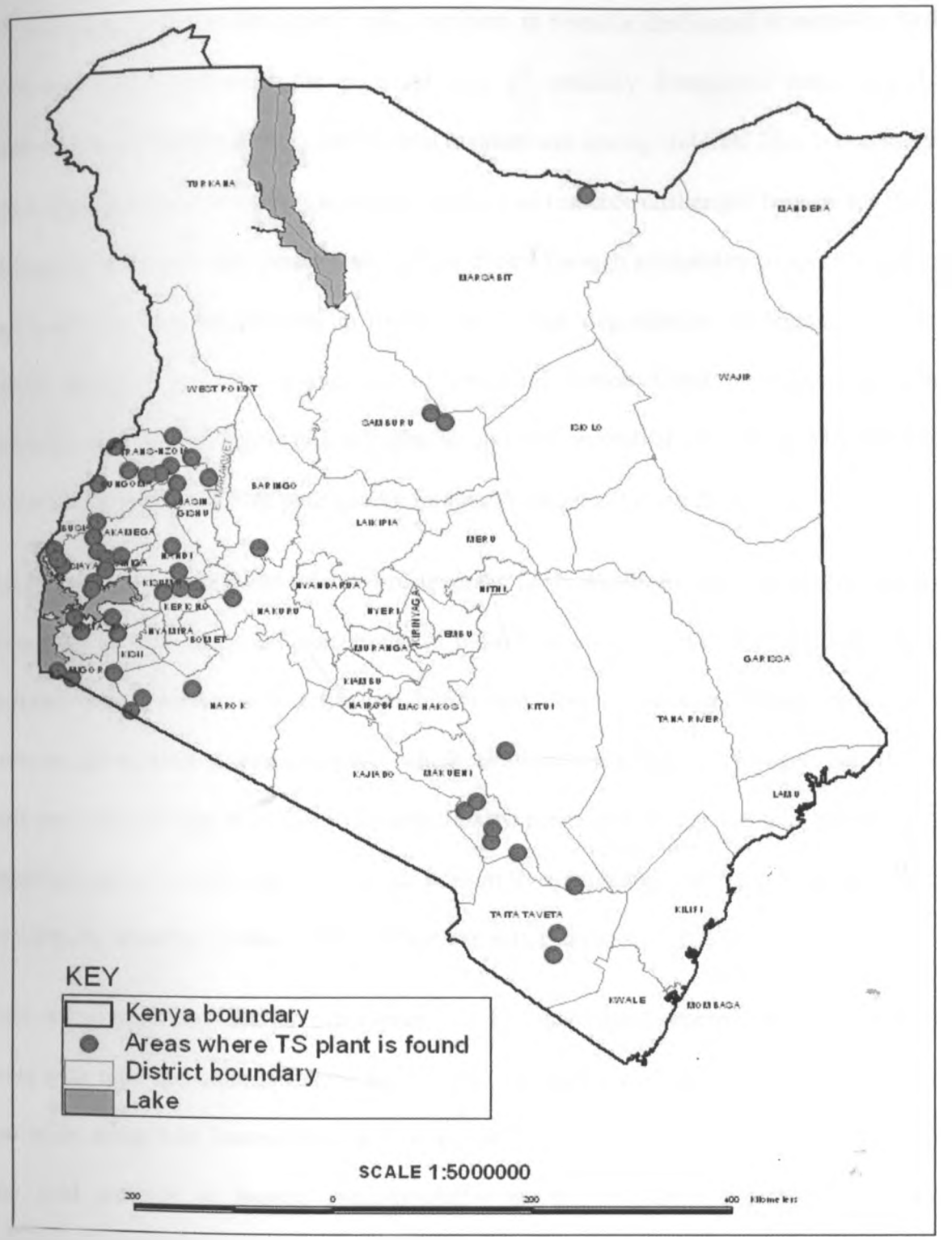


Figure 2 3 Map showing distribution of Tylosema fassoglense plant in Kenya

Animal based protein sources tend to be of superior quality compared to plant sources (Halver, 1986); however, access to the same is still a problem in resource challenged households. Saskia and Bloem (2009) reviewed the potential role of specially formulated foods and food supplements in preventing protein and mineral malnutrition among children. This is necessary to mitigate against protein malnutrition among children in resource challenged households besides providing alternatives to the commercial feed producers through availability of specific essential amino acids that may be deficient in routine diets. High degradability of legume seeds may represent an advantage for degradation of structural carbohydrates by supplying enough fermentable energy and degradable nitrogen to optimize microbial activity thereby extending their use in supplementation of poor quality forages (Yáñez-Ruiz *et al*, 2009).

Amino acids play significant roles as precursors in the biosynthesis of biomolecules which are important for normal body function. Some of these biomolecules and their functions include; glutathione which serves as a sulfhydryl buffer and detoxifying agent (Fang, *et al*, 2004); glutathione peroxidase, a selenoenzyme which catalyzes reduction of hydrogen peroxide and organic peroxides (Stryer *et al*, 2007); porphyrins synthesized from glycine and vital in oxygen transport (Stryer *et al*, 2007) and nitric oxide (a short lived messenger) derived from arginine that plays a role in ensuring normal cardiovascular integrity (Stryer *et al*, 2007)

Some amino acids may also have therapeutic or pharmacological actions; tryptophan is used in patients with mild insomnia to induce sleep while formulations enriched with branched chain amino acids along with lowered amounts of aromatic amino acids are used to improve plasma amino acid patterns in hepatic encephalopathy patients (Yoshimura and Harper, 1993). McKnight *et al*(2010) have also shown that, arginine supplementation may be a novel therapy for obesity and metabolic syndrome, acting via decreased plasma levels of glucose,

homocysteine, fatty acids, dimethylarginines and triglycerides as well as improved whole body insulin. J-A Ye *et al* (2010) reported that milk protein and yield tend to be higher in cows fed methionine- and lysine-supplemented pellets when compared with other groups not given the supplemented pellets. Characterization of seed oil and nutrient assessment of *adenanthera pavonina*-an underutilized legume revealed; CP, 29.4%, CO, 18%, ash 2.37%, Cu, 22.8mg/kg; Zn, 45.6mg/kg and iron 102.5mg/kg, essential amino acids (EAA) constituted 32.39% of total amino acids while fatty acid profile revealed that linoleic acid (53.5%) and oleic acid (17.2%) as major fatty acids of the lipid extract. These results points towards possible utilization as a useful supplement for both protein and oil in animal feeding (Ezeagu *et al*, 2004). Low methionine in poultry feed impairs feather condition and reduces egg weight while decrease in plumage condition results in body heat loses and therefore greater feed intake by poultry (Jönsson L and Elwinger K, 2009).

Protein quality of plant based sources remains a concern due to lack of essential amino acids and presence of anti-nutritional factors (Mathews, 1989). This leads to the need to assay relative proportion of the essential amino acids as a measure of the estimated protein quality (El-Adawi and Taha, 2001). Data obtained from amino acid profile analysis may be used to establish the amino acid score of the protein that may eventually give an indication of its nutritional value or quality (Friedman M and Brandon D L, 2001). Studies on red sea bream juvenile reveal that ideal amino acid profile for fish may correspond to that of whole body protein and this may be the most appropriate dietary amino acid pattern for optimum growth (Alam *et al, 2005*).

The oil content of major legume seeds typically range from 1 to 47 % with about half of this made up of polyunsaturated fatty acids (PUFA) (Sridhar and Seena, 2006). Oils from a variety of seeds play a vital role in world trade largely because of their nutritional importance (Potter *et al*,

2007). Omega three (ω3) long chain fatty acids provide the structural basis for development of brain and central nervous system in early stages of growth as well as maintenance of long term health and prevention of some chronic illnesses (Burlingame *et al*, 2009, Thiebaut *et al*, 2009). PUFAs reduce absorption of Cu and Zn in animals while SFAs enhance Fe uptake (Ching *et al*, 2008). The seed from members of leguminosae family contain nutritional components such as crude protein, crude oils and mineral elements and therefore can be used directly as raw material for animal feed formulation or human food (Mathews, 1989, Vadivel and Janardhan, 2005, Viano *et al*, 1995).

Fats supply calories for energy and essential polyunsaturated fatty acids like linoleic acid. The latter cannot be adequately synthesized by animals and as such must be obtained from dietary sources (Belitz and Grosch 2009). Samplels *et al* (2010) reported a good relationship between addition of crushed linseed to feed and α-linoleic acid (ALA) in reindeer meat leading to proposals to supplement animals with long chain polyunsaturated fatty acids (LC-PUFA) where they do not have access to natural pasture.

Good sources of linoleic acids include grains and seed oils from nuts. Soybean seed which is a member of leguminosae family is rich in essential fatty acids comprising 64% polyunsaturated fatty acids (PUFAs) and 21.5% monounsaturated fatty acids (MUFAs) (Mathews, 1989, USDA, 2009). Quality and compositional studies on some edible leguminosae seed oils in Botswana also reveal that MUFAs make upto 52%, PUFAs, 26% with the remainder being SFA at 22% of the *Tylosema esculentum* seed oil (Ketshajwang. et. al, 1998). The role of polyunsaturated fatty acids (PUFAs) in infant nutrition has been investigated with the benefits of docosahexaenoic acid (DHA) to infants clearly stated (Uauy-Dagach *et al*, 1994, Oski, 1997). Steffens *et al* (1995) and Kim *et al*, (1995) investigated the effects of adding various oils to the diet on growth, feed

conversion and chemical composition of *Cyprinus carpio*(carp) reporting superior weight gain, feed conversion ratio and protein utilization in all groups of carp that received fat enriched high energy diets. Gonzalez C.A and Salas-Salvado J (2006) investigated the potential of nuts in the prevention of cancer in human beings showing possibility of protective effect on rectum and colon.

Iron occurs at the active center of biomolecules responsible for oxygen and electron transport. It forms part of metalloenzymes such as oxidases, hydrogenases, reductases, dehydrogenases, deoxygenases and dehydrases (Cotton *et al*, 2008). In biological systems, three well characterized iron complexes exist which include proteins that contain porphyrin units such as haemoglobin, myoglobin and cytochrome P_{450}, a diverse group of proteins that contain non-heme iron but made up of the iron-sulphur clusters like nitrogenase, rubredoxin, and ferredoxins and thirdly, the non heme diiron oxo-bridged species such as hemerythrin, methane monoxygenase, phytoferritins and ribonucleotide reductase (Cotton *et al*, 2008, Guanghua, 2010). It has been reported that plants utilize iron stored in seed vacuoles to support their growth (Briat *et al*, 2010). Mineral assay of various plant seeds confirms this distribution even though the amounts might be influenced by the environments in which the plants grow (Sridhar K.R. *et al*, 2006; El-Adawi *et al*, 2001; Karr-Lilienthal *et al*, 2004, Thavarajah *et al*, 2010).

Copper on the other hand is found in metal containing proteins in both plant and animal tissues; it is an essential element as it plays an important role in the formation of reactive oxygen species in biological processes. (Theophanides and Anastassopoulou, 2002). Copper presence in the ligand binding sites of proteins implies that it exists as a complex with amino acids. Levels of copper in legume seeds have been reported in previous studies though they are quite varied; for

example 2.0-100mg/kg in *canavalia spp* and 14-29.4 mg/kg in soybean (Sridhar *et al*, 2006; Karr-Lilienthal *et al*, 2004).

Zinc is also recognized as an essential mineral element to all forms of life and a large number of diseases and congenital disorders in both humans and animals have been traced to zinc deficiency. It is a constituent of enzymes involved in protein metabolism and nucleic acid synthesis. Its role at the active sites of proteins may vary from cofactor to purely structural support or stabilization of DNA binding proteins. In zinc containing peptides, sulphur and nitrogen atoms of cystein and histidine residues are the most frequent ligating atoms. Metallothionines are also known to serve as a good storage for zinc and their presence in seeds might account for detection of zinc in the same (Cotton *et al*, 2008, Trzaskowski *et al*, 2008).

Indigestible components of plant materials provide roughage and bulk to ensure healthy condition of the intestine (Potter and Hotchkiss, 2007). Cellulose, hemicellulose, lignins, pectins and other plant materials that are not readily digestible are collectively referred to as fibre (Potter and Hotchkiss, 2007). All these substances hold water, tend to soften stools and decrease stool transit time through the large intestine (Potter *et al*, 2007). Seed processing through grinding may affect the physical properties like particle size and in turn the water holding capacity of the fibre. Fibre may also bind minerals making them unavailable for absorption which might lead to mineral imbalance and deficiency (Sridhar K.R *et al*, 2006). This may be attributed to presence of hydroxyl functional groups which are likely to complex the mineral elements hence making them unavailable to the animals. It is therefore important to note that high amounts of fibre in a diet may lower bioavailability of essential mineral elements. Thus, a proper balance must be established so that efforts to fortify food or feeds achieve the intended goals and deliver the required nutrients. Legumes are typically low in fibre and therefore seeds from this family can be

potential candidates in the food fortification campaigns geared towards addressing nutrient deficiency worldwide.

2.1 Problem Statement

The outbreak of Bovine spongiform encephalopathy (BSE) commonly referred to as mad cow disease in Europe triggered a ban on the use of animal based protein sources in ruminant feed formulation as a measure to limit spread of the disease (WHO/FAO, 2001); this in turn exposed the local industry to loses since it relied heavily on animal based sources of protein in feed formulation hence severe shortage of protein source for animal feed formulation targeting the European market (Brookes, 2001).

Detection of melamine in specially formulated infant foods in China (Sunday Times, 2008) and subsequent ban on importation of infant foods into the local market from China by the Kenya government through KEBS highlights the likelihood of exposure of the local consumer to unsafe products. This is due to the the fact that the current tools used in the analysis of protein by the local regulatory authorities showed the products meeting and exceeding the protein requirements as set out in the local regulations(KEBS, 2008).

Protein and mineral malnutrition is still a global concern due to prevalence of preventable diseases in the local population attributed to their deficiency in local diets (WHO, 1993, Iqbal *et al*, 2006)

Recommendations in parts of Europe (Scandinavian countries) that feed sources rich in high quality protein will be prerequisite in organic production i.e. A regulation stating that only diets with 100% of approved raw feed materials will be permitted in organic diets comes into force in the year 2012 (Jönsson L and Elwinger K, 2009, EC, 2007).This implies that Kenyan exporters to this market are likely to face technical barriers on feed exports due to lack of data on protein quality of feed hence need to profile crude protein to establish the quality status.

2.2 Justification

Bans on animal based feeds in the European Union (Brookes, 2001) have pushed the local industry to look for alternative sources of protein in ruminant feed formulation. This in turn has created renewed interest in alternative sources to complement the traditional ones currently in use such as wheat bran, cotton seed cake, maize bran and pulses just to mention a few.

Food fortification campaign is also gaining momentum in the local market to address deficiency of essential nutrients such as minerals and protein especially in food formulation. This calls for reliable information on the nutritional composition of the target fortificant to ensure that only materials with proven nutritional value are utilized in the fortification process.

There is also need to improve on the current kjeltec method of determination of protein, this will in the long run help in protecting the consumer against industry malpractice (like melamine spiking of products) by accurately determining amino acid profile of the crude protein reported. Identification of additional protein sources besides the traditional ones like soybean and *phaseola vulgaris* to boost the fight against protein malnutrition as well as improving on the food security situation in the country is of high importance (FAO, 2004).

2.3 Objectives of the study

2.3.1 Main objective

To evaluate nutritional components of *Tylosema fassoglense* seed for animal feed formulation.

2.3.2 Specific objectives of the study

- To determine and characterize crude protein and oil content of the TS seed
- To determine crude fibre content of the TS seed.
- To determine mineral content of the TS seed {Cu, Fe, Zn}
- To determine calorific value of the TS seed

2.4 Instrumentation

2.4.1 Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES)

2.4.1.0 Introduction to ICP-OES

Atomic emission spectrometry (AES), atomic absorption spectrometry (AAS) and atomic fluorescence spectrometry (AFS) are among the branches of analytical spectrometry that derive analytical information from atomic spectra in the optical region of the electromagnetic spectrum (Fifield, 1995, Skoog, 1985). The optical region refers to the ultra violet region (UV), visible region, and the near infra red (Skoog, 1985). Atomic spectra in this region originate from energy transitions in the outer electronic shells of free atoms or ions.

Atomic emission spectrometry is often denoted 'optical emission spectrometry (OES) in particular to differentiate it from Auger Electron spectroscopy which has similar acronym AES In OES, the sample must be atomized or dissociated into free atoms and or ions (Fifield, 1995, Harris, 2007, Skoog, 1985). This is accomplished through an excitation source which furnishes not only energy for atomization of the sample but also for the excitation of atoms and ions of the element to be determined (analytes). Inductively coupled plasma (ICP) is chiefly used as excitation source for OES although flame and furnaces have also been used on several occasions (Taylor, 2001, Fifield, 1995). A common feature of all excitation sources is the presence of a flowing or stationary gas, the support or discharge gas, the flame gases, the surrounding atmosphere or an artificial, controlled atmosphere. Samples must be brought to the system in a form that can easily be evaporated and atomized by interacting with the hot gases from the excitation source (Fifield, 1995, Skoog, 1985, Taylor, 2001).

The radiation emitted from the excitation source encompasses contribution from all components i.e. the gaseous atmosphere, the free atoms, ions and molecules of the sample, any species formed between the constituents of the gaseous atmosphere mutually or with the sample constituents (Skoog, 1985, Fifield 1995, Taylor 2001). The emission spectra of the source then consists of line and band spectra superimposed on continua that result from (i) various types of interactions including recombination between free electrons and ions (ii) recombinative interaction between atoms and (iii) thermal emission from incandescent solid particles (Skoog 1985, Taylor, 2001). Each of the species present emits a characteristic spectrum from which one or more lines may be chosen to identify the element (qualitative) and also quantify it (Taylor, 2001). Identification of an analyte will entail establishing the presence of one or more sensitive lines of the element while at the same time ascertaining that the lines found don't originate from other sources or elements that emit lines at wavelengths close or similar to the most sensitive lines of the analyte (Fifield, 1995, Skoog, 1985, Harris, 2007). Quantitative analysis derived from this technique use an empirically developed relationship between the intensity of a spectral line of the analyte and its concentration in the sample (calibration curve) (Fifield, 1995, Harris, 2007). It is worth mentioning that the ICP-OES technique has a multielement capability and therefore can perform simultaneous analysis of several analytes all at once and with relatively good precision. A schematic diagram of ICP-OES is shown in Figure 2.4 below:

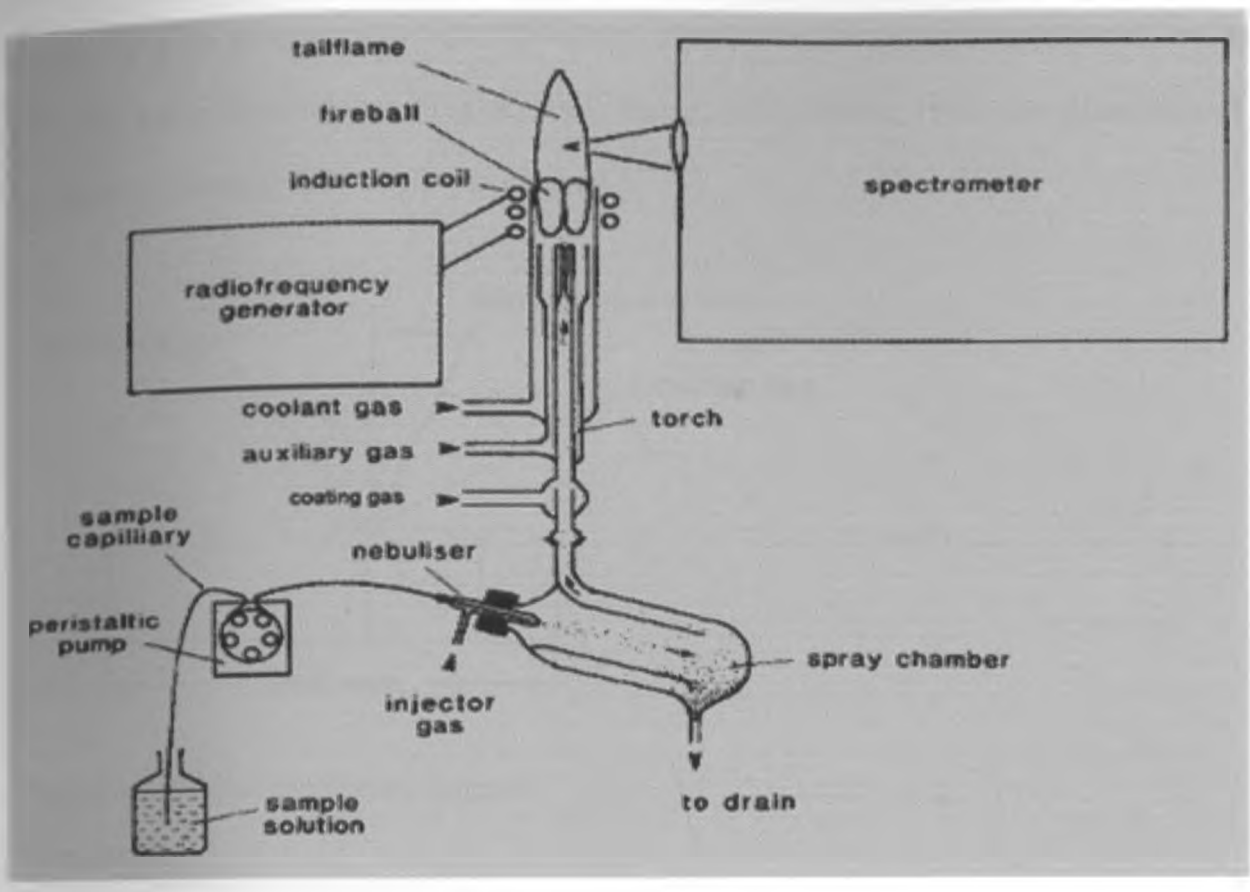


Figure 2.4: ICP-OES schematic diagram

2.4.1.1 Major components of the ICP-OES instrument

Monochromator

Monochromators have a high flexibility of line choice; principally any wavelength for line and background measurement is accessible. A monochromator enables a specific band to be selected and it is the separation of a specific line that ensures good precision of analytical results

Typical monochromators commonly used are diffraction gratings. Diffraction gratings are dispersive optical components with grooves or lines parallel to each other. Interferometric gratings are considered superior to ruled gratings as they have very high stray light rejection and lines are better distributed across the surface of the substrate. Because interferometric gratings

have negligible groove errors, high resolution is possible i.e. the higher the number of lines per mm the better the resolution (Taylor, 2001, Skoog, 1985, Fifield, 1995). An illustration of a diffraction grating is shown below in Figure 2.5.

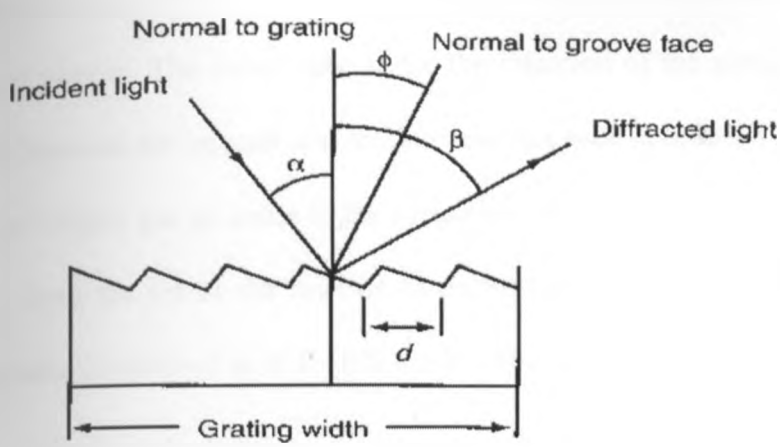


Figure 2.5: Diffraction grating diagram

Generator

The generator consists of a water cooled coil of a radiofrequency (RF) generator. The RF is switched on at high power and the gas in the coil region is made electrically conductive by Tesla sparks. Plasma will then be formed provided the magnetic strength is high enough and the gas streams follow a particular rotationally symmetrical pattern. The RF currents flowing in the coil generate oscillating magnetic fields with lines of force axially oriented inside the coil. These induced magnetic fields generate in turn high annular electric currents in the conductor which is then heated as a result of its ohmic resistance (Taylor, 2001).

The Torch

It is used to contain and assist in maintaining the plasma. Torches are typically made of materials transparent to RF radiation and therefore they do not attenuate the field generated by the load coil/antenna. A typical quartz torch suitable for the formation of atmospheric pressure argon

plasma consists of three concentric quartz tubes. A coolant gas (argon) is introduced into the space between the outer and centre tubes at a tangential direction relative to the longitudinal axis of the torch creating a vertical flow. This gas stream (a) isolates the plasma from the internal wall of the outer quartz tube preventing melting. (b) It encourages the formation of toroidal (annular) shaped plasma. The centre tube is for the injection of the sample aerosol into the plasma. The space between the injector and the intermediate tube is used for the introduction of the auxiliary flow of Argon gas to assist in the formation of the plasma and ensure that the plasma is forced away from the tip of the injector to prevent it from melting (Fifield, 1995, Taylor, 2001). A schematic diagram of an ICP-OES torch is shown in Figure 2.6 below:

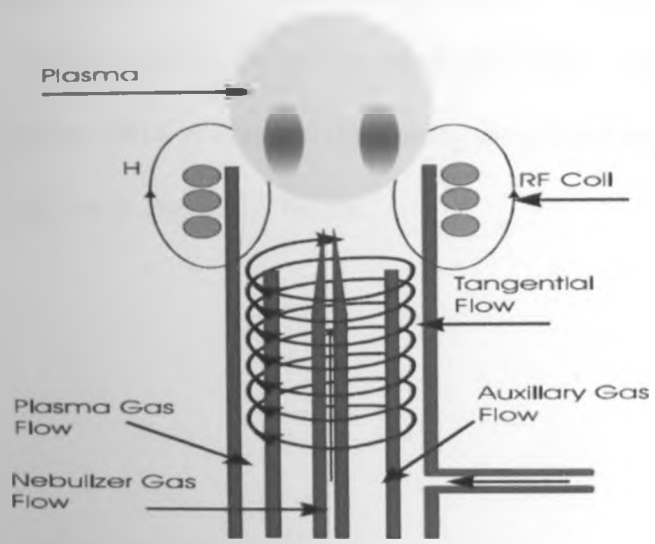


Figure 2.6: Schematic diagram of an ICP torch

Plasma

Plasma is a distinct phase of matter, separate from the traditional solids, liquids, and gases; it is a collection of charged particles that respond strongly and collectively to electromagnetic fields, taking the form of gas-like clouds or ion beams (Fifield, 1995, Skoog, 1985, Taylor, 2001). Since

the particles in plasma are electrically charged (generally by being stripped of electrons), it is frequently described as an "ionized gas." (Fifield, 1995, Skoog, 1985, Taylor, 2001).Inductively coupled plasmas are formed by coupling energy produced by RF generator to the plasma support gas with an electromagnetic field. The field is produced by applying RF power (700-1500 W) to an antenna/load coil constructed from 3 mm Cu tubing wrapped in a two or three turn 3cm diameter coil positioned around the quartz torch assembly designed to configure and confine the plasma. The plasma is generated by a few 'seed' electrons generated from the spark of a tesla coil to the flowing support gas in the vicinity of the load coil. After creation, the plasma is sustained by inductive coupling i.e. seed electrons are accelerated by the RF field collisions with neutral gas atoms to create the ionized medium of the plasma. These collisions produce additional electrons and the cascading effect creates and sustains the plasma (Skoog, 1985, Taylor, 2001). A diagram illustrating the plasma and the various heating zones within the plasma is shown in Figure 2.7 below.

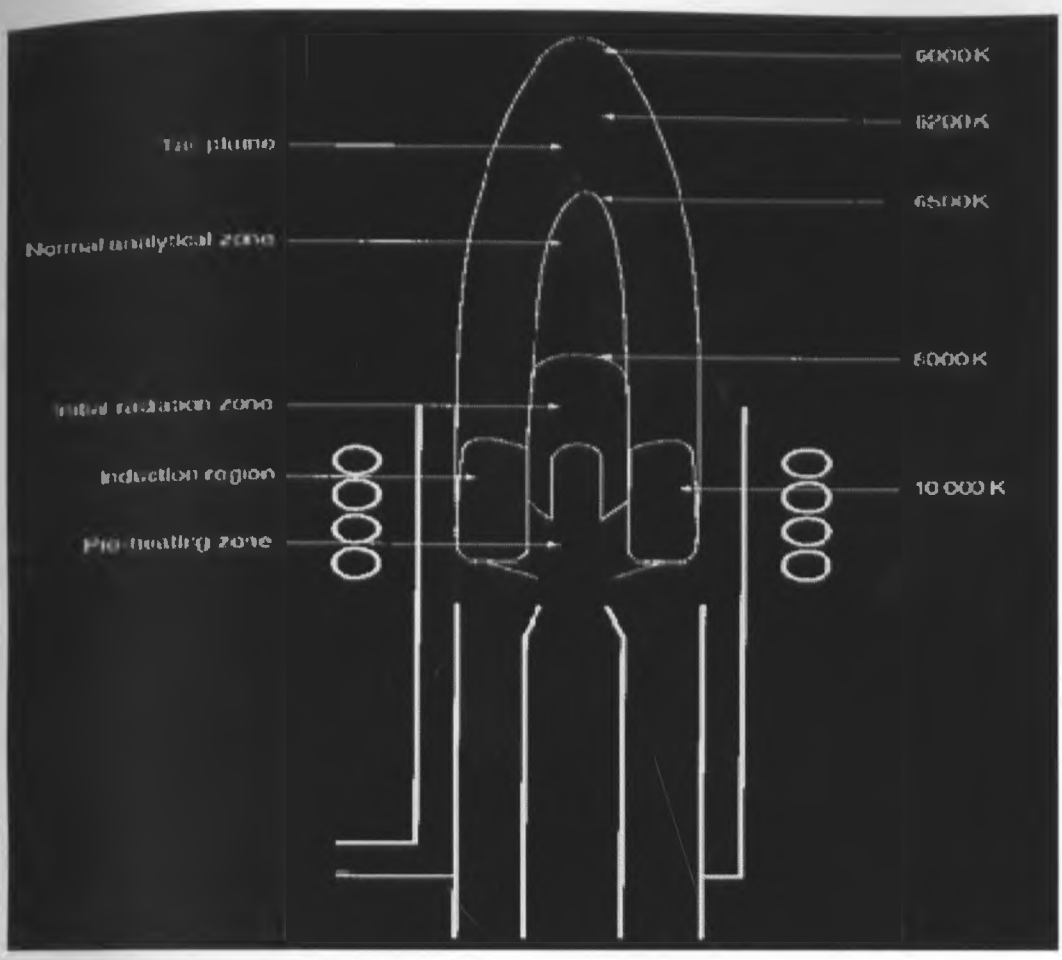


Figure2.7: The plasma diagram

Sample introduction design

The sample in liquid state is pumped into a nebulizer using a peristaltic pump. The nebulizer then converts the liquid sample into an aerosol consisting of finely divided droplets which are suspended in the plasma carrier gas. The flowing gas creates the aerosol that is finally carried to the plasma through the spray chamber for excitation.

The most common type of nebulizer used is the concentric pneumatic type illustrated in Figure 2.8 below.

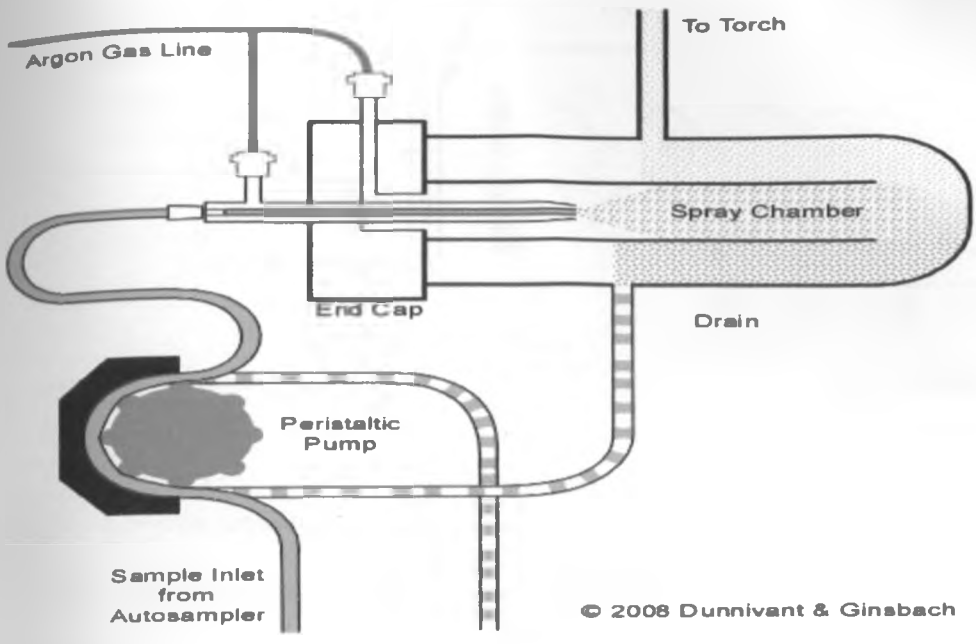


Figure 2.8: Sample introduction chamber with pneumatic concentric nebulizer

Detector

Once an emission line has been isolated by a spectrometer, a detector is used to measure the intensity of the emission line. Single or multi-channel detectors can be used. Photomultiplier tubes are the most commonly used single channel detectors because of their internal amplification and larger dynamic range. They are capable of converting radiation intensities directly into electronic signals of sufficiently high levels for further handling through their dynode systems.

The most commonly used multi-channel detector is the photographic plate. In this case, the number of channels is determined by the grain size of the emulsion and the plate is capable of recording thousands of spectral lines in a single exposure. However, it should be noted that the photographic plate has low sensitivity, non linear response characteristic and time consuming readout as its major disadvantages. A photomultiplier tube (PMT) is illustrated in Figure2.9 below:

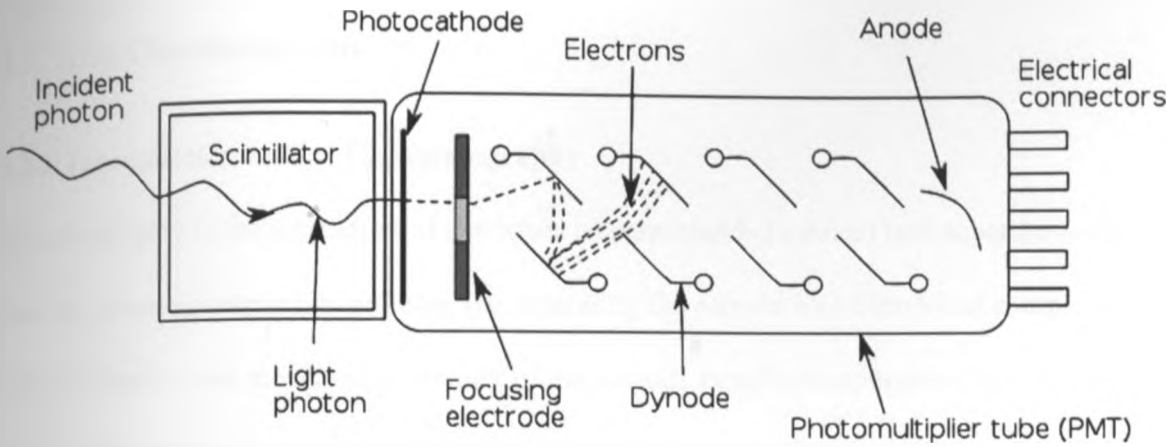


Figure 2.9: Photomultiplier tube diagram

In this project ICP-OES was used to determine the trace elements in axial view mode with Indium as an internal standard after appropriate sample preparation.

2.4.2 Gas Chromatography

2.4.2.0 Introduction to Gas Chromatography

Chromatography is the separation of a mixture of compounds (solutes) into separate components based on solute adsorption to column. By separating the sample into individual components, it is easier to identify and measure the amount of the various sample components (Harris, 2007). There are numerous chromatographic techniques and corresponding instruments. Gas chromatography (GC) is one of these techniques. It is estimated that 10-20 % of the known compounds can be analyzed by GC. To be suitable for GC analysis, a compound must have sufficient volatility and thermal stability (Harris, 2007, Willard *et al*, 1981). Compounds stable and existing as gas or vapour at 400- 450 ^{o}C or below can be analyzed by GC.

One or more high purity gases are supplied to the GC. One of the gases (called the carrier gas) flows into the injector, through the column and then into the detector. A sample is introduced into the injector usually with a syringe or an exterior sampling device. The injector is usually heated to 150-250°C which causes the volatile sample solutes to vaporize. The vaporized solutes are transported into the column by the carrier gas. The column is maintained in a temperature controlled oven (Harris, 2007, Willard *et al*, 1981).

The solutes travel through the column at a rate primarily determined by their physical properties, and the temperature and composition of the column. The various solutes travel through the column at different rates. The fastest moving solute exits (elutes) the column first then is followed by the remaining solutes in corresponding order. As each solute elutes from the column, it enters the heated detector. An electronic signal is generated upon interaction of the solute with the detector. The size of the signal is recorded by a data system and is plotted against

elapsed time to produce a chromatogram. (Harris, 2007, Willard *et al*, 1981)

The ideal chromatogram has closely spaced peaks with no overlap of the peaks. Any peaks that overlap are called co-eluting. The time and size of a peak are important in that they are used to identify and measure the amount of the compound in the sample. The size of the resulting peak corresponds to the amount of the compound in the sample. A larger peak is obtained as the concentration of the corresponding compound increases. If the column and all of operating conditions are kept the same, a given compound always travels through the column at the same rate. Thus, a compound can be identified by the time required for it to travel through the column (called the retention time) [Harris, 2007, Skoog, 1985].

The identity of a compound cannot be determined solely by its retention time. A known amount of an authentic, pure standard sample of the compound has to be analyzed and its retention time and peak size determined. This value can be compared to the results from an unknown sample to determine whether the target compound is present (by comparing retention times) and its amount (by comparing peak sizes) [Harris, 2007, Skoog, 1985, Willard *et al*, 1981].

If any of the peaks overlap, accurate measurement of these peaks is not possible. If two peaks have the same retention time, accurate identification is not possible. Thus, it is desirable to have no peak overlap or co-elution. (Fifield, 1995, Harris, 2007). A schematic diagram of a typical gas chromatograph is shown in Figure 2.10 below:

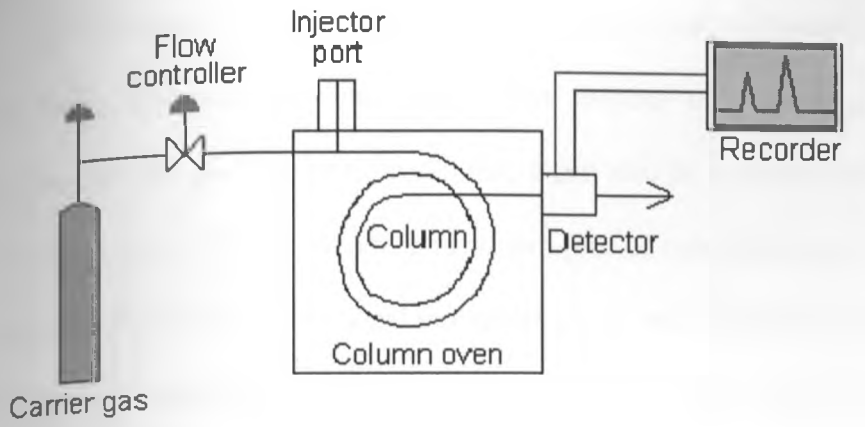


Figure 2.10: Schematic diagram of typical Gas Chromatograph

2.4.2.1 Gas Chromatography (GC) instrumentation

Columns

This is where the actual separation takes place after injection of the sample into the port where it evaporates and is swept through by the carrier gas. Most analyses use long narrow open tubular columns made of fused silica and coated with polyimide. Open tubular columns offer higher resolution, shorter analysis time and greater sensitivity than packed columns though they have less sample capacity. Wall coated columns feature approximately 0.5 μm thick film of liquid phase on the inner wall of the column while support coated column has solid particles with stationary liquid phase attached to the inner wall. In porous layer type of columns, the solid particles are the active stationary phase (Willard *et al*, 1981, Harris, 2007). Due to their higher surface area, support coated columns can handle larger samples as compared to wall-coated columns. Their performance is intermediate between those of wall coated columns and packed columns (Harris, 2007).

Narrow columns provide higher resolution than wider columns but they do require higher operating pressures and less sample. The number of theoretical plates(N) on a column is proportional to the length of the column; it can also be stated that resolution is also proportional to square root of N and therefore to square root of column length. Solids used for porous layer open tubular columns are typical molecular sieves with cavities into which small molecules enter and are particularly retained e.g. alumina (Al_2O_3). Packed columns contain fine particles of solid support coated with non volatile liquid stationary phase. They provide greater sample capacity but give broader peaks, longer retention times and less resolution. They are usually made of stainless steel or glass. Smaller particles will improve column efficiency though more pressure would be required to force the mobile phase through the column (Fifield, 1995, Harris, 2007).

Sample injection design

Sample injection can be manual or automatic and may be done in a split, split-less or column mode using a syringe. Split injection introduces a sample into the oven where it is passed through a mixing chamber before a portion is pushed into the column while the remainder goes to the vent. In split-less mode, there is no mixing chamber and the volatiles are pushed direct to the column. On-column injection involves introducing the solution directly into the column without going through a hot injector. The initial column temperature is low and column is heated gradually to initiate chromatography (Harris, 2007).

It is worth noting that in all the sample injection modes described, the initial temperature on injection shall always be lower than boiling points of solutes of interest. An illustration of a split/split less injector system is given in Figure 2.11 below:

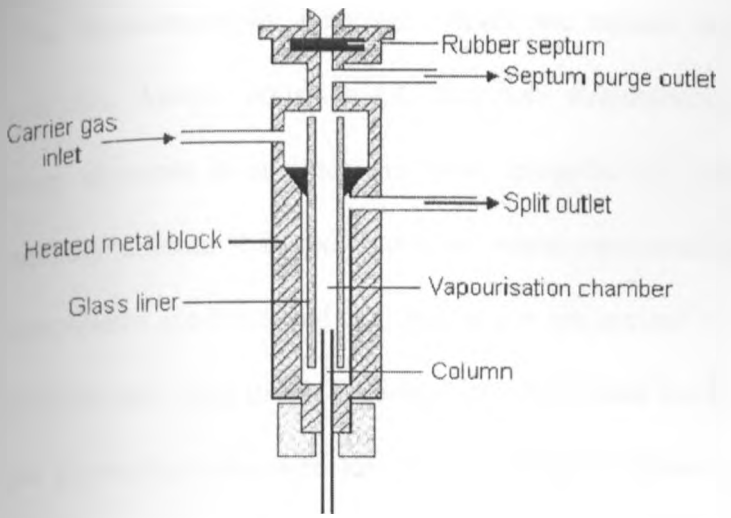


Figure 2.11: The split/splitless injector diagram

Detectors

Gas chromatography detectors (GC detectors) identify solutes as they exit a chromatographic column. As solutes are eluted from the column they interact with the gas chromatography detector. Gas chromatography detectors convert this interaction into an electrical signal that is sent to the data system. The magnitude of the signal is plotted from the time of injection and a chromatogram is generated. Gas chromatography detectors use one of several technology types to identify solutes as they exit the column. Choices include flame ionization, atomic emission, electron capture, photoionization, flame photometric, chemiluminescence spectroscopy, and nitrogen phosphorous detectors (Harris, 2007).

Thermal conductivity and flame ionization are two types of gas chromatography detectors. Thermal conductivity GC detectors have an electrically-heated wire or thermistor. The temperature of the sensing element depends on the thermal conductivity of the gas flowing around it. Changes in thermal conductivity cause a temperature rise in the element, which is sensed as a change in resistance (Willard *et al*, 1981, Harris, 2007).

Gas chromatography detectors which use atomic emissions or electron capture are also available. Atomic emission GC detectors simultaneously determine the atomic emissions of many elements in analytes that elute from the GC capillary column. As the eluant exits the capillary column it is fed into a microwave-powered plasma (or discharge) cavity where the components are destroyed and their atoms are excited by the energy of the plasma. These excited particles emit light that is separated into individual lines via a photodiode array. Electron capture gas chromatography detectors use a radioactive Beta emitter (electrons) to ionize some of the carrier gas and produce a current between a biased pair of electrodes. When organic molecules contain electronegative functional groups such as halogens, phosphorous, and nitro, groups pass by the GC detector and capture some of the electrons while reducing the current measured between electrodes (Harris, 2007).

Photoionization gas detectors, chemiluminescence spectroscopy gas detectors, and nitrogen phosphorous gas detectors are all types of gas chromatography detectors. Photoionization GC detectors use ultraviolet light as a means of ionizing an analyte exiting from a GC column, allowing electrodes to collect the ions. The generated current is a measure of the analyte concentration (Harris, 2007).

Chemiluminescence spectroscopy gas chromatography detectors use quantitative measurements of the optical emission from excited chemical species to determine analyte concentration. Typically, the emission is measured from energized molecules rather than excited atoms. The bands of light determined by this technique emerge from molecular emissions and are broader and more complex than bands which originate from atomic spectra (Willard *et al*, 1981, Harris, 2007).

Nitrogen phosphorous gas chromatography detectors burn the compound in the plasma surrounding a rubidium bead is supplied with hydrogen and air. Nitrogen and phosphorous-containing compounds produce ions that are attracted to the collector. A signal is generated from the measurement of the number of ions hitting the collector.

Flame ionization gas chromatography detectors consist of hydrogen or an air flame and a collector plate. The effluent from the GC column passes through the flame, which breaks down organic molecules and produces ions. A collector electrode attracts the negative ions to the electrometer amplifier producing an analog signal which is connected to the data system input. In this project, the FID detector was used in the analysis of fatty acid profile and it is illustrated in the Figure 2.12 below.

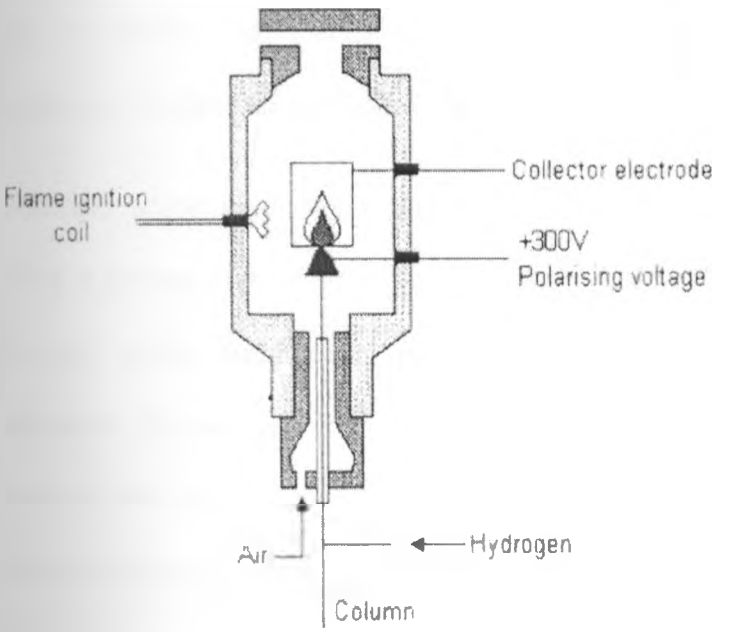


Figure 2.12: Flame ionisation detector diagram

2.4.3 Ion Exchange Chromatography

Typical set up of Ion exchange chromatograph advanced single purpose amino acid analyzer consists of buffer solution reservoirs, a reservoir for regeneration solution and a set of solenoid valves that are operated by a programmer. A buffer pump and autoloader ensure sample application and elution of separated amino acids. Separation is carried out in a thermostated ion-exchange column. From a ninhydrin reservoir, the reagent is delivered to a mixing block through a separate ninhydrin pump; the eluate mixed with the reagent passes through the reaction coil and detector (Nollet, 1992). The recorder and the programmer are attached to the data system unit, essentially a computing integrator that produces reports of retention times, peak areas, calibration factors and concentration units.

Generally amino acids are separated in their native form on a sulphonated polystyrene resin using a system of sodium or lithium citrate buffers. In ion exchange separations, pellicular, strongly acidic, sulphonated polystyrene based cation exchangers are used, for example; ultropac8, Kyowa gel, Perkin Elmer high speed amino acid analysis column, LKB high performance column and Hitachi AAA Standard packed PH column are some of the commercially available columns in the market (Nollet, 1992, Harris, 2007).

In a classical ninhydrin analyzer, separation is effected stepwise, rather than gradient. Elution and chromatography can be further optimized by the careful control of the temperature of the analytical column. Hydrolysates of protein and foodstuff samples can be separated on a three- or four-buffer sodium system while physiological fluids require use of five lithium buffers.

Continuous gradient elution e.g. sodium citrate (pH 3.2) to Sodium borate (pH 9.8) can be used for amino acid analysis by HPLC with ion exchange chromatography. The post column ninhydrin reaction proceeds in a reaction coil at elevated temperatures (130 - 135 °C). Ninhydrin reacts with primary amino acids to give a chromophore with a wavelength of maximum absorption of 570 nm (Nollet, 1992). With secondary amino acids, it forms a yellow complex with different absorption characteristics and therefore detection at 570 nm and 440 nm is normally used.

When a mixture of amino acids percolates through a column of sulphonated polystyrene, the individual amino acids separate into discrete zones. The resolution is effected by ionic and hydrophobic interactions between resin matrix, amino acid molecules and by the flow of buffers through the column. In acid hydrolysates of proteins, the commonly observed order of elution of the amino acids from a column of sulphonated polystyrene is as follows: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine (Nollet, 1992). These can be further grouped into three general classes according to the nature of their side chains: acidic and hydroxyl amino acids (aspartic through glutamic acid), neutral amino acids with aliphatic and aromatic chains (proline through phenylalanine) and basic amino acids (lysine through arginine) [Nollet, 1992]. The negatively charged resin repels the molecules with negatively charged side chains whereas it strongly binds basic amino acids in which the side chains are positively charged at the pH of the eluting buffers. (Hancock, 1989, Nollet, 1992)

The net charge on molecules may also explain the elution order of some similar amino acids, for example, the carboxyl groups of aspartate are stronger acids than those of glutamate as revealed by the isoelectric point for aspartate of pH 2.76 compared with pH 3.17 for glutamate. At the pH

of the first eluting buffer, aspartate behaves as a slightly stronger acid than glutamate and elutes first implying that elution order follows a pattern of increasing isoelectric points (Nollet, 1992).

Another factor affecting elution order is one of hydrophobic interactions that may form between non-polar side chains and the cross-linked polystyrene resin. Amino acids with short side chains elute before those with long side chains, for example valine emerges before leucine. As branching in the side chains is increased, elution time decreases thus, the order of elution of 6-carbon aliphatic amino acid is: isoleucine, lucine, and norleucine (Nollet, 1992). Hydroxylation accelerates elution for example, serine appearing before alanine, tyrosine before phenylalanine, hydroxyproline before proline and hydroxylysine before lysine. (Nollet, 1992)

Post column derivatization has proven reliable in the analysis of amino acids in various food products and even physiological fluids. Ninhydrin or orthophthaldehyde (OPA) is pumped into the reaction coil maintained at about 49 ^{o}C where it reacts with eluates from the cation exchange resin column. The complex formed is purple or yellow and can be detected at 540 nm or 440 nm. Typical run times are anywhere between 30 minutes to 90 minutes (Hancock, 1989, Nollet, 1992).

In this project, ion exchange chromatography was used to separate the amino acids and post column ninhydrin derivatization for detection of the amino acids present in the protein hydrolyzate.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Materials/ Equipment and Reagents Used

3.1.1 Reagents

Acetone, AR grade, purity, >99.5%, Sigma-Aldrich,

Sodium hydroxide (NaOH), AR grade, Purity, 98%, Panreac,

1.5%m/v Sodium hydroxide: 2.5grams of NaOH pellets were dissolved in 100mL of distilled water transferred to a 200mL volumetric flask then topped to the mark using distilled water.

Concentrated Sulphuric acid, AR grade, Purity, 98%, Rankem

1.25%(v/v) Sulphuric acid: 2.5 mL of Conc. H_2SO_4 was added to 100mL of distilled water in a 200mL volumetric flask then topped to the mark using distilled water

Kjeltabs, Thomson and Capper

Petroleum ether (40-60 ^{o}C), AR grade, purity, 99.5% min, Sigma-Aldrich

Hydrochloric acid, AR grade, purity, 37%, Panreac Ltd

0.3M Hydrochloric acid

Sodium citrate buffer, pH 3.2: Dissolve 1.7040g of Citric acid and 0.5558g of sodium citrate in one litre of water.

Sodium citrate buffer, pH 3.3: Dissolve 1.6555g of Citric acid and 0.0.6237g of sodium citrate in one litre of water.

Sodium citrate buffer, pH 4.0: Dissolve 1.3115g of Citric acid and 1.1051g of sodium citrate in one litre of water.

Sodium citrate buffer, pH 4.9: Dissolve 0.8231g of Citric acid and 1.7889g of sodium citrate in one litre of water.

Ninhydrin solution (Concentrated and 50%v/v)

Ethanol, AR grade, purity, 99.5%, Merck

10%v/v Ethanol, 10 mL of ethanol was dissolved in 100mL of distilled water in a volumetric flask

Sodium hydroxide, AR grade, purity, 97%, CDH

Sodium hydroxide-GR grade, Purity, 99-100%, Lobachemie

Methanol –GR grade, Purity, 99.8%, Lobachemie

Boron Trifluoride (BF_3)-10% in methanol, puriss grade, Sigma- Aldrich

Isooctane, 100%, Burdik and Jackson

Sodium chloride-GR, Purity, 99-100%, Lobachemie

Sodium hydroxide (NaOH), AR grade, purity, 98%, Panreac

Alcoholic sodium hydroxide 0.5M (2g of NaOH dissolved in methanol then made to 100mL using methanol)

Saturated sodium chloride (NaCl) solution (36gNaCl dissolved in 100mL distilled water)

Hydrofluoric acid (HF), AR grade, purity, 48%, Sciencelab

Nitric acid (HNO_3), AR grade, purity, 69.8%, Mallinckrodt

1.5%v/v Nitric acid (1.5%v/v HNO_3): 15mL of Nitric acid was added to about 400mL of distilled water then topped to 1 litre using distilled water.

SRM 1573a Tomato Leaves, NIST

SRM 1566b Oyster Tissue, NIST

SRM 3114 Copper Standard Solution, concentration, 9.993±0.016mg/g, NIST

SRM 3126a Iron Standard Solution, Concentration, 10.001±0.023 mg/g, NIST

SRM 3168a Zinc Standard Solution, Concentration, 9.998 ±0.026 mg/g, NIST

Indium solution (1.0mg/g), NIST

3.1.2 Equipments/ Materials

Pyrex Weighing bottles 30mL capacity

AT 261 Delta Range Mettler Analytical balance, capable of weighing ± 0.00001g

Analytical balance, Mettler Toledo model capable of weighing ± 0.0001g

Muffle furnace, Heraeus model capable of maintaining a temperature of at least 500 °C

Foss Fibre crucibles, class B porosity 2

Foss Fibretech equipment, Serial No. 2457

Heraeus Muffle furnace capable of maintaining a temperature of at least 500 °C

Foss Acid digestion block

Foss Acid digestion vessels

Foss Kjeltec analyzer, Serial No. 24101

Foss Sohxlet extraction unit, Serial Number 663

Oil bath

Hitachi L-8900 protein hydrolyzate Amino Acid Analyzer fitted with (i) Hitachi packed guard column, 4mm IDx5mm and (ii) Hitachi AAA standard protein hydrolyzate Column, 4mm IDx60mm

Gas Chromatograph (Shimadzu GC-14®) with capillary injection system, fused silica column (25m, 0.20-0.35mm i.d) with chromosorb-waw 80 / 100 mesh GC packing material.

Carrier gas-helium

Volumetric flasks-25 and 100mL

Pasteur type pipettes

Volumetric pipettes-1 and 2mL

Dry nitrogen source

ICP-OES- Perkin Elmer Optima DV 5500 model fitted with an auto sampler.

CEM®Microwave oven digestor

Teflon digestion vessels

Teflon beakers (125 mL) with lids

Nalgene® LDP bottles (30 mL, 50mL, and 120mL capacity)

Hot plate (Capable of heating up to 200 ^{o}C)

Test tubes (30 mL capacity compatible with ICP-OES auto sampler)

3.2 Sampling

500gm of dry *Tylosema fassoglense* seeds were harvested from mature plants in Nduru, Bugo and Uuna areas of Masumbi Sub-Location, South East Alego Location in Siaya County. The seeds were harvested as follows: Nduru area (200gm), Bugo area (150gm) and Uuna area (150gm).

The seeds were mixed, packed in a zip lock polythene bag then transported to the laboratory for further processing. Ten seed samples were taken from the lot and measured for mass and dimensions to provide information on the approximate size of the seed.

The seeds were shelled using a pestle and mortar to reduce the size then milled in a mini lab mill to approximately 100 microns. The powdered sample was then packed in a 250 mL Nalgene LDP bottle for preservation, sealed and stored pending chemical analysis.

3.3 Experimental

3.3.1 Loss in Mass on Drying (Moisture)

1.5 grams of the sample was transferred into a weighing bottle, the masses recorded and placed into a desiccator containing magnesium perchlorate. The samples were left in the desiccators and masses taken after five days. The certified reference materials were treated similarly and final masses taken after five days. The loss in mass on drying was calculated based on the drop in mass after the five day analysis period.

3.3.2 Total Ash Content

0.5 grams of the sample was transferred into platinum crucibles, placed in a muffle furnace and ignited at 600 °C to constant mass. After ignition, the samples were transferred in a desiccator, cooled then weighed and the ash content calculated as the percentage loss on mass after ignition at 600 °C. The measurement conditions for total ash determination are summarized in Table 3.1 below:

Table 3.1: Total ash measurement program

Step	Temperature (^{0}C)	Heating rate (^{0}C/minute) to next step	Remarks
1	90	10	
2	130	2	
3	240	5	
4	615[a]	3	Held for one hour at 615 ^{0}C

[a] Setting at 615 ^{0}C to compensate for the +15 ^{0}C temperature difference as documented in the calibration certificate (NIST, 2009)

3.3.3 Crude Protein

Exactly 0.3 grams of the sample was weighed directly into the digestion tube, Kjeltabs were added followed by 10 mL of conc. H_2SO_4 then loaded into the pre-heated digestion block and digested for about 30 mins till a green solution formed. The digested sample solution was then cooled and transferred to the Kjeltec analyzer; steam distilled with 40% (m/v) NaOH.The nitrogen evolved was collected on boric acid receiver solution and

finally titrated with 0.5 M HCl. The amount of crude protein in the sample was then calculated based on titre volume and standard Kjeldahl conversion factor of 6.25.

3.3.4 Crude Oil

2 grams of the sample was hydrolyzed in 0.3M HCl, filtered then dried in an oven at 103 °C. The residue was then transferred into an extraction thimble covered with defatted cotton wool and loaded into the extractor. Pre-weighed fat cups containing petroleum ether were fitted into the unit and the oil extracted at about 60 °C. After the extraction process the fat cups were released, dried in an oven maintained at 103 °C, cooled in a desiccator and weighed. The amount of crude oil was determined as the gain in mass recorded after the final weighing of the fat cup.

3.3.5 Crude Fibre

1 gram of the sample was weighed into a fibre crucible, loaded into the cold extraction unit and defatted with acetone. The defatted samples were then loaded into the Fibretech analyzer and treated with about 150 mL of hot 1.25 % m/v H_2SO_4, digested for 30 minutes in boiling 1.25 % H_2SO_4 then rinsed three times with 50 mL portions of hot distilled water. After the rinsing, the sample was treated with 150 mL of hot 1.25 % m/v NaOH, digested for 30 minutes then rinsed with three 50mL portions of hot distilled water. The crucibles were rinsed with acetone, dried in an oven maintained at 130 °C for two hours, cooled then weighed. The crucibles were transferred to a muffle furnace, heated gradually to 525 °C till white ash formed, cooled then weighed using the Mettler Toledo Analytical balance. The amount of crude fibre was then calculated from the loss of mass recorded after ignition.

3.3.6 Amino Acid Profile

100 µl of 6 M HCl and 0.2 µl of 1 nM of norvaline in 0.2 % phenol (as the first internal standard) was added to approximately 2 mg of the sample in a hydrolysis tube, tube was capped then incubated/hydrolyzed at 116 °C for 16 hours.

The tubes were cooled and dried, analyte dissolved in 100µl of sodium citrate buffer (pH 3.3), 2 µl of 2 nM taurine in 0.02M HCl (as a second internal standard) added then loaded into amino acid analyzer (Hitachi L8900 Amino Acid Protein Hydrolyzate Analyzer) for amino acid separation and quantification using ion exchange chromatography technique. The measurement condition for amino acid determination is summarized in Table 3.2 below:

Table 3.2: Amino Acid measurement conditions

Gradient							Post column derivatization			
Time	%B1[a]	%B	%B3	%B4	Flow	Temperat	%R1[b]	%R2	%R3	Flow
0.0	100	0	0	0	0.320	57	50	50	0	0.032
1.0	100	0	0	0						
1.1	0	100	0	0						
11.0	0	80	20	0						
35.0	0	0	100	0						
47.0	0	0	100	0		85	50	50	0	
47.1	0	0	0	100						
51.0	0	0	0	100						
51.1	100	0	0	0						
59.0	100	0	0	0					100	
59.1	100	0	0	0		57	50	50	0	
69.0	100	0	0	0						

[a]Buffers: B1 0.2N Sodium citrate pH 3.3

B2 0.2N Sodium citrate buffer pH 3.2

B3 0.2N Sodium citrate buffer pH 4.9

B4 0.2N Sodium hydroxide column regenerant, pH 11

[b]R1: Concentrated ninhydrin solution

[c]R2: Ninhydrin dilution solution

[d]R3: 10% ethanol (place holder)

3.3.7 Fatty Acid Profile

Oil was extracted from the seed using isooctane. 25 milligrams of oil extract from the sample was then weighed into a culture tube, treated with 1.5 mL of 0.5M NaOH, sealed then saponified at 100 °C for five minutes. The analyte was cooled, treated with 2 mL of BF_3 /methanol reagent, capped, mixed then derivatized at 100 °C for thirty minutes and cooled to about 40 °C. It was later treated with 1 mL of isooctane then shaken vigorously while still warm. 5 mL of saturated NaCl solution was added, tube sealed and agitated and left to stand so as to separate. The isooctane layer was transferred to a clean glass tube; the methanol/water phase was extracted again with isooctane and the extracts combined. 1 µl of the extract was injected into the gas chromatograph under appropriate conditions for separation and quantification of the fatty acids. The fatty acid measurement conditions are summarized in Table 3.3 below:

Table 3.3: Fatty acid Measurement conditions

SNO	PARAMETER	CONDITION
1	Injection port temperature, ^{0}C	250
2	Oven	
	i) Initial temperature, (^{0}C)	270
	ii) Initial hold time (minutes)	0
	iii) Program rate (^{0}C/minute)	1.0
	iv) Final temperature, (^{0}C)	225
	v) Final hold time (minutes)	0

3.3.8 Mineral Element Analysis

0.5 grams of the sample, SRM 1573a, SRM 1566b and eight procedural blanks were placed into respective Teflon vessels. It was treated with 10 mL HNO_3, 2 mL HF and 0.1mL of 10 mg/g Indium solution then left in the fume hood for pre-digestion overnight. The vessels were later removed, sealed and placed in the microwave oven for digestion. On completion of digestion, they were left to cool and finally vented free of NO_2 fumes in a fume hood. The solution was transferred to the Teflon beakers and heated on a hot plate with surface temperature of about 180 °C to near dryness. The solutions were diluted to 50 grams with 1.5 % v/v HNO_3 then transferred to LDPE Nalgene bottles.

To determine Copper, two 8 gram aliquots were taken from each dilution and transferred to weighed 30 mL polyethylene bottles. A 0.25 gram spike was taken from a spike solution containing 2µg/g Copper and was added to one aliquot for each sample. The spike solution was prepared from SRM 3114 Copper Standard Solution.

To determine iron, two 12 gram aliquots were taken from each dilution and transferred to weighed 30 mL polyethylene bottles. A 0.2 gram spike was taken from a spike solution containing 25 µg/g iron and was added to one aliquot for each sample. The spike solution was prepared from SRM 3126a Iron Standard Solution.

To determine Zinc, two 12 gram aliquots were taken from each dilution and transferred to weighed 30 mL polyethylene bottles. A 0.2 gram spike was taken from a spike solution containing 27µg/g Zinc and was added to aliquot for each sample. The spike solution was prepared from SRM 3168a Zinc Standard Solution.

Both the spike and unspiked solutions were loaded onto the autosampler and analysed. The readings were recorded in an MS-Excel sheet for further processing to final results.

The trace metal measurement conditions are summarized in Tables 3.4 and 3.5 shown below:

Table 3.4: Microwave Settings for Digestion of Elements in *TS* Seed

Step	**Power (W)**	**Power**	**Ramp Time**	**T (°C)**	**Hold Time (min)**
1	800	100	25:00	140	20:00
2	0	0	0	0	20:00
3	800	100	25:00	190	20:00
4	0	0	0	0	25:00

Table 3.5: Measurement parameters of mineral composition of TS seed

Element	Wavelength (nm)	Plasma View	Integration Time (s)	Read Time (s)	Number of Runs
Cu	324.752	Axial	0.100	1.000	2
Fe	259.939	Axial	0.100	1.000	2
Zn	213.857	Axial	0.100	1.000	2

3.3.9 QUALITY CONTROL AND ASSURANCE

3.3.10 Proximate Analysis

The balance used for weighing the sample for crude oil, crude protein and crude fibre was calibrated and regularly monitored for stability using standard masses maintained by Testing Services Department of KEBS. The ovens and furnace used in crude oil, crude fibre tests and the digestion block for crude protein analysis were all calibrated for the respective operating temperatures and regularly monitored for performance and stability using calibrated thermocouples maintained by Testing Department of KEBS.

The balance used for mass measurements during moisture study and total ash determination was calibrated and regularly monitored for performance using standard masses maintained by Inorganic Chemical Metrology group at NIST. The muffle furnace used for determination of total ash was calibrated and regularly monitored for performance using calibrated thermocouples maintained by Inorganic Chemical Metrology Group at NIST.

3.3.11 Amino Acid Profile

The quality control tool used in this test was based on two internal standards and temperature regulation of the column during the chromatography process. The results were assured based on retention times of a known mixture of standard amino acids (Crawford J.M, 2009).

3.3.12 Fatty Acids Profile

The quality control tool employed in this technique was the use of updated retention times besides the in house documented precision and accuracy criteria of acceptance of results (AOCS 1b-89(2009).

3.3.13 Mineral Element Analysis

The balance used for mass measurements during moisture study and total ash determination was calibrated and regularly monitored for performance using standard masses maintained by Inorganic Chemical Metrology group at NIST. Indium was used as an internal standard during sample preparation to improve on the precision of ICP-OES measurements. Certified Standard Solutions (SRM 3168a, 3126a and 3114) were used for spiking of samples for instrumental measurements. Two CRMs (SRM 1573a and SRM 1566b) were used to assure on the final test results.

CHAPTER FOUR

4.0 RESULTS

4.1 General

Typical *Tylosema fassoglense* seed weighs an average of 2.64 ± 0.35 grams. The seed has a longitudinal length of about 2.32 ± 0.18cm and a lateral diameter of 1.94 ± 0.21 cm as outlined in Table 4.1 below:

TABLE 4.1: *Tylosema fassoglense* seed mass and dimensions measurements

SEED	Mass, g	d1[a], cm	d2[b], cm
Mean [c]	2.64	2.32	1.94
SD	0.35	0.18	0.21

[a] longitudinal diameter

[b] lateral diameter

[c]Mean of ten measurement results

4.2 Proximate analysis

Table 4.2 shows the mean moisture, crude protein, crude oil, total ash and crude fibre content of the *Tylosema fassoglense* seed determined as outlined in sections 3.3.1, 3.3.2, 3.3.3, 3.3.4 and 3.3.5. The carbohydrate content and calorific value were calculated based on moisture, oil, protein, fibre and ash contents. Of the nutritional components, crude protein content was the highest followed by crude oil content.

TABLE 4.2: Proximate composition of *Tylosema fassoglense* seed

SNO	PARAMETER	UNIT	RESULT, g/100g	SD
1	Moisture	g/100g	6.169	0.057
2	Crude Protein (CP)	g/100g	24.19	0.41
3	Crude Oil (CO)	g/100g	22.02	0.27
4	Crude Fibre (CF)	g/100g	13.38	0.12
5	Total ash	g/100g	3.26	0.047
6	Carbohydrates*	g/100g	31.23	-
7	Calorific value**	kJ/100g	208.14	-

*Based on calculation from the formula: 100-(CP+CF+CO+Ash+Moisture)

** Energy equivalent

4.3 Amino acid profile

The results of amino acid profile determined as outlined in section 3.3.6 are shown in Table 4.3 and Figure 4.1. The essential amino acids average about 44 % while the non-essential amino acids constitute about 56 %. The data shows that methionine is the limiting essential amino acid. Aspartic acid and asparagine could not be separated and are therefore reported as a mixture. Similarly glycine and glutamic acid could not be

separated and are reported as a mixture of both. Cysteine and tryptophan residues could not be determined due to method limitations.

TABLE 4.3: Amino acid profile of *Tylosema fassoglense* seed

SNO	AMINO ACID	UNIT	RESULT[a]	SD
	ESSENTIAL AMINO ACIDS			
1	Isoleucine	g/100g	4.688	0.0346
2	Leucine	g/100g	6.176	0.0518
3	Lysine	g/100g	6.007	0.0498
4	Methionine	g/100g	0.760	0.0199
5	Phenylalanine	g/100g	4.844	0.0229
6	Threonine	g/100g	3.046	0.0435
7	Valine	g/100g	5.216	0.0554
8	Tyrosine	g/100g	13.746	0.1189
Sub Total		**g/100g**	**44.483**	
	NON ESSENTIAL AMINO ACIDS (NEAA)			
1	Asparagine/aspartic acid (asx)*	g/100g	10.684	0.1068
2	Serine	g/100g	4.238	0.1148
3	Glutamine/glutamic acid (glx)*	g/100g	15.666	0.1383
4	Glycine	g/100g	5.372	0.0616
5	Alanine	g/100g	3.309	0.0429
6	Histidine	g/100g	2.696	0.0141
7	Arginine	g/100g	6.928	0.1589
8	Proline	g/100g	6.625	0.2155
Sub Total		**g/100g**	**55.518**	
GRAND TOTAL (EAA+NEAA)		**g/100g**	**100**	

[a]Mean of three determinations

* Could not be separated and therefore reported as a mixture.

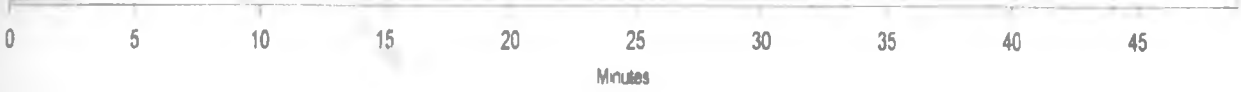
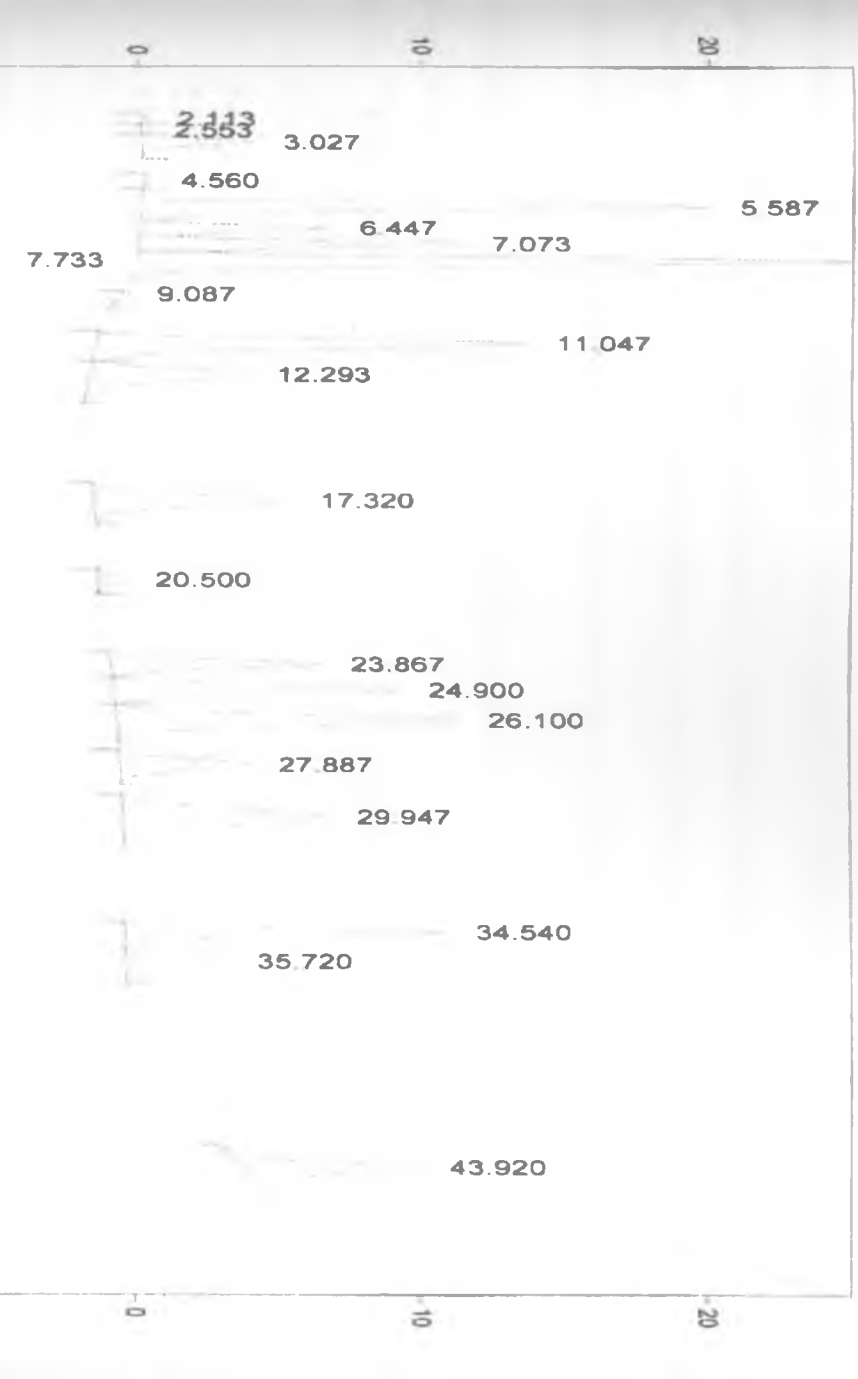


Figure 4.1: Amino acid chromatogram


2.113
2.553
3.027
4.560
5.587
6.447
7.073
7.733
9.087
11.047
12.293
17.320
20.500
23.867
24.900
26.100
27.887
29.947
34.540
35.720
43.920

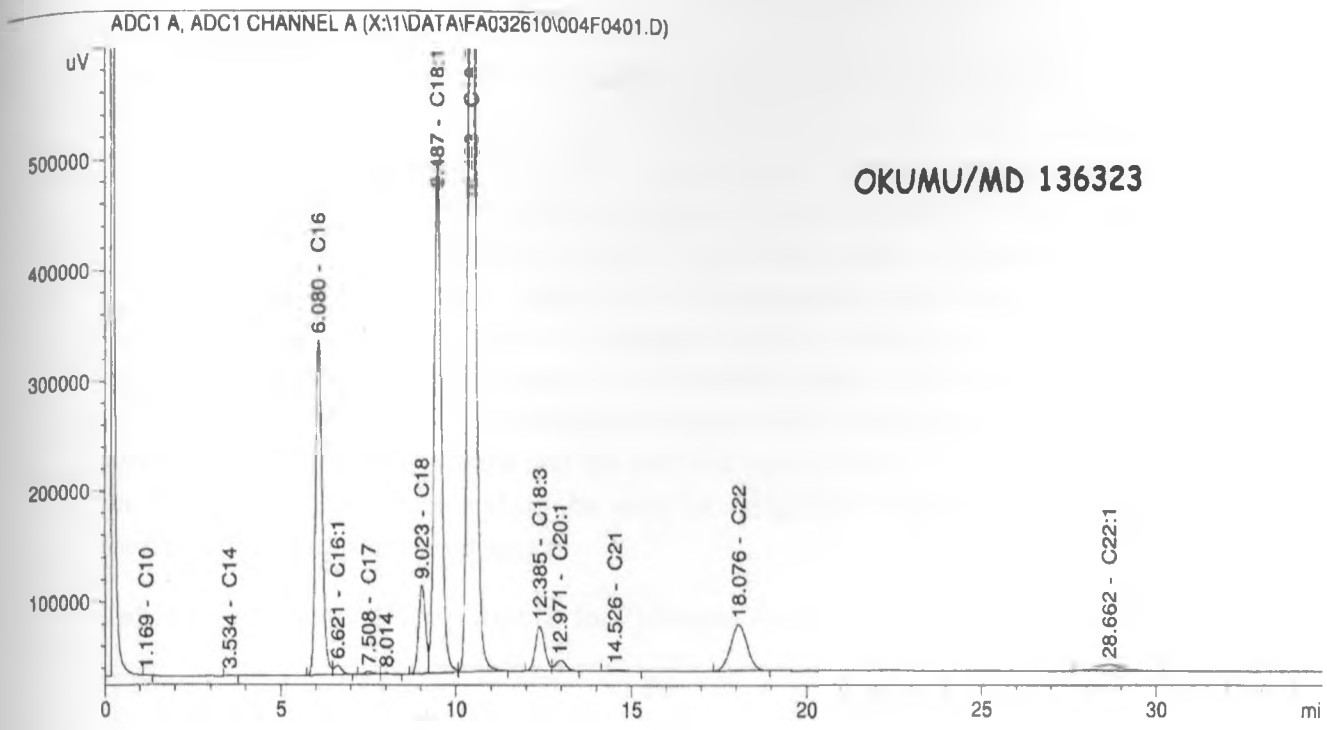
4.4 Fatty acid profile

The results of the fatty acid composition of the TS seed determined as outlined in section 3.3.7 are shown in Table 4.4 and Figure 4.2. Linoleic acid is the dominant fatty acid (46.64%) followed by oleic acid (23.5%) and palmitic acid (15.18%) respectively. The saturated fatty acids make up to 24 % of the total fatty acids while the unsaturated fatty acids form 76 % of the total fatty acid content. The unsaturated fatty acids are made up of 35 % MUFA and 65 % PUFA.

Table 4.4: Fatty acid profile of *Tylosema fassoglense* seed

[a]Mean of three determinations.

SNO	Fatty Acid	UNIT	RESULT[a]	SD
	Saturated Fatty Acids (SFA)			
1	C14:0(Myristic acid)	g/100g	0.04	0.0033
2	C16:0(Palmitic acid)	g/100g	15.18	0.0821
3	C17:0(heptadecanoic acid)	g/100g	0.14	0.0067
4	C18:0(Stearic acid)	g/100g	3.77	0.1250
5	C21:0(Heineicosanoic acid)	g/100g	0.15	0.01
6	C22:0(Docosanoic acid)	g/100g	5.08	0.0252
Sub Total		**g/100g**	**24.36**	
	Mono-Unsaturated Fatty Acids (MUFA)			
1	C16:1(Palmitoleic acid)	g/100g	0.45	0.0219
2	C18:1(Oleic acid)	g/100g	23.50	0.7034
3	C20:1(Eicosenoic acid)	g/100g	0.75	0.0033
4	C22:1(Docosenoic acid)	g/100g	1.44	0.1017
Sub Total		**g/100g**	26.14	
	Poly-Unsaturated Fatty Acids (PUFA)			
	C18:2(Linoleic acid)	g/100g	46.64	0.7157
	C18:3(Linolenic acid)	g/100g	2.75	0.0567
Sub Total		**g/100g**	49.39	
	Other	g/100g	0.05	0.0088
Grand Total (SAF+MUFA+PUFA+other)		g/100g	99.94	



OKUMU/MD 136323

Figure 4.2: Fatty acids chromatogram

4.5 Mineral elements

Table 4.5 shows the results with respective uncertainties of trace minerals in *Tylosema fassoglense* seed determined as outlined in Section 3.3.8. The iron content was the highest among the three mineral elements tested followed by zinc and finally copper. The relatively high mineral element content results can be attributed to the high total ash (3.26 g/100g) content which is a crude estimate of the overall mineral composition. Tables 4.6 and 4.7, show mineral element results with corresponding uncertainty (JCGM 100:2008, Taylor *et al,* 1994) of the certified reference materials (SRM 1573a and SRM 1566b) used for quality assurance. The results are illustrated further in Figures 4.3, 4.4, 4.5, 4.6, 4.7 and 4.8. The measurement uncertainty components are summarized in Table 4.8. The agreement between SRM results and the certified values imply that the trace metal values for TS seed are true values and can be used for assigning of values to any plant material for use as a quality assurance sample.

Table 4.5: Mineral element Results for *Tylosema fassoglense* Seed

Item	**Cu**	**Fe**	**Zn**
Units	µg/g	µg/g	µg/g
$\bar{X}$ =	16.869	57.42	27.39
sd =	0.758	2.27	0.95
u_c	0.258	0.698	0.821
U	0.557	1.537	1.857

Table 4.6: Results for Control SRM 1573a Tomato Leaves

Item	**Cu**	**Fe**	**Zn**
Units	µg/g	µg/g	µg/g
Result[a]	4.442	364.873	29.308
Sd	0.183	48.863	0.485
u_c	0.287	21.925	1.22
Certified Value U	4.70 0.14	368 7	30.9 0.7

[a]Mean of four measurements

Table 4.7: Results for Control SRM 1566b Oyster Tissue

Item	**Cu**	**Fe**	**Zn**
Units	µg/g	µg/g	µg/g
Result[a]	71.834	198.845	1425.0
Sd	2.54	18.208	0.5
u_c	1.51	10.23	1.24
Certified Value U	71.6 1.6	205.8 6.8	1424 46

[a]Mean of three measurements

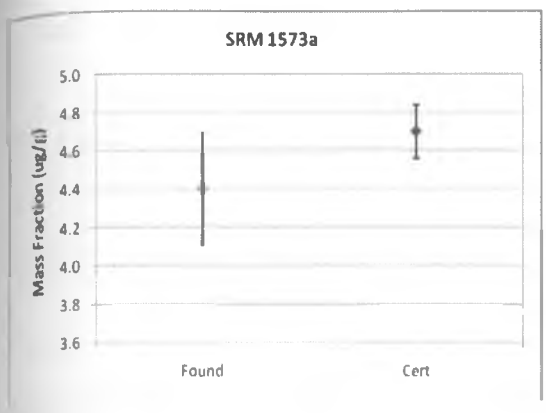


Figure 4.3: Cu SRM result

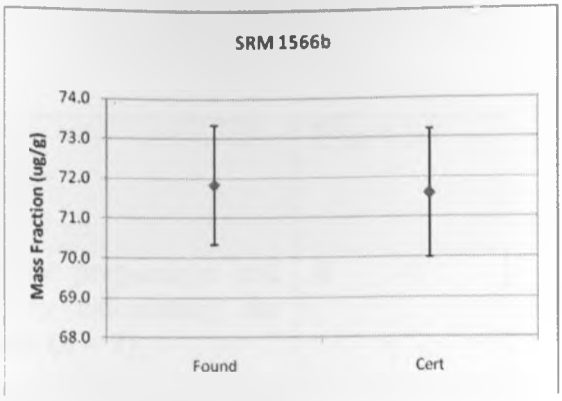


Figure 4.4: Cu SRM result

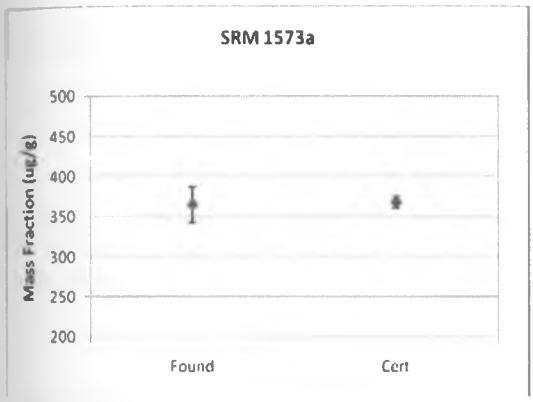


Figure 4.5: Fe SRM result

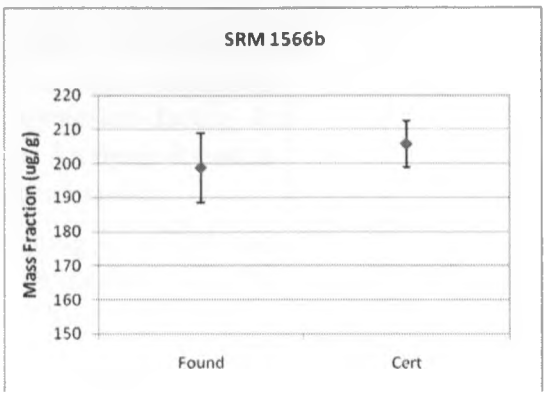


Figure 4.6: Fe SRM result

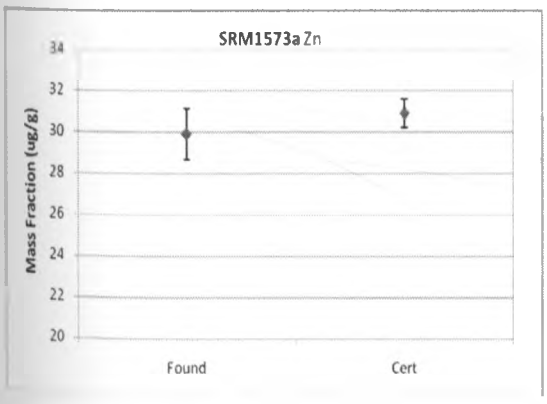


Figure 4.7: Zn SRM result

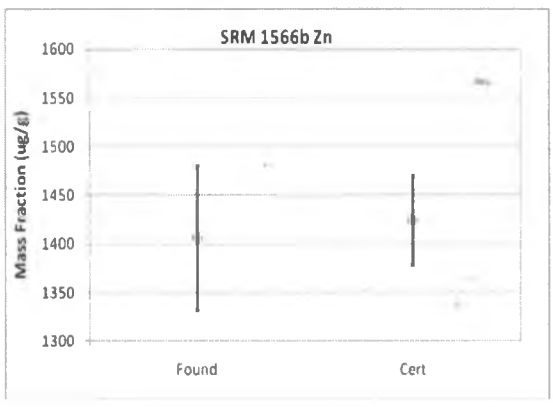


Figure 4.8: Zn SRM result

Table 4.8. Descriptions of Components of Measurement Uncertainty

Uncertainty	Basis	Type	DF
Sample Replication, S_{sample}	The uncertainty due to sample preparation and measurement is estimated by calculating the standard deviation of the mean. ($n = 7$)	A	6
Blank Replication, S_{blank}	The uncertainty due to blank preparation and measurement is estimated by calculating the standard deviation of the mean. ($n = 8$)	A	7
Moisture Correction $S_{moisture}$	The uncertainty due to the moisture correction is estimated by calculating the standard deviation of the mean. ($n = 4$)	A	3
Primary Standard, u_s	The uncertainty associated with the primary standards is calculated to be the expanded uncertainty divided by the expression factor, k, obtained from the Certificate of Analysis for each SRM used as the standard addition spike.	B	∞
Weighing of Standards, u_{b1}	The uncertainty for each weighing is ± 0.01 mg based on the certificate of calibration for the balance (NIST, 2009). This estimate is normalized by division by $\sqrt{3}$.	B	∞
Weighing of Samples, u_{b2}	The uncertainty for each weighing is ± 0.01 mg based on the certificate of calibration for the balance (NIST, 2009). This estimate is normalized by division by $\sqrt{3}$.	B	∞

CHAPTER FIVE

5.0 DISCUSSION

5.1.0 Proximate Analysis

Figure 5.1 below show an illustration of the comparative amounts of crude protein, crude oil, carbohydrates, crude fibre and total ash of *Tylosema fassoglense, soybean, pea bean, chick pea, lima beans,lentils, peanuts and garden beans.* . Data in Table 5.1 shows respective proximate composition in other legumes.

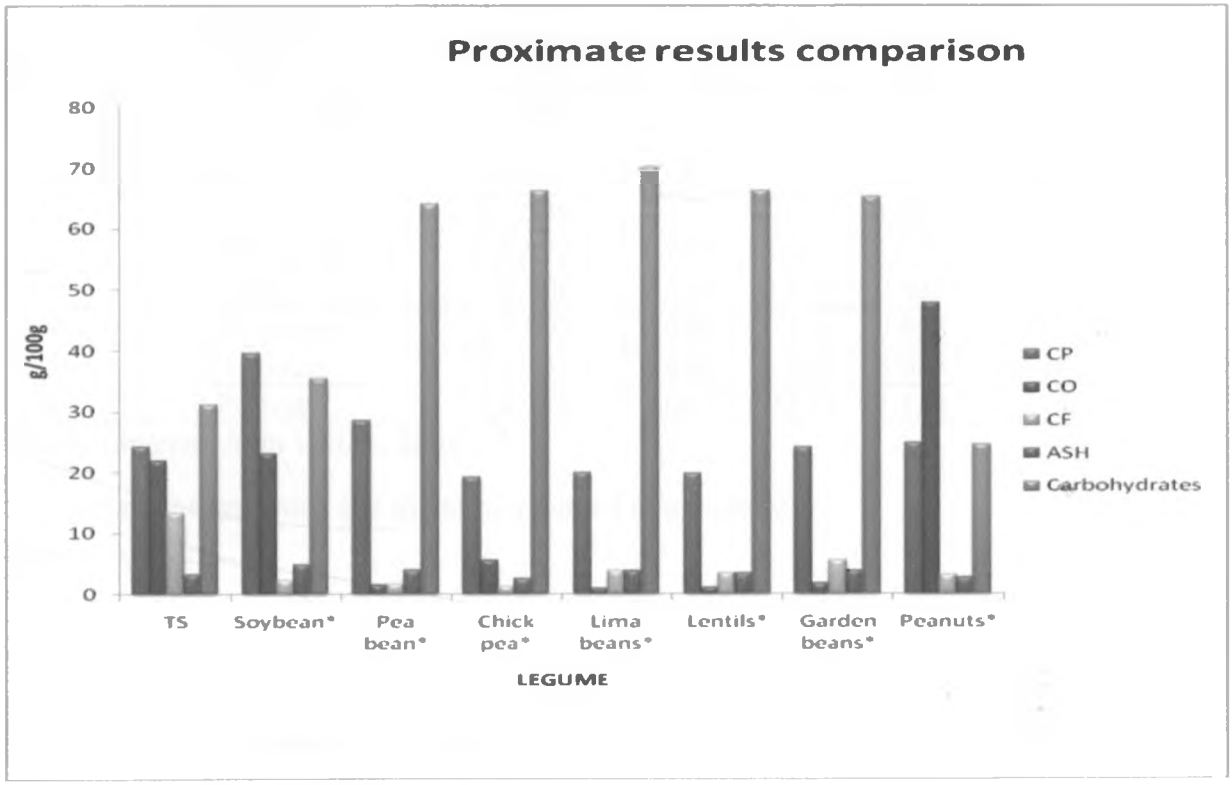
TABLE 5.1: Proximate analysis data of common legumes

LEGUME FLOUR	CP % m/m	CO % m/m	CF % m/m	ASH % m/m	Carbohydrate % m/m
Soybean[a]	39.7	23.1	2.2	4.8	35.5
Pea bean[a]	28.6	1.6	1.7	4.0	64.1
Chick pea[a]	19.2	5.6	1.3	2.6	66.3
Lima beans[a]	20.0	0.9	3.8	3.8	70.3
Lentils[a]	19.8	1.1	3.4	3.4	67.3
Pea nuts[a]	24.8	47.9	3.1	2.7	24.6
Garden beans[a]	24.1	1.8	5.5	3.9	65.2

[a] data obtained from USDA, 2009.

It is evident that *Tylosema fassoglense* seed crude protein content compares favourably with those of other legumes (garden beans, pea bean, chick peas, lima beans, lentils and peanuts) with the exception of soybean which is almost twice as high. The crude oil

content of *Tylosema fassoglense* compares favourably with that of soybean but much higher than other legumes such as garden beans, chick beans, lentils, Lima beans. The amount of oil in peanuts is much higher at about double the amount in *Tylosema fassoglense* seed. The carbohydrate content compares well with that of soybean, lentils and peanuts but about half the amount in other legumes (pea bean, chick pea, garden beans and lima beans). The total ash content compares well with the amounts in other legumes (pea bean, chick pea, garden beans and lima beans). The crude fibre content of *Tylosema fassoglense* seed is quite high compared to that of other legumes (soybean, chick beans, pea bean, lima beans, garden beans and peanuts).



* Data obtained from USDA, 2009.

Figure 5.1: Proximate Analysis comparison

5.1.1 Amino Acid profile

The amino acid composition of *Tylosema fassoglense* (Table 4.3) seed shows considerable variation when compared with the content of amino acids composition of other legumes (soybean, lima beans, garden beans, peanuts and sunflower) indicated in Table 5.2.

Table 5.2: Amino Acid Profile of other Legumes

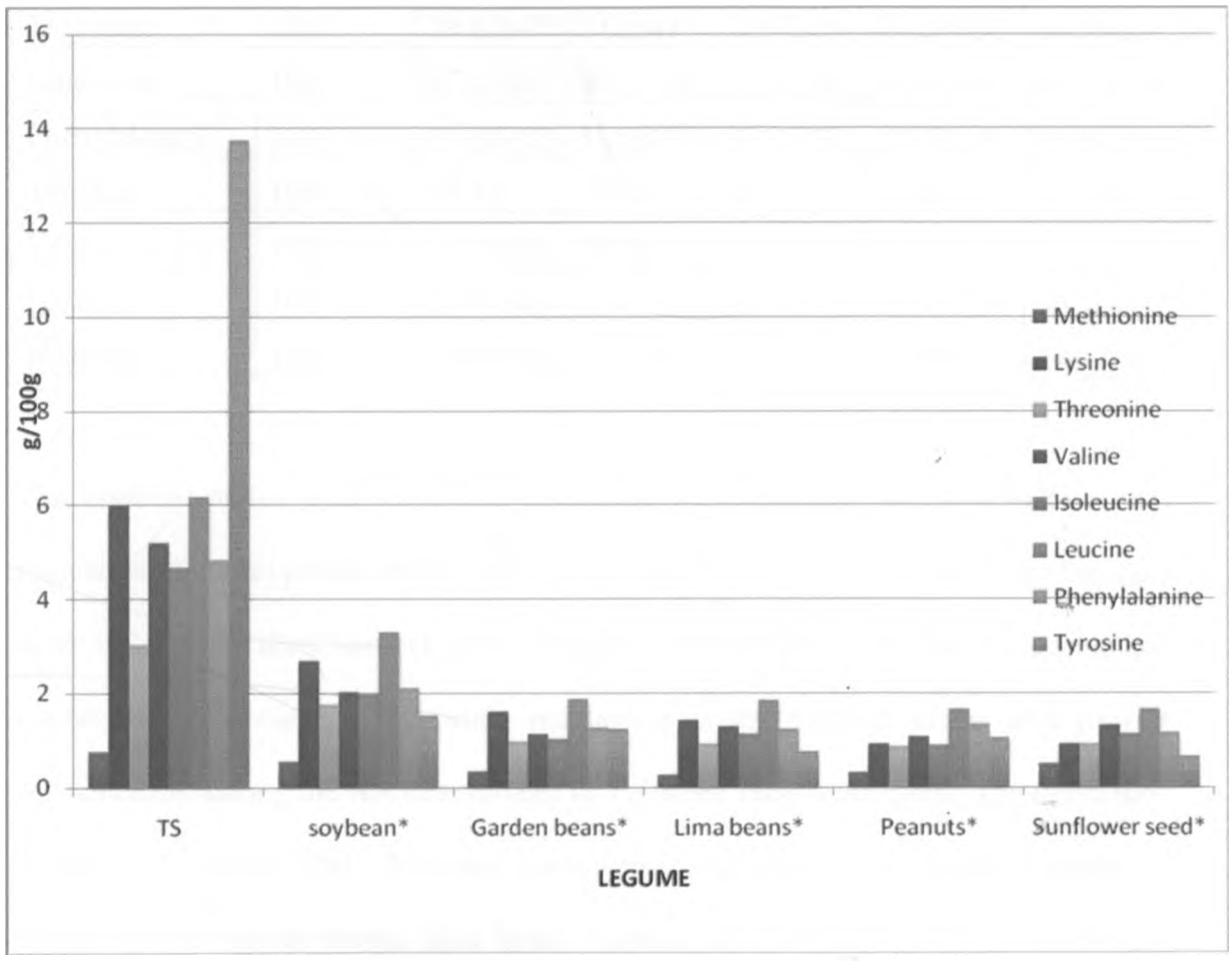
SNO	AA	Soybean [a]	Garden beans[a]	Lima beans[a]	Peanuts[a]	Sunflower seed[a]
1	Asparagine/aspartic acid (asx)*	5.112	2.852	2.767	3.146	2.446
2	Threonine	1.766	0.992	0.927	0.883	0.928
3	Serine	2.357	1.282	1.428	1.271	1.075
4	Glutamine/glutamic acid (glx)*	7.874	3.595	3.038	5.39	5.579
5	Glycine	1.880	0.920	0.906	1.554	1.461
6	Alanine	1.915	0.988	1.095	1.025	1.117
7	Valine	2.029	1.144	1.291	1.082	1.315
8	Methionine	0.547	0.355	0.271	0.317	0.494
9	Isoleucine	1.971	1.041	1.129	0.907	1.139
10	Leucine	3.309	1.882	1.850	1.672	1.659
11	Tyrosine	1.539	1.233	0.759	1.049	0.666
12	Phenylalanine	2.122	1.275	1.236	1.337	1.169
13	Lysine	2.706	1.618	1.438	0.926	0.937
14	Histidine	1.097	0.656	0.656	0.652	0.632
15	Arginine	3.153	1.460	1.315	3.085	2.403
16	Proline	2.379	1.000	0.975	1.138	1.182

[a] Data obtained from USDA, 2009

*Could not be separated and therefore reported as a mixture

Tyrosine content was the highest among individual amino acids of *Tylosema fassoglense* seed. Among the essential amino acids, *Tylosema fassoglense* contained the highest amount of tyrosine when compared with other legumes (soybean, garden beans, lima beans, peanuts and sunflower seed) as shown in Figure 5.2.Glutamic acid/glutamine and

asparagine/aspartic acid were found to be the major non essential amino acids in *Tylosema fassoglense* seed (Table 4.3). The results for amino acid composition of *Tylosema fassoglense* seed are in fair agreement with those of lentils, green pea, cow pea and chick pea (Iqbal *et al*, 2006). The essential amino acid to non essential amino acid ratio for *Tylosema fassoglense* seed is 0.80 which compares quite well with that of green pea and lentils (Iqbal *et al*, 2006). This ratio shows that *Tylosema fassoglense* can be treated as a good source of essential amino acids (EAA) for both human beings and animals that require EAA for normal body functions but cannot synthesize the same.



* Data obtained from USDA, 2009

Figure 5.2: Essential amino acids comparison

5.1.2 Nutritional quality of proteins

The nutritional quality of protein for *Tylosema fassoglense* seed, garden beans, lima beans, peanuts and sunflower seed were assessed and compared (Table 4.3, 5.3 and Figure 5.3).

Table 5.3: Calculated Amino Acid Scores

AA	Reference	Garden beans	Lima beans	Peanuts	Sunflower	TS
Lysine	100	27.89655	24.7931	15.96552	16.15517	103.569
Cystine	100	14.2	10.84	12.68	19.76	30.4
Threonine	100	29.17647	27.26471	25.97059	27.29412	89.58824
Isoleucine	100	37.17857	40.32143	32.39286	40.67857	167.4286
Phenylalanine	100	39.80952	31.66667	37.87302	29.12698	76.88889
Tyrosine	100	49.32	30.36	41.96	26.64	549.84
Valine	100	32.68571	36.88571	30.91429	37.57143	149.0286
Leucine	100	28.51515	28.0303	25.33333	25.13636	93.57576
Histidine	100	34.52632	34.52632	34.31579	33.26316	141.8947

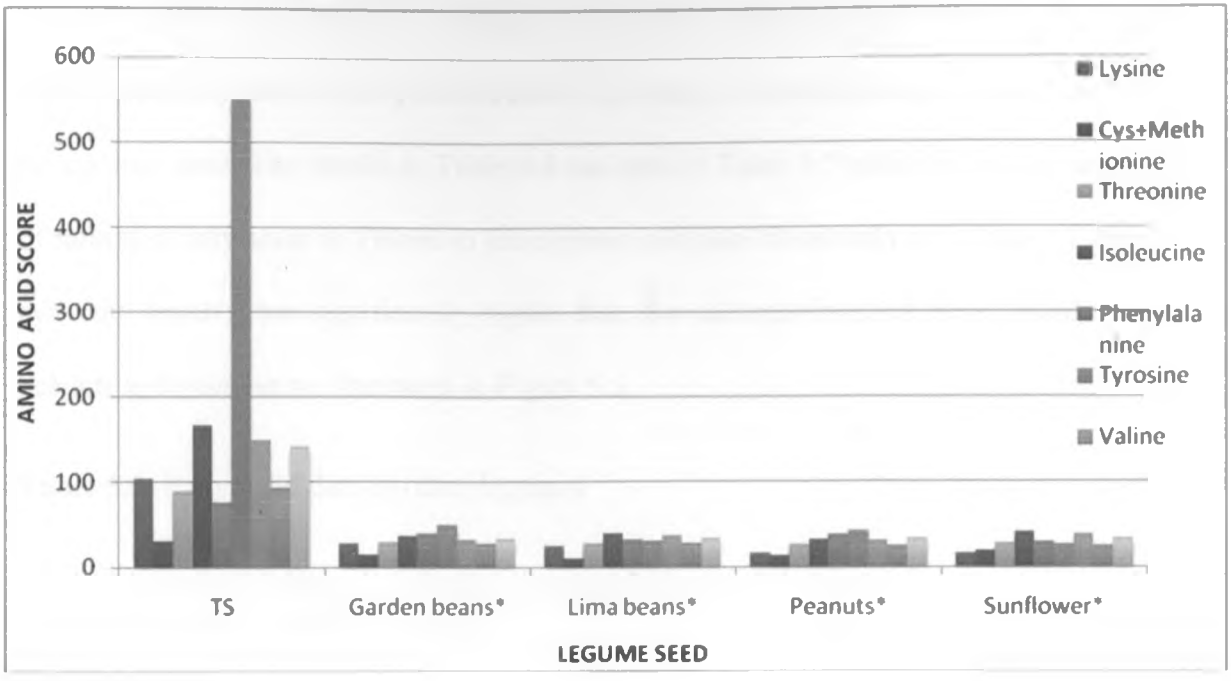
The essential amino acid score was calculated with reference to FAO/WHO (1985) standard amino acid profile established for humans (FAO/WHO, 1985) whereby the ideal score is 100. The illustration (Figure 5.3) shows that histidine, tyrosine, isoleucine and valine are in excess amounts while methionine is the limiting amino acid (lowest concentration among the AA determined) in *Tylosema fassoglense* seed. The illustration (Figure 5.3) shows that *Tylosema fassoglense* seed has better protein quality in comparison to garden beans, lima beans, peanuts and sunflower. Data in Table 5.4 indicate that *Tylosema fassoglense* seed meets the recommended levels of essential amino

acids for children and adults with the exception of leucine level in the 1 year old group (FAO/WHO, 1985)

Table 5.4: TS AA results against FAO/WHO[a] recommended AA acid intake values.

		Suggested human Amino Acid Requirements, g/100g			
EAA	TS Result in g/100g	1yr	2-5yrs	10-12yrs	Adult
Threonine	3.046	4.3	3.4	2.8	0.9
Cys+meth	0.76	4.2	2.5	2.2	1.7
val	5.216	5.5	3.5	2.5	1.3
Ile	4.688	4.6	2.8	2.8	1.3
Leu	6.176	9.3	6.6	4.4	1.9
Tyr+phe	18.59	7.2	6.3	2.2	1.9
His	2.696	2.6	1.9	1.9	1.6
Lys	6.007	6.6	5.8	4.4	1.6
Trp	-	1.7	1.1	0.9	0.5

[a]FAO/WHO, 1985



* Data obtained from USDA, 2009.

Figure 5.3: Amino acid Scores

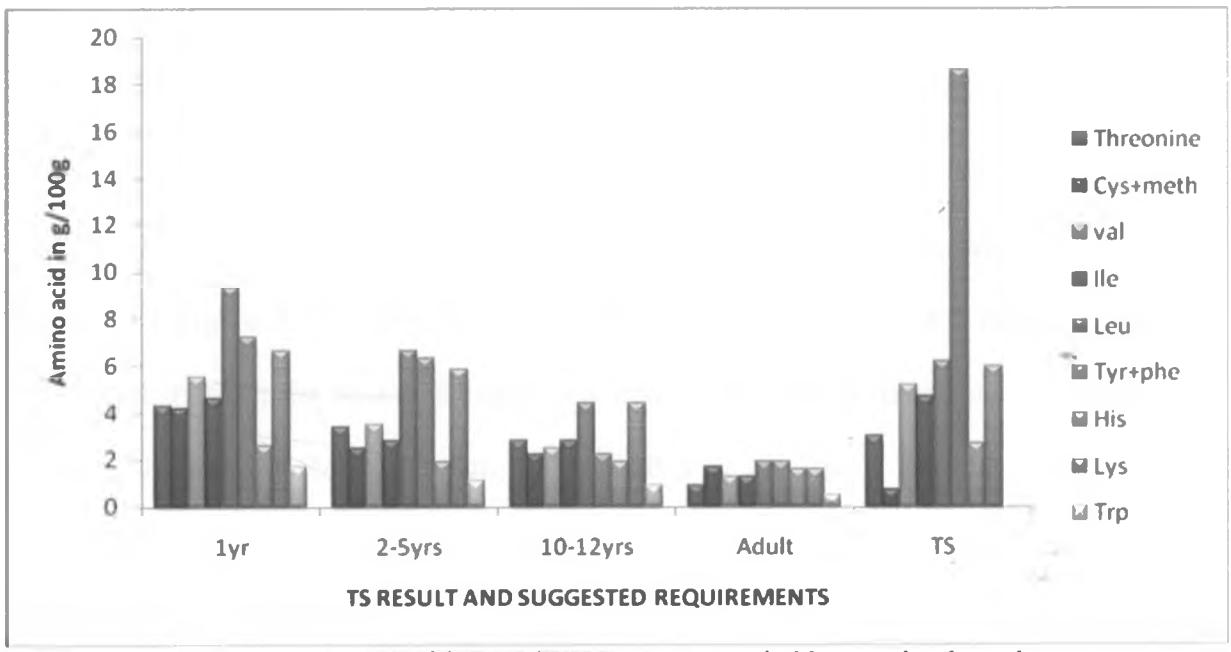


Figure 5.4: TS EAA compared with FAO/WHO recommended human intake values

5.1.3 Fatty acid composition

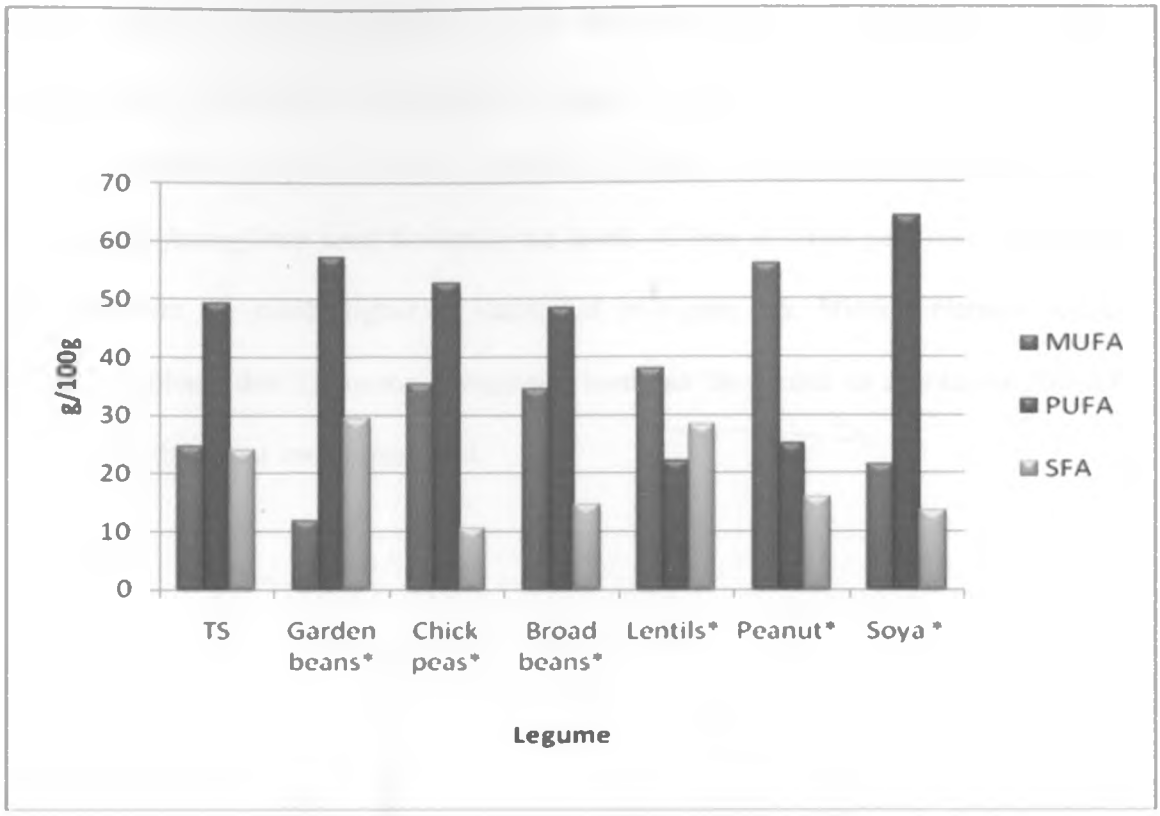
The data shown in Table 4.4 indicates that linoleic acid and oleic acid are the major unsaturated fatty acids while palmitic acid is the major saturated acid present in *Tylosema fassoglense* seed. The results in Table 4.4 and data in Table 5.5 indicate that the amounts of saturated fatty acids in *Tylosema fassoglense* compare favourably with those of garden peas and lentils; but significantly higher than the contents in chick peas, broad beans, peanuts and soybean as illustrated in Figure 5.5.

Table 5.5: Fatty Acid data of other legumes

FA	Garden beans[a]	Chick peas[a]	Broad beans[a]	Lentils[a]	Peanut[a]	Soya[a]
MUFA	11.83	35.45	34.5	38.05	56	21.5
PUFA	57.2	52.8	48.5	22.2	25	64
SFA	29.4	10.5	14.62	28.22	16	13.5

[a] Data obtained from USDA, 2009.

The total amount of unsaturated fatty acids in *Tylosema fassoglense* seed compares well with the amounts in other legumes (garden beans, broad beans, chick peas, peanut and soybean) (Figure 5.5)(USDA,2009). The SFA / (UFA), C18-1/C18-2 ratios compare quite well with similar results obtained in a study conducted in Argentina and India on some wild legumes (Maestri *et al*, 2002, Sridhar *et al*, 2006). Results in Table 4.4 indicate therefore that *Tylosema fassoglense* can be treated as a good source of unsaturated fatty acids.



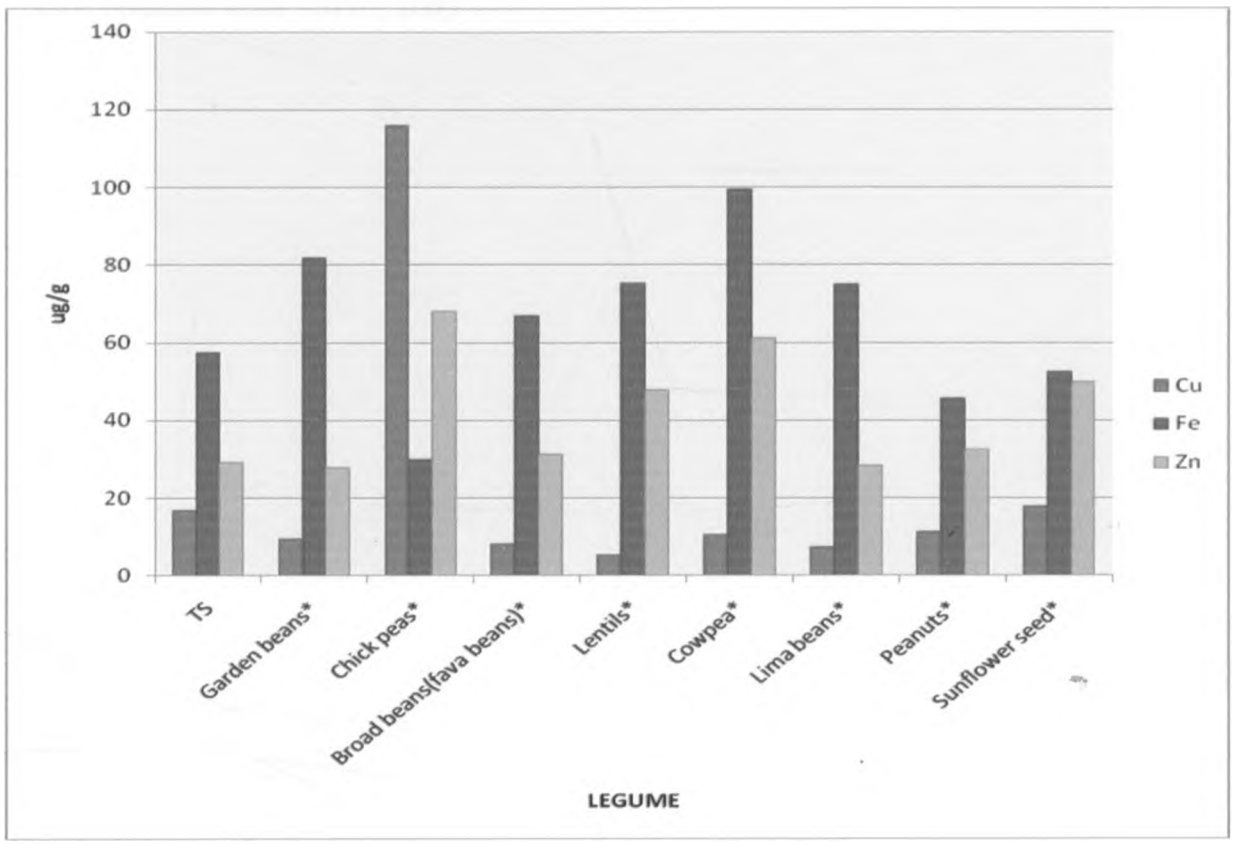
*Data obtained from USDA, 2009.

Figure 5.5: FA Comparison

5.1.4 Mineral Elements

The results in Table 4.5 indicate that *Tylosema fassoglense* seed contains the three nutritional elements (copper, iron, and zinc) tested with iron being the most abundant among the three. The levels of copper in *Tylosema fassoglense* seed compares well with those of sunflower seed, cowpea and peanuts but relatively higher than levels in other legumes (garden beans, broad beans and lima beans) as illustrated in Figure 5.6. The copper levels in other legumes (lentils and chickpeas) are about six times higher than the amount of copper in *Tylosema fassoglense* seed (Figure 5.6). The amount of iron in *Tylosema fassoglense* seed compares well with that in broad beans, peanuts and sunflower seed; the levels are significantly higher than the amount in chick peas by

about a factor of two and relatively lower than the amount in garden beans, lentils, cowpeas and lima beans as illustrated in Figure 5.6 and Table 5.6. The zinc levels in legumes (garden beans, broad beans, lima beans, peanuts) compares well with the amount in *Tylosema fassoglense* seed; however, the levels of zinc in chick pea, cow pea, lentils and sunflower are much higher as illustrated in Figure 5.6. Mineral element results therefore indicate that *Tylosema fassoglense* seed can be treated as a potential mineral source in both human and animal feed.



* Data obtained from USDA, 2009.

Figure 5.6: Mineral composition comparison

Table 5.6: Mineral Elements Data for other legumes

LEGUME	Cu, mg/kg	Fe, mg/kg	Zn, mg/kg
Garden beans[a]	9.58	82	27.9
Chick peas[b]	116	30	68
Broad beans[a] (fava beans)	8.24	67	31.4
Lentils[a]	5.19	75.4	47.8
Cowpea[a]	10.59	99.5	61.1
Lima beans[a]	7.4	75.1	28.3
Peanuts[a]	11.44	45.8	32.7
Sunflower seed[a]	18	52.5	50

[a]Data obtained from USDA, 2009

5.1.5 Feed quality

Data in Table 5.7 below indicate that *Tylosema fassoglense* seed complies with the Kenya Standard Specification for Poultry and Pig Feeds in Crude Protein but exceeds the requirements for Dairy Meal. Similarly, the *Tylosema fassoglense* seed crude protein level is within standard requirements for commonly used raw materials in animal feed compounding such as maize bran, sunflower seed cake and cotton seed cake but outside the range for the limits in Wheat bran. The results in Table 5.7 indicating compliance with Kenya Standard specifications for poultry and pig feed(KS0-138, KS 01-61) imply that the seed may be used as an animal feed or raw material in the animal feed compounding process.

TABLE 5.7: TS RESULTS AGAINST ANIMAL FEED REQUIREMENTS

FEED	Crude protein g/100g	Crude oil/fat, g/100g	Crude fiber, g/100g	Total ash, g/100g	Energy, Kcal/kg	KS
TS	24.2	22.0	13.4	3.3	485.0	-
Dairy meal	14-16	3-6	12	NS	NS	01-62
Pig finisher	12min	2-10	8 max	8 max	NS	01-138
Sow weaner	16min	2-10	8 max	8 max	NS	01-138
Chick marsh	18min	NS	NS	NS	2600	61
Layers marsh	15min	NS	NS	NS	2600	61
Broiler starter	20min	NS	NS	NS	3000	61
Broiler Finisher	18min	NS	NS	NS	3000	61
Wheat bran	13.5-15.0	2.5 min	12.0 max	NS	NS	01-952
Maize bran	6.0 min	8.0 max	12.0 max	NS	NS	01-1085
Sunflower seed	35 max	8.0 max	15.0 max	8.0 max	NS	01-673
Cotton seed cake	33.0 max	8.0 max	17.0 max	7.0 max	NS	01-673

'NS' means not specified

CHAPTER SIX

6.0 CONCLUSION

Results of the current study show that TS seed contains 24.19% crude protein made up of various amino acids of which 44% are essential amino acids (EAA) with methionine appearing as the limiting amino acid. Crude oil content of TS seed is 22.02% made up of 24.7%SFA, 49.39% PUFA and 24.03%MUFA respectively. The most abundant fatty acids were found to be linoleic acid (46.64%), oleic acid (23.5%) and palmitic acid (15.18%) respectively.

The TS seed contains 16.9mg/kg, 57.4mg/kg and 29.3mg/kg of Copper, Iron and Zinc respectively of the mineral elements tested. Crude fibre was found to be 13.38%, Carbohydrate content 31.23% and total ash 3.26%.

The levels of protein, amino acids, lipids and fatty acids, essential minerals (Copper, Iron and Zinc) in TS seed in substantial quantities may support its use in food or feed fortification targeting mineral and protein deficiency as well as its use as direct food or raw material in the animal feed compounding process.

Based on the current study findings, *Tylosema fassoglense* seed may be treated as a potential food/feed and might in future find utilization in the fight against protein and nutrient malnutrition on the global stage.

6.1 RECOMMENDATIONS

There is need to quantify sulphur based amino acids such as tryptophan and cystein owing to limitation of the method used in this study. There is need to develop better techniques to separate the amino acids reported as mixtures (such as aspartic acid and asparagine, glutamine and glutamic acid). The results of such a study will definitely complement the current amino acid results and yield vital additional information to be used in any efforts geared towards addressing protein quality in food and feed industry. The sugars present may also need to be characterized to give an overview of its profile and potential benefits.

Presence of anti-nutrients in plant based food sources is always a drawback on availability of essential nutrients to human beings and animals. It is therefore necessary to carry out further research into anti-nutritional compounds that may be present in this seed. This in turn may lead to identification of appropriate technologies that may be necessary to refine the product should there be need.

There is also need to conduct further research to determine mechanisms through which dietary fat and fatty acids affect mineral metabolism in animals. This kind of work may lead to a better approach towards setting standard specifications for minerals in plant based food/feed products where the mineral levels might appear naturally high.

Multidisciplinary collaboration among chemists, plant breeders, biochemists, nutritionists, food technologists etc in research on this seed is likely to unravel the full potential of this plant in as far as addressing dietary, health, social and economic needs of the local population and as such should be encouraged.

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APPENDIX

APPENDIX 1: FATTY ACID CHROMATOGRAMS

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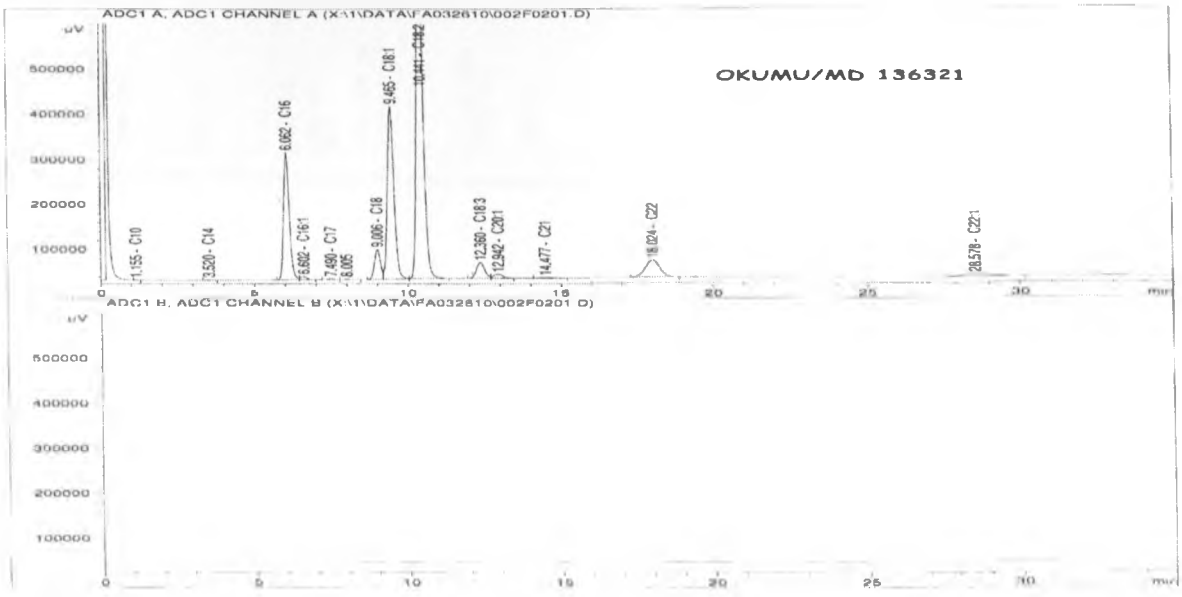

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Acq. Instrument	: FAP	Inj Volume	: Manually
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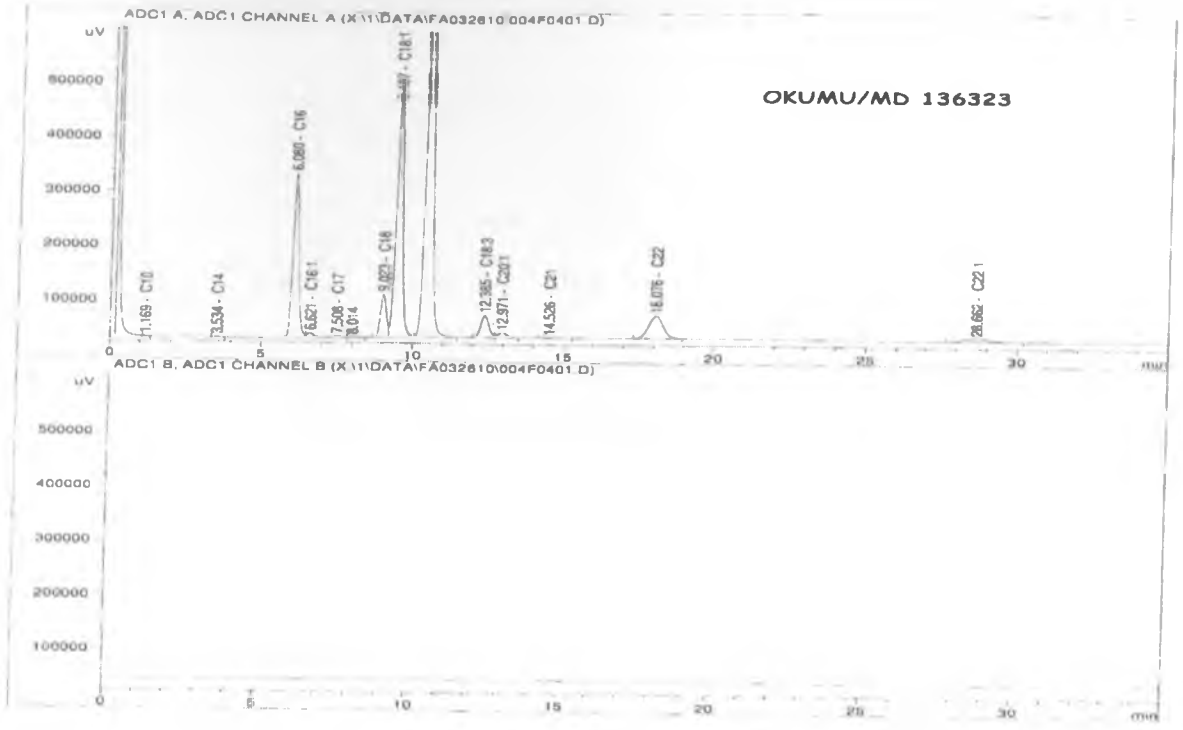

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Page 1 of 1

APPENDIX 2: AMINO ACID REPLICATE DATA

Amino Acid	HHH2323	nmoles aa	µgrams	mole percent	# residues (If 10K* protein)
cysac	0.0000	0.000	0.000	0.0%	0.0
cmcys	0.0000	0.000	0.000	0.0%	0.0
asx	0.9366	0.937	0.108	10.3%	9.1
thr	0.3153	0.315	0.032	3.5%	3.1
ser	0.4738	0.474	0.041	5.2%	4.6
glx	1.2417	1.242	0.160	13.6%	12.1
gly	0.9906	0.991	0.057	10.9%	9.6
ala	0.4911	0.491	0.035	5.4%	4.8
val	0.5520	0.552	0.055	6.0%	5.4
met	0.0602	0.060	0.008	0.7%	0.6
ileu	0.4324	0.432	0.049	4.7%	4.2
leu	0.5700	0.570	0.064	6.2%	5.5
tyr	0.8516	0.852	0.139	9.3%	8.3
phe	0.3398	0.340	0.050	3.7%	3.3
lys	0.4893	0.489	0.063	5.4%	4.8
his	0.2031	0.203	0.028	2.2%	2.0
trp	0.0000	0.000	0.000	0.0%	0.0
arg	0.4354	0.435	0.068	4.8%	4.2
pro	0.7467	0.747	0.073	8.2%	7.3
% injected	100%			total residues:	89

	nmol protein		µg.	
Analyzed	0.1029		1.029	
Sample total	102.8778		1028.778	

in 2.1 mg

*10k refers to 10 kilodaltons (assumed theoretical molecular weight of the protein

Amino Acid	HHH2324	nmoles aa	µgrams	mole percent	# residues (If 10K* protein)
cysac	0.0000	0.000	0.000	0.0%	0.0
cmcys	0.0000	0.000	0.000	0.0%	0.0
asx	1.0336	1.034	0.119	10.7%	9.4
thr	0.3214	0.321	0.032	3.3%	2.9
ser	0.5450	0.545	0.047	5.6%	5.0
glx	1.3551	1.355	0.175	14.0%	12.3
gly	1.0186	1.019	0.058	10.5%	9.3
ala	0.5022	0.502	0.036	5.2%	4.6
val	0.5680	0.568	0.056	5.9%	5.2
met	0.0661	0.066	0.009	0.7%	0.6
ileu	0.4504	0.450	0.051	4.7%	4.1
leu	0.5909	0.591	0.067	6.1%	5.4
tyr	0.9312	0.931	0.152	9.6%	8.5
phe	0.3634	0.363	0.053	3.8%	3.3
lys	0.5074	0.507	0.065	5.2%	4.6
his	0.2136	0.214	0.029	2.2%	1.9
trp	0.0000	0.000	0.000	0.0%	0.0
arg	0.4990	0.499	0.078	5.2%	4.5
pro	0.7172	0.717	0.070	7.4%	6.5
% injected	100%			total residues:	88

	nmol protein		µg.	
Analyzed	0.1098		1.098	
Sample total	109.7840 (w/o Cys, Trp)		1097.840	

in 1.7 mg

*10k refers to 10 kilodaltons (assumed theoretical molecular weight of the protein

Amino Acid	HHH2325	nmoles aa			
			µgrams	mole percent	# residues
cysac	0.0000	0.000			(If 10K* protein)
cmcys	0.0000	0.000	0.000	0.0%	0.0
asx	0.7693	0.769	0.000	0.0%	0.0
thr	0.2512	0.251	0.089	10.6%	9.3
ser	0.4147	0.415	0.025	3.4%	3.0
glx	0.9885	0.989	0.036	5.7%	5.0
gly	0.7701	0.770	0.128	13.6%	12.0
ala	0.3808	0.381	0.044	10.6%	9.3
val	0.4325	0.433	0.027	5.2%	4.6
met	0.0454	0.045	0.043	5.9%	5.2
ileu	0.3400	0.340	0.006	0.6%	0.6
leu	0.4495	0.450	0.038	4.7%	4.1
tyr	0.7015	0.702	0.051	6.2%	5.5
phe	0.2688	0.269	0.114	9.6%	8.5
lys	0.3860	0.386	0.040	3.7%	3.3
his	0.1631	0.163	0.049	5.3%	4.7
trp	0.0000	0.000	0.022	2.2%	2.0
arg	0.3734	0.373	0.000	0.0%	0.0
pro	0.5503	0.550	0.058	5.1%	4.5
% injected	100%				
				total residues:	88

	nmol protein		µg.		
Analyzed	0.0824		0.824		
Sample total	82.4482		824.482		in 1.5 mg

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