Structural, nutritional and protein functional properties of *Trichilia emetica* and *Trichilia dregeana* seeds

by

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PREFACE

The work described in this thesis was carried out in the Department of Dietetics and Human Nutrition, School of Agricultural, Earth and Environmental Sciences, University of KwaZulu-Natal; under the Supervision of Prof Muthulisi Siwela and Dr Obiro Wokadala.

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As the candidate's supervisors we agree to the submission of this thesis.

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Prof Muthulisi Siwela (Supervisor)

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Dr Obiro Cuthbert Wokadala (Co-Supervisor)

DECLARATION

I, Gugu Felicity Tsomele declare that:

1. The research work reported in this thesis, except where otherwise stated, is my original work.
2. This thesis, or any part of it, has not been submitted for any degree or examination at any other University.
3. This thesis does not contain any other persons' writing, unless specifically acknowledged as being sourced from them.
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ABSTRACT

Food and nutrition insecurity are prevalent in developing regions, especially in the predominantly low-economic status of sub-Saharan Africa. Yet, several edible domesticated and wild plants are grossly under-utilised as food sources. The woody plants *Trichilia* species are indigenous to Africa and are also found in other developing countries. Although the oily seeds of *Trichilia* species seem to have great potential for contributing towards the alleviation of malnutrition in the developing regions, they are grossly under-utilised and there is a dearth of scientific literature regarding their nutritional potential. To improve the utilisation of *Trichilia* seeds on a commercial scale, it is vital to study their physical characteristics and the nutritional and food-related (functional) physico-chemical properties of their components. In this study, the structural and chemical composition of *Trichilia emetica* (*T. emetica*) and *Trichilia dregeana* (*T. dregeana*) seeds, in addition to the nutritional and functional properties of their proteins, were investigated and compared with the soybean.

The results of the seeds analysis showed that *Trichilia* seeds had difficulty sliding on surfaces due to their oblate shape. *Trichilia* seeds showed lower sphericity (0.66) than soybean (0.99), with higher bulk density (645.9 kg/m^3 and 433.6 kg/m^3, respectively) and lower true density (875.8 kg/m^3 and 950.4 kg/m^3, respectively). The porosities for *T. emetica* and *T. dregeana* were significantly lower (55.07% and 54.38%), with a higher geometric mean diameter (29.7 mm and 16.9 mm) than soybean. *Trichilia* species showed similar shapes, but different localisation of the seeds' protein bodies when compared with soybean. *Trichilia* seeds' protein bodies were larger in size and more round in shape than those of the soybean.

The other investigations in this study determined the nutritional properties of the flour and protein of *T. emetica* and *T. dregeana* seeds, and compared the chemical structure and functional properties of protein in the *Trichilia* seeds with the soybean. The results showed that *T. emetica* and *T. dregeana* seeds contained substantial protein (25.6% and 17.3%, respectively) and high concentrations of fat (49% and 51.5%, respectively). Potassium, calcium, iron and zinc were the major minerals in the *Trichilia* seeds. *T. emetica* and *T. dregeana* protein had substantial concentrations of Phenylalanine, Leucine, Isoleucine, Valine and Lysine and Methionine, which were comparable with those of the soybean. The concentrations of essential amino acids in *Trichilia* seeds, similar to their concentrations in soybean, were above the recommended Food and Agricultural Organisation standards for adults. The proportion of essential amino acids to total amino acids of *T. emetica* and *T. dregeana* seeds was similar to that of the soybean. The *Trichilia* spp protein had more β-conformation than α-helices (21%) comparable with soybean protein. The protein concentrates of *T. emetica* seed had higher Water holding Capacity, Foaming Capacity and Foaming Stability with lower Oil holding Capacity and Emulsion activity Index when compared with soybean and *T. dregeana* seeds.

The findings of the current study indicate that the physical and nutritional properties of *T. emetica* and *T. dregeana* seeds and their protein properties had comparable properties to existing commercial oilseeds. This could improve the utilisation of the seeds as complementary foods and can cause an increase in the number of available food sources for food and nutrition security, thereby improving the livelihoods of individuals. However, there would be a need to modify the existing technologies for drying, milling, packaging and transportation to accommodate the *Trichilia* seeds. Furthermore, the bioavailability of the minerals and protein of the *Trichilia* seeds should be assessed.

DEDICATION

To GOD Almighty, My Creator, My Anchor and My Heavenly Father.

"I would not have done it without You LORD"

To my late Grandfather, Dr FD Mashele

who used to say,

"It's supposed to be hard, if it was easy everyone would be doing it"

May His Soul rest in Peace

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1. INTRODUCTION

Trichilia species *(Trichilia emetica* and *Trichilia dregeana)* are indigenous plants that flourish in well-drained, rich sandy soil and in areas with high rainfall (Orwa *et al*., 2009a; Uamusse & Yeboah, 2016). They naturally grow throughout Sub-Saharan Africa (Germishuizen & Meyer, 2003). The *Trichilia* seeds have a high mineral content, specifically Magnesium (Mg), Potassium (K), Phosphorous (P), Iron (Fe) and Sodium (Na) (Saka & Msonthi, 1994) similar to peanuts (Jonnala *et al*., 2005) and cotton seeds (Beyzi *et al*., 2019). *Trichilia* seeds are oil-rich seeds with a good quality oil known as mafura oil, and they are also rich in fat. Traditionally, these seeds are used for cosmetics and medicinal purposes due to their high phytochemical content (Komane *et al*., 2011 & Vermaak *et al.*, 2011).

Due to high malnutrition rates in African rural areas, the *Trichilia* seeds, especially *Trichilia emetica*, are used as a complementary food to control malnutrition (Komane *et al*., 2011; Van Wyk B *et al*., 2000; Saka & Msonthi, 1994). Therefore, the *Trichilia* seeds have great potential as an alternative food source to other sources of oilseeds. However, *Trichilia* is under-utilised and under-researched which results in the seed not being commercially utilised or cultivated. The physicochemical characteristics, amino acids and protein chemical structure of the *Trichilia* seeds are unknown. This lack of information on amino acid profile, chemical structure and physicochemical properties further promotes the underutilisation of the *Trichilia* seeds in the food industry.

Trichilia seeds can be used as a food source to improve food and nutrition security, thereby increasing its utilisation. However, to determine the potential use of *Trichilia* seeds as a food ingredient, knowledge of the seed's nutritional composition, physicochemical properties and protein chemical structure, as well as its functional properties, can provide ways to improve the usage of the seeds and reduce malnutrition in rural African communities.

1.1. Definitions, Acronyms and Abbreviations

AAA: Aromatic amino acids

AACC: American Association of Cereal Chemists

AAS: Amino acids score

AgriLASA: Agri-Laboratory Association of Southern Africa

Ala: Alanine

ANOVA: One-way analysis of variance

AOAC: Association of Official Analytical Chemists

AR: Aspect ratio

ARC-TSC: Agricultural Research Council- Tropical and Subtropical Crops

ARC-GC: Agricultural Research Council- Grain Crops

Arg: Arginine

Asp: Aspartic acid

B: Boron

BCAA: Branched chain amino acids

BFLM: Bright field light microscope

Ca: Calcium

CD: Circular dichroism

CLSM: Confocal laser scanning microscope

Cu: Copper

Cys: Cysteine

DIAAS: Digestible indispensable amino acids

EAA: Essential amino acids

EAI: Emulsion activity index

ES: Emulsion stability

E/T: Proportion of essential amino acids to total amino acids

FAO: Food and Agricultural Organisation

FC: Foaming capacity

Fe: Iron

FS: Foaming stability

FTIR: *Fourier*-transform infrared spectroscopy

GI: Globoid inclusions

GMD: Geometric mean diameter

Glu: Glutamic acid

Gly: Glycine

HAA: Hydrophobic amino acids

HCL: Hydrochloric acid

His: Histidine

Ile: Isoleucine

K: Potassium

Leu: Leucine

LP: Lipid bodies

LSD: Least significant difference

Lys: Lysine

Met: Methionine

Mg: Magnesium

Mn: Manganese

NaOH: Sodium hydroxide

NCAA: Negatively charged amino acids

NEAA: Non-essential amino acids

NMR: Nuclear magnetic resonance

OHC: Oil holding capacity

P: Phosphorus

PB: Protein bodies

PCAA: Positively charged amino acids

PDA: Photodiode array

PEM: Protein energy malnutrition

Phe: Phenyalanine

Pro: Proline

RDA: Recommended daily intake

RH: Relative humidity

S: Sulphur

SANBI: South African National Biodiversity Institute

SEM: Scanning electron microscope

Ser: Serine

SOY: Soybean

TD: *Trichilia dregeana*

TE: *Trichilia emetica*

TEM: Transmission electron microscope

Thr: Threonine

Trp: Tryptophan

Tyr: Tyrosine

UPLC: Ultra-Performance Liquid Chromatography

UV: Ultra-violet Spectroscopy

V_1: Unit volume

Val: Valine

WHC: Water holding capacity

WHO: World Health organization

XRD: X-ray diffraction

Zn: Zinc

1.2. Outline of the thesis structure

The structure of this thesis is as follows:

Chapter 1: Introduction, which includes the problem statement, definitions, acronyms and abbreviations; and an outline of the thesis structure

Chapter 2: Literature review

Chapter 3: Hypotheses, and an outline of the study and methodology

Chapter 4: *Trichilia emetica* and *Trichilia dregeana* seeds macro- and microstructure, and their physical properties

Chapter 5: Nutrient and anti-nutrient content of flour and protein concentrate of *Trichilia emetica* and *Trichilia dregeana* seeds

Chapter 6: Chemical structure and functional properties of *Trichilia emetica* and *Trichilia dregeana* seed protein

Chapter 7: General discussion

Chapter 8: Conclusions and recommendations

References

The referencing style used in this thesis is according to the guidelines used at the Dietetics and Human Nutrition department, University of KwaZulu-Natal, Pietermaritzburg,

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2. LITERATURE REVIEW

This Literature review is divided into four parts. The first part of the review is on global food and nutrition insecurity. The second part is on the *Trichilia* seed and plant ecology, the importance of the indigenous seeds, and the nutritional and physical properties of oilseeds. The third part is on the seed microstructure and protein composition. The fourth part of the review is on the secondary structure of oilseed protein, functional properties and protein digestibility.

2.1. Food and nutrition insecurity as a global problem

Food and nutrition insecurity are when an individual or household has limited or uncertain access and availability to safe and nutritious food to meet their daily needs (FAO, 2004; Govender *et al*., 2016 & Cafiero *et al*., 2018). Food insecurity is associated with poverty and leads to undernutrition or malnutrition. Elements contributing to food insecurity include access to food, food availability, utilisation and stability (FAO, 2008). Food insecurity can be affected by many factors such as increased conflict, climate-related events, economics and geographic location (Drysdale *et al*., 2019). In terms of geographic location, food and nutrition insecurity have been found to differ between rural and urban areas (Harris *et al*., 2019 & Drysdale *et al*., 2019). Garrett and Ruel (1999) found that in Mozambique food prices are higher in rural communities compared to urban areas; however, Walsh and Van Rooyen (2015) had found the opposite in South Africa. Therefore, higher food prices in rural areas can limit access to nutritional food in rural communities.

Globally, food insecurity affects more than 26.4% of the world population with Sub-Saharan Africa having higher numbers, and the United States reflecting a lower percentage of approximately 11.1% (Drysdale *et al*., 2019 & Huizar *et al*., 2020). In the year 2014 to 2016, severe food insecurity increased between 23.4-26.6% in Africa; however, in developed continents like Europe, America and Asia severe food insecurity is decreasing (Cafiero *et al*., 2018). In rural areas, there is an inadequate intake of nutrients associated with food insecurity due to poor food availability, which leads to increased rates of stunting and underweight (Drysdale *et al*., 2019 & Harris *et al*., 2019). Individuals in rural communities opt for cereals, legumes, fruits and vegetables as their staple food due to the high cost of commercially processed complementary foods found on store shelves (Harris *et al*., 2019). Staple foods such as maize, rice, sorghum, millet and wheat are rich in energy and fibre but lack essential amino acids (Lysine, Leucine, Valine and Tryptophan) and minerals such as calcium (Ca), iron (Fe) and zinc (Zn) (Kulamarva *et al*., 2010). Maize meal-based complementary food is a vital part of the diet; however, maize contains high levels of phytates that can inhibit protein, Ca, Fe and Zn absorption (Gibson *et al*., 2010).

Plant proteins such as legumes (soybean) and indigenous seeds (Bambara and cowpea) with a similar nutritional profile are used as a food or protein source (Fasoyiro *et al*., 2012). The oilseeds and indigenous seeds are affordable alternatives that can be used to make value-added products at reduced costs, especially in rural areas of Africa (Jain *et al*., 2009). Indigenous seeds have appealing nutritional qualities that can help alleviate food and nutrition security in Africa. However, the different *Trichilia* seeds' nutritional properties and chemical structures have never been entirely researched, and are therefore underutilized.

2.2. **Ecology of *Trichilia* plants**

Trichilia is an indigenous plant that is evergreen and available all year round. This plant can adapt to widely different agroecological conditions (Orwa *et al*., 2009 & Vermaak *et al*., 2011). *Trichilia* grows well in areas with high rainfall and rich, well-drained sandy soil (Komane *et al*., 2011). The *Trichilia* plants flower from August to November and bear fruits between April to June each year (Yeboah, 2016 & SANBI, 2004). This South African indigenous plant can grow up to 20 to 35 metres in height and can start bearing fruits 5 to 6 years after planting (Grundy & Campbell, 1993). The leaves of this indigenous plant are dark or less glossy green on the upper surface, and covered with brownish curly hairs on the lower surface. However, *T. dregeana* is hairless on the undersurface of the leaves (Orwa *et al*., 2009). The flowers are small, creamy to pale yellow-green in colour and are fragrant; however, other plants have white flowers (Vermaak *et al*., 2011). *Trichilia* genus belongs to the *Meliaceae* family (Mahogany family) with about 260 species worldwide (Usman *et al*., 2018) and these species include *T. emetica*, *T. hirta*, *T. dregeana*, *T. americana* amongst others. The *Trichilia* genus name is derived from the word 'tricho' referring to the 3-lobe fruits of the plant (Shakkeel *et al*., 2015). They are widespread across America, Asia (exotic range) and sub-Saharan African countries (native range of the species) such as Zimbabwe, Sudan, Cameroon, Uganda, Malawi and Swaziland (Fig 2.1) (Komane *et al*., 2011; Germishuizen & Mayer, 2003). However, *T. emetica* and *T. dregeana* are the most commonly found species in South Africa. In South Africa, the plants are mainly found in Mpumalanga, Limpopo, KwaZulu Natal and the Eastern Cape (Komane *et al*., 2011). *Trichilia emetica* is commonly called Natal Mahogany or *Mkhuhlu* (Siswati) or *mutuhu* (Tshivenda) or *umathunzini* (IsiZulu) in South Africa. The *T. dregeana* is called Forest Mahogany or Cape Mahogany (Vermaak *et al*., 2011); however, in other languages such as IsiZulu and Siswati the *T. dregeana* is called the same name as *T. emetica*.

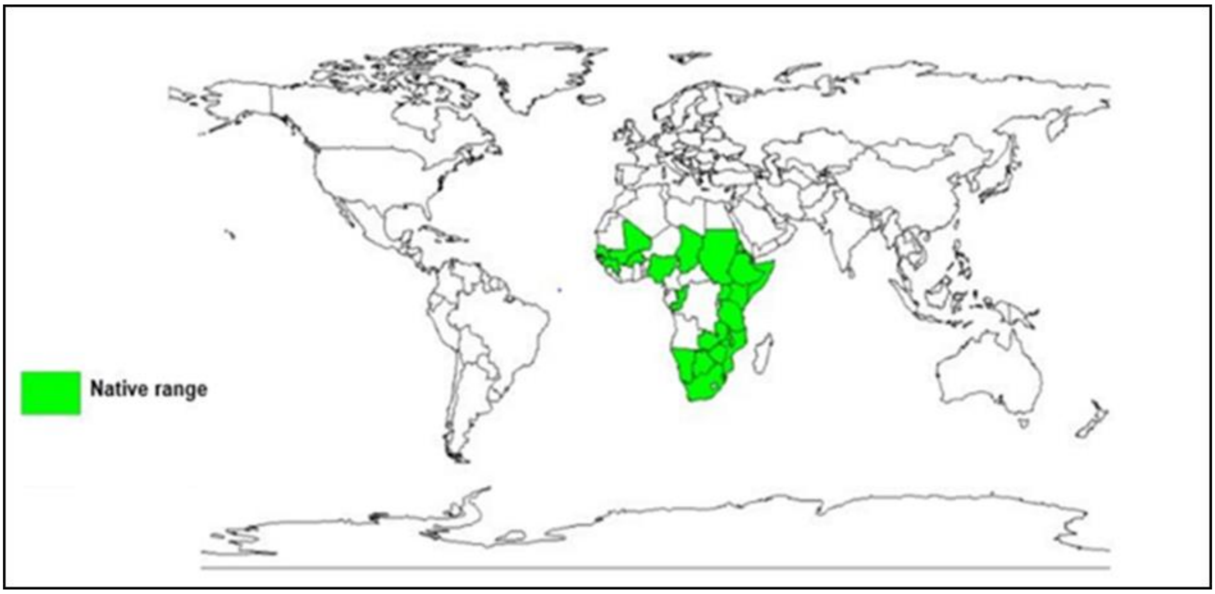

Fig 2.1: Geographical distribution of native *Trichilia* species on the African continents (Orwa *et al*., 2009)

Even though the two species are called by similar names and look identical, there are differences between the *Trichilia* plants. The *T. emetica* differs from the *T. dregeana* in fruit morphology, stem attachment, plant leaves and fruit size (Table 2.1) (SANBI, 2004).

The *T. dregeana* has compound leaves of about 21 cm in length with eight to nine widely spaced pairs of lateral veins and acute to acuminate tips, while the *T. emetica* has elliptic to oblong-elliptic leaves with about thirteen to sixteen closely spaced pairs of lateral veins with more rounded tips. The fruits for both *T. emetica* and *T. dregeana* are creamy brown; however, *T. dregeana* fruits split into three or four valves and *T. emetica* fruits split into two or three valves. The *T. dregeana* bark is grey-brown with a smooth texture and sometimes with segments around the base of the stem. However, the *T. emetica* bark is dark grey to brown with a smooth to rough stem (Palgrave, 2002; Orwa *et al*., 2009 & Vermaak *et al*., 2011).

Table 2.1: Different characteristics of *T. emetica* and *T. dregeana*

Characteristics	***T. emetica***	***T. dregeana***
Fruit Stem attachment	Attached with a distinct stripe to the fruits stem	Grows directly from the leafstalk
Fruit Colour	Black, with less of a glossy orange-red scarlet colour	Black with glossy red scarlet colour
Leaves	Elliptic in shape, less glossy and a light green colour	Compound in shape and a glossy, dark green colour
Fruit size[a]	18-25mm in diameter	3-15mm in diameter

[a] Orwa *et al*., 2009

2.2.1. Importance of Indigenous *Trichilia* plants and seeds

Utilisation of the *Trichilia* spp remains domesticated, despite the growth of these indigenous food crops being of paramount importance for sustaining the life of humans in terms of acting as a supplement to the current food source (Akinola *et al*., 2020 & Van Wyk, 2011). In the past, *Trichilia* plants were used for ornamental purposes or as a food source for animals. Currently, *T. emetica* is used as a complementary food to alleviate malnutrition in African rural areas (Van Wyk *et al*., 2000 & Komane *et al*., 2011). The seeds can be soaked in water to produce a milky juice which is then mixed with vegetables such as spinach, sweet potatoes or squash to make delicious dishes, and for soup preparation (Komane *et al*., 2011 & SANBI, 2004). In addition, *Trichilia* seeds can be used as an active ingredient in cosmetics manufacturing and personal care products based on their revitalising and nourishing properties. *Trichilia* seeds are used to produce two types of oils known as mafura oil from the seed, and mafura butter from the kernel (Komane *et al*., 2011). *Trichilia* oil is edible, and is used to preserve food products and to cook food in rural communities (Komane *et al*., 2011). Traditionally, *Trichilia* oil is often used as a hair oil, while the butter is used in the production of natural soaps, candles, hair oil, body ointment and lip balm (Komane *et al*., 2011). The bark of the tree is used to treat intestinal ailments (Grundy & Campbell, 1993). The leaves are eaten by cattle and goats (Mashungwa, 2007). The roots are crushed to a powder and used to treat various health issues such as cirrhosis, river blindness and ascariasis, as well as to act as a remedy for colds and pneumonia (Komane *et al*., 2011 & Sanogo, 2011). Wood from this tree is used to make furniture, musical instruments and firewood (Tropical database plants, 2019); however, some people also chew the wood in order to maintain their oral health (Komane *et al*., 2011). *Trichilia* spp are also valued in Africa for their traditional medicinal purposes, such as treating leprosy, various skin ailments, kidney problems, jaundice, stomach complaints, fractures, malnutrition control, malaria, insomnia, asthma, pneumonia, colds and backaches; however, the part of the plant used to treat these conditions is not specified in the literature (Vermaark *et al*., 2011, Komane *et al*., 2011 & SANBI, 2004).

2.3. Nutritional composition and physical qualities of oilseeds

Oilseeds such as bambara groundnuts, peanuts, sunflower seeds, cottonseeds, soybean and canola seeds are found to have great nutritional potential (Table 2.2). Certain oilseeds like sunflower seeds and *T. emetica* have a lower protein content, while soybean, marama (gemsbok beans), and peanuts have a higher protein content with a lower oil/fat content than sunflower seeds. There is limited knowledge of the different *Trichilia* species' nutritional and physical properties. However, Saka & Msonthi (1994) observed that the *T. emetica* had a crude protein content of about 17.0%, crude fat content of 22.9% and an ash content of about 4.5%. In addition to oilseeds containing an extensive amount of protein and fat, they are also rich in certain micronutrients such as Fe, Ca and K, but are poor in P and Mg (Kumar & Shankar, 2013). *Trichilia emetica* was previously observed to have high K (13.017 mg/100 g), Mg (1.129 mg/100 g) and P levels (3.164 mg/100 g); however, Fe was low (0.043 mg/100 g), and Ca was absent (Saka & Msonthi, 1994). Sunflower seeds and peanut seeds have a lower K content (1088 mg/100 g and 564-614 mg/100 g, respectively) compared with cottonseeds and rapeseeds (1110 mg/100 g and 1149-2232 mg/100 g, respectively) (Anjum *et al.*, 2012; Jonnala *et al*., 2005; Beyzi *et al*., 2019 & He *et al*., 2013). The variations noted in the chemical compositions of oilseeds can be related back to different agricultural practices, environmental conditions and genotypes (Ratajczak *et al.*, 2017 & Maharjan *et al*., 2019).

Table 2.2: Chemical composition of different oilseeds

Element	**Sunflower**	**Soybean**	**Peanut**	**Marama**	***T. emetica***
Crude Protein (%)	20.8	32.5	24.90	32.3	17
Crude Fat (%)	51.6	14.9	39.10	40.0	22.9
Moisture (%)		7.9	4.11	5.3	N/D
Fibre (%)	8.6	8.6	2.91	N/D	8.1
Ash (%)	3.0	4.8	4.55	3.0	4.55
Reference	Anjum *et al*., 2012	Amonsou *et al.,* 2011	Kumar & Shankar, 2013	Mosele *et al*., 2011	Saka & Msonthi, 1994

Oilseeds can be a potential food source due to their enhanced nutritional profile. Knowledge of their nutritional and physical qualities may help in the improved utilisation of oilseeds and indigenous seeds. Physical properties of oilseeds including particle size, shape, mass, bulk density, true density, porosity, surface area and geometric mean diameter (GMD) are vital in determinimg the cleaning, separation, milling, cooling, storage, transportation and sorting of the seeds (Unal *et al*., 2009). The GMD, surface area, unit volume and sphericity are determined using length, width and thickness. Therefore, knowing these physical properties can assist in designing the processing equipment required, such as grading and sorting machines. These are essential in determining the required size reduction operations and the quality of the final product (Prada & Aguilera, 2007). The way food material responds to processing procedures provides essential quantitative knowledge for the rational design of methods and machinery. Rapeseeds have been found to have a sphericity of between 0.91-0.93 (Unal *et al*., 2009); however, oilseeds like castor seeds have a sphericity of about 0.67 (Perea-Flores *et al*., 2011). The sphericity of approximately 0.67 or less means that the seed may have difficulty rolling but could easily slide on the surface (Perea-Flores *et al*., 2011). The sphericity is also interrelated to the porosity and GMD of the seeds. Porosity is dependent upon the geometry and surface properties of the material. The porosity is also affected by the moisture content concentration of the seed. Moisture content must be taken into consideration when investigating porosity. Adebowale *et al*. (2011) observed that reconstituted seeds with 10-30% moisture can cause an increase in porosity with an increase in moisture concentration. Porosity is the percent void of the unconsolidated weight of seeds often needed in airflow and heat flow studies (Unal *et al*., 2017). This is used in calculating the rate of aeration and cooling, heating and drying, and in the design of heat exchange methods. GMD is related to the mass, or energy and heat transfer rates through the surface of the seeds (Niveditha *et al*., 2013). GMD can help to lower the energy costs in the industry during the different stages of milling, cooling, drying and in heating operation designs. Oilseeds such as rapeseeds have a lower GMD of about 1.96-2.11 mm while soybean seeds have a higher GMD value (12.6 mm) (Unal *et al*., 2009).

Bulk density is used to determine the seed quality during storage (Unal *et al*., 2009), with higher bulk density being favored in the storage, packaging and transportation of food materials (Bhattachanya, 2011). However, lower bulk density is advantageous in the drying process, particularly in the tunnel and fluidized bed drying. The lower bulk density also enables rapid airflow into the food material. This can help in ensuring physiological changes in food materials or seeds remains consistent (Maskan, 2000 & Zhang *et al*., 2006).

2.4. **Microstructure of oilseeds**

The microstructure of oilseeds is vital in the extraction, processing and utilisation of the oilseed protein (Aguilera, 2005; Parada & Aguilera, 2007). The microstructure helps in improving nutrient bioavailability, and determines the rate and extent of nutrient release during digestion (Aguilera, 2022). The seed microstructure for oilseeds such as soybean, almond, castor, peanuts and marama bean has been extensively studied (Lott & Buttrose, 1978; Young *et al*., 2004b; Perea-Flores *et al*., 2011; Young *et al*., 2004a & Mosele *et al*., 2011a). The protein bodies of the seeds consist structurally of proteinaceous matrix material surrounded by a continuous network of lipid bodies. The protein bodies and lipid bodies may appear circular, spherical or oval. Physical localization of protein bodies relative to the lipids has been found to influence protein extractability and functional properties (Amonsou *et al*., 2014). The protein bodies of different oilseeds can be similar in shape but different in size, and there may also be an absence or presence of crystalline inclusion called globoids or druse crystals (Amonsou *et al*., 2011).

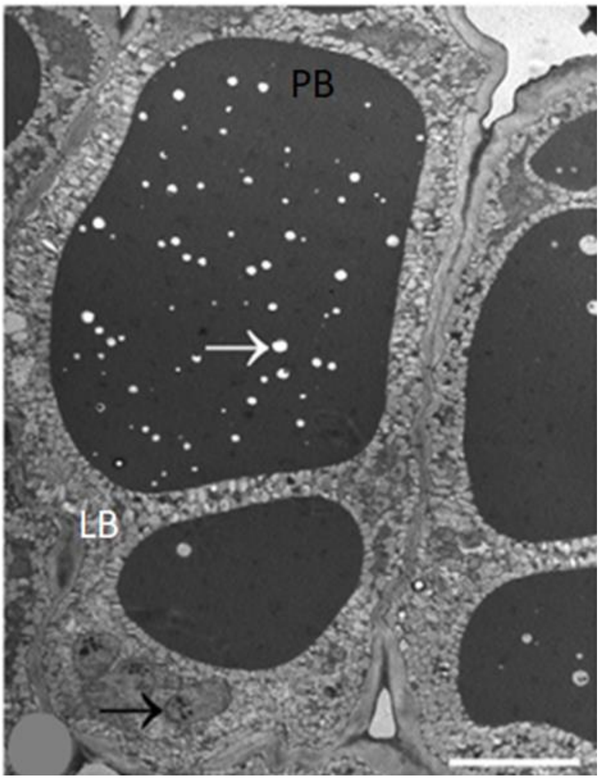

Fig 2.2: Seed microstructure of mature marama bean seeds. The marama bean protein showing large protein bodies with numerous globoids (white arrow) (Mosele et al., 2011a)

Globoids are inclusions characterised by their high phytate content. The globoid inclusions have a spherical or oval shape similar to the protein bodies. Globoid inclusion is a storage site for P, Mg, K and Ca deposited in insoluble phytate (Madsen & Brinch-Pedersen, 2020). Calcium found in the phytate can be in an insoluble form, or more soluble in legumes or oilseeds. This soluble state is due to the high K to Mg and Ca ratio during globoid formation (Madsen & Brinch-Pedersen, 2020). The phytate is said to be rich in minerals such as Ca, P, K and Mg (Madsen & Brinch-Pedersen, 2020); however, other oilseeds such as almond seeds lack Ca (Young *et al*., 2004b). The globoid inclusions can be small and rare, or large and frequent. Work done on peanut, almond and marama bean seeds showed that those seeds had a protein body size of about 8µm; 12µm and 13µm; respectively (Young *et al*., 2004(a & b) & Mosele *et al*., 2011a). The fixation or sectioning of the samples during sample preparation can cause loss of globoid inclusions. Globoids can be identified by using an electron microscope or laser scanning microscope. Marama bean seeds have been found to contain larger protein bodies with numerous globoids when observed under TEM even at lower magnification (Fig 2.2). Other researchers have found that soybean may have globoid inclusions present, or they may be absent. The presence or absence of protein body inclusions in soybean microstructure may be due to the preparation methods used, which include the fixation of the cells. It is suggested that high pH fixation can lead to the lack of globoid inclusion in oilseeds such as soybean protein bodies compared to when using a low pH for fixation (Amonsou *et al*., 2011).

The seed microstructure of some underutilized indigenous seeds, namely marama bean and Bauhinia, has been studied before. The microstructure is vital in understanding the food material functionality of the seed, and thus plays an important role in seed utilisation (Aguilera, 2005; Parada & Aguilera, 2007). Awareness of the physical location of storage protein in the seed is also important as it may affect functional properties such as protein digestibility. Seed microstructure has been found to influence the physical properties of the seeds such as grain hardness (Aguilera, 2005; Aguilera & Stanley, 1999). This grain hardness is vital in milling operations and equipment design for processing (Aguilera, 2005; Aguilera & Stanley, 1999).

2.4.1. Storage protein in oilseeds and their amino acids

Storage proteins are proteins that are found in large amounts in the seeds; they are tissue-specific, only occuring in the seeds. These proteins are synthesized during seed development, sequestered in protein bodies and hydrolyzed to constituent amino acids during germination and early seedling growth, eventually possessing a high level of nitrogen-rich amino acids (Capriotti *et al*., 2014 & Vitale *et al*., 2017). The protein can also act as a storage reservoir for nitrogen, carbon and sulphur (Madsen & Brinch-Pedersen, 2020). Plant proteins have been used for years in the production of foods such as bakery products, beverages, dairy, egg substitutes, salad dressings, sauces and flavourings (Guo *et al*., 2010). However, the use of plant protein concentrate is still limited due to the presence of anti-nutritive compounds such as phytic acids or polyphenols (Arntfield, 2018 & Arrutia *et al*., 2020), and other plant proteins, such as canola protein, contribute to undesirable flavours when added as a food ingredient to food products (Hald *et al*., 2019). Protein concentrates can be used to improve the nutritional value of food products, they can act as an emulsifier to stabilise and form an oil-in-water emulsion, and they improve sensory perception and enhance texture characteristics such as gumminess, chewiness and hardness (Zhang *et al*., 2016; Arrutiaet *et al*., 2020; Aider & Barbana, 2011).

The improved sensory perceptions of the food product, due to the addition of plant protein isolates, may be caused by the products of globulin proteolysis or the plant protein concentrate may contain typical patterns of aroma precursors formed by the degradation of the globulins via the enzyme proteases (Voigt *et al*., 1994). The use of vegetable or plant proteins, instead of meat proteins, as food ingredients can help in bioactive peptide formation during digestion. These peptides can also have a positive effect on the regulation of the cardiovascular and immune systems (Aider & Barbana, 2011).

2.4.2. Amino Acids in oilseeds

Amino acids are organic compounds that are composed of an amino group (NH2), carboxyl group (COOH) and a side chain (R) (See Fig 2.3). Each amino acid is distinguished from other amino acids by its side chain. Protein amino acids composition is the most important factor determining the protein quality. Proteins from different plant sources contain different amino acids profiles that describe different characteristics and health benefits (Kumar et al., 2022). Plant proteins extracted from grains, seeds, legumes, nuts and pulses are mostly low or deficient in essential exogenous amino acids such as lysine (Lys), sulphur-containing amino acids (methionine (Met) and cysteine (Cys)), isoleucine (Ile) and threonine (Thr) (Kumar et al., 2022). Oilseeds contain high concentrations of Leu, Ile, Val, Lys, Phe and Tyr (Table 2.3). Most of the oilseeds had lower sulphur-containing amino acids and Trp, except from sunflower and chia seeds (Table 2.3).

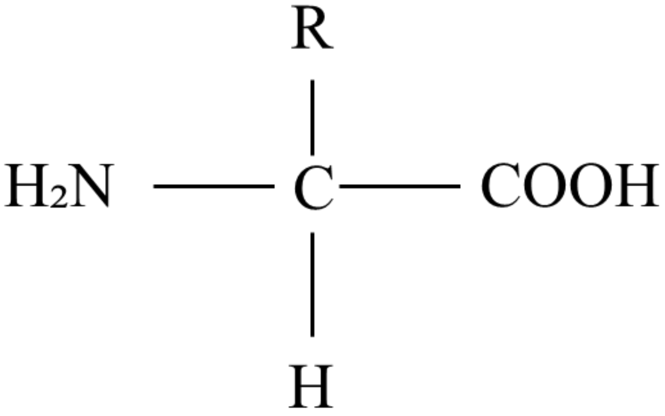

Fig 2.3: The general structure of an amino acid (Hettiarachchy et al., 2012)

Even though soybean protein has been denoted as complete protein; however, the soybean protein has shown to have low concentration of essential amino acids than the chia, cottonseed, peanut and sunflower (Table 2.3) (Carrera et al., 2020). The commercial rapeseed/canola protein amino acids was characterised by high levels of sulphur-containing amino acids which exceed the requirements established by WHO/FAO (2007) standards (Chmielewska et al., 2021).

Table 2.3.: Different amino acids content found in oilseeds

Amino Acids	**Soy**	**Flax**	**Hemp**	**Rapeseeds**	**Cotton seed**	**Sunflower**	**Peanut**	**Chia**
Ile	1.97	0.87	1.29	1.25	2.8-3	44.5	3.5	33.4-33.7
Leu	3.47	1.18	2.16	2.51	5.2-5.6	85	7.0	72-74
Lys	2.37	0.75	1.28	2.04	4.2-4.6	36.5	3.8	60.3
Met	0.59	0.32	0.93	0.47	1.3	31.5	1.3	34-36
Cys	0.80	0.32	0.67	0.59	1-1.1	6.0	0.3	7.8-10.5
Phe	2.25	0.95	1.45	1.44	4.7-4.9	37.5	5.4	64-65
Tyr	1.35	0.53	1.26	0.99	2.5-2.7	25	3.4	37-40
Thr	1.63	0.72	1.27	1.59	2.9-3.1	37	2.2	36
Val	1.94	1.07	1.78	1.55	4.2-4.6	61.5	3.9	44-45
Trp	0.54	0.30	0.37	0.43	1.0-1.2	6.5	0.7	ND
Reference	1	2	4	3	7	5		6

1. Carrera *et al.*, 2011; 2. Klimel-Kopyra *et al.*, 2013
3. Mejicanos & Nyachoti, 2018; 4. USDA, 2016; 5. Bautista *et al.*, 1996; 6. Coelho & Salas-Mellado, 2018; Kumar et al., 2022

The differences in amino acids content may be caused by plant variety and genetic diversity, different cultivation conditions and geographical latitudes (sun exposure, rainfall, temperature, soil fertility), growing conditions, seed maturity and the time of harvest as well as the application of various analytical methods (Kotecka-Majchrzak *et al.*, 2020). Leucine (Leu) and Val are present in higher concentrations compared with sulphur-containing amino acids and hydrophobic Tryptophan. The addition of oilseed proteins rich in sulphur-containing amino acids can improve the nutritional value of the food product (Kotecka-Majchrzak *et al*., 2020). Aspartic acid (Asp), Arg (Arginine), Tyr, Phe, Ile and Leu are found in globulins at high concentrations and Glu (Glutamine), Val and Lys is found in albumins at high concentrations (Aider & Barbana, 2011). The storage proteins for oilseeds can be determined by using SDS gel page which could be in a 2D or 3D format.

2.4.3**. Protein secondary structure of oilseeds**

Plant proteins are comprised of 20 or more amino acids that are chemically bonded together by peptide bonds (Hettiarachchy *et al*., 2012). The protein structure is divided into four levels; primary, secondary, tertiary and quaternary structures (Fig 2.4). The primary structure is made up of extended polypeptide chains, while the secondary structure is comprised of α-helices, β-sheets, β-turns and random coils through hydrogen bonding (Hettiarachchy *et al*., 2012). The tertiary structure is comprised of the polypeptide folded into a 3-dimensional structure. This structure is the spatial disposition between linear chains, segments on assumed optimization of hydrophobic, electrostatic and Van der Wall's interaction in the protein-water (Hettiarachchy *et al*., 2012). The quaternary structure is the spatial disposition of protein with many peptide chains due to protein-protein interactions (Hettiarachchy *et al*., 2012).

The secondary structure of a protein is very sensitive to the location of both Amide I and II bands because the H-bonding between the different elements of the secondary structure involves both C=O and N-H bonds (Carbonaro & Nucara, 2010). Amide I and II are the characteristic bands found in the infrared (IR) spectra of protein arising from amide bands that link the amino acids together (Gallagher, 2009). The formation of the Amide I band peak is due to the stretching vibration of C=O bonds and Amide II is due to the absorption that leads primarily to the bending vibration of the N-H bond (Gallagher, 2009; Sadat & Joye, 2020). Amide II absorption indicates that not all of the protein peptides hydrogen atoms have been exchanged for deuterium or heavy hydrogen. This may be most likely due to the inaccessible compact form of some native, folded protein concentrates (Achouri *et al*., 2012).

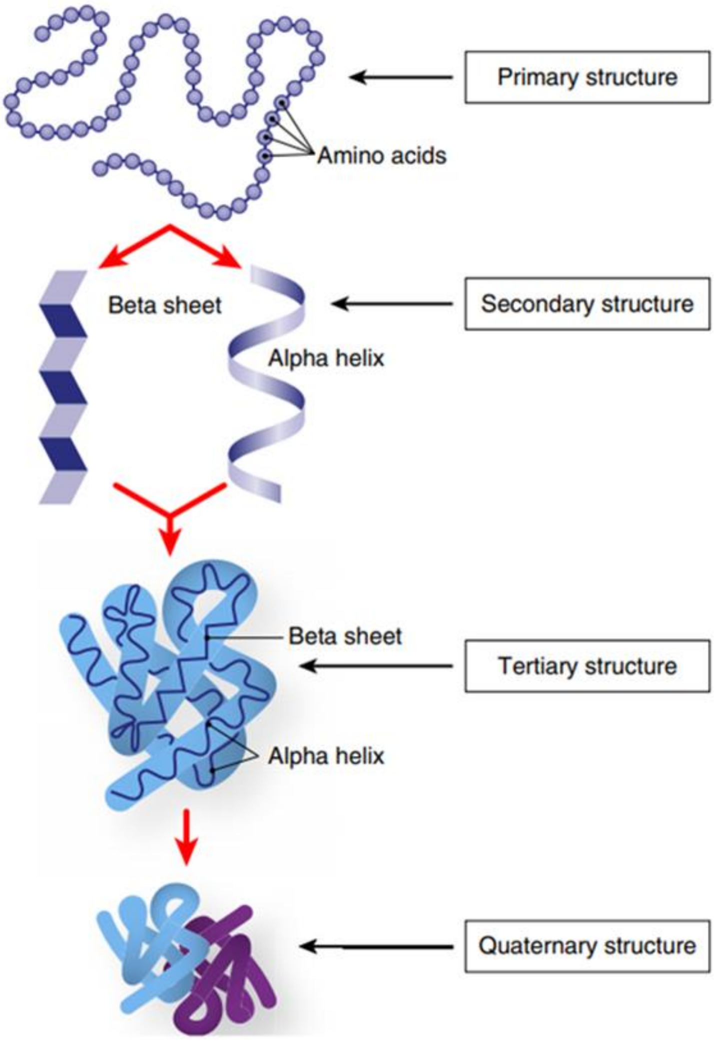

Fig 2.4: The different levels of the storage protein structure (Hettiarachchy *et al*., 2012)

The secondary structure and major functional group of the storage protein can be determined using Fourier-transform infrared spectroscopy (FTIR) (Zhang *et al*., 2015), Raman (Wang *et al*., 2017), Circular dichroism (CD) (Chandrapala *et al*., 2012), X-ray diffraction (XRD) (Jenkins *et al*., 2013) and UV-fluorescence (Barrios-Peralta *et al*., 2012), to name a few methods. FTIR provides a valuable method for studying conformational changes of protein in solid, crystal and solution states (Achouri *et al*., 2012). This procedure may be limited by the type of protein material used. The protein materials usually studied are protein extracts that include isolates, concentrates and meals; these extracts are composed of different individual proteins rather than purified fractions (Achouri *et al.*, 2012). The FTIR is used to identify amide bands in the protein structure. The Amide bands are generally found around 1610-1642 cm^{-1} for the β-sheet, 1643-1649 cm^{-1} for the random coil, 1605-1662 cm^{-1} for the α-helix and 1662-1682 cm^{-1} for the β-turn (Zhang *et al.*, 2020; Sadat & Joye, 2020). The FTIR for cottonseed protein showed that the protein IR spectra was approximately 1658.78 cm^{-1} for Amide band I and 1546.91 cm^{-1} for Amide band II (See Fig 2.5 below) (Kumar *et al.*, 2021). The canola protein concentrate has been reported to have a β-sheet in the region of 1618 cm^{-1} and 1688 cm^{-1}, a random coil of approximately 1645 cm^{-1}and α-helix of about 1651 cm^{-1}. Sunflower seeds have been found to have a β-sheet between 1630.5-1631.0 cm^{-1}, which is a little bit higher than the soy protein concentrate (Gerzhova *et al.*, 2015; Bandara *et al.*, 2018; Zhang *et al.*, 2013). Sesame protein shows a random coil of about 1648 cm^{-1}, α-helix in the region of 1661 cm^{-1} and a β-turn of approximately 1672 cm^{-1} (Achouri *et al.*, 2012). These results for sesame protein were similar to soy protein concerning the random coil; however, the α-helix and β-turn for sesame were slightly higher than the soy protein. The FTIR for rapeseeds previously indicated high β-sheet content and a strong band rear of 1065 cm^{-1} that is typical of inter-molecular sheet interactions and thus supports the oligomeric nature of the protein (Aider & Barbana, 2011). The pronounced decrease in band intensity and slight shift towards lower wave numbers suggest extensive unfolding, and thermal denaturation of the protein that is not reversible during cooling (Achouri *et al.*, 2012). The localization of the Amide band I for the oilseeds is somehow like most of the seeds. The Amide band I peak localization may be affected due to the processing techniques (heating and high pH) and the presence of anti-nutrients in other oilseeds, such as phenolic compounds in sunflower seeds. The presence of phenolic compounds and carboxylic groups in the protein concentrates may cause the carbonyl (C=O) stretching vibration to shift the peak to higher values (Aider & Barbana, 2011). Heating and increased pH can result in the denaturation of the proteins secondary structure, leading to the loss of the β-sheet (Zhang *et al*., 2020).

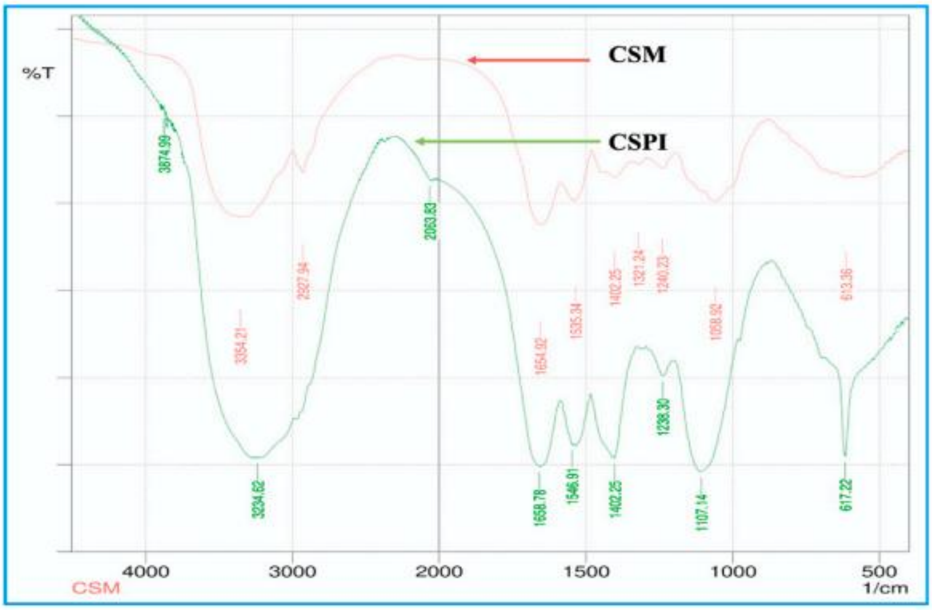

Fig 2.5: FTIR for cottonseeds protein (CSPI) demonstrating the different Amide bands location (Kumar *et al*., 2021).

2.5. Functional properties of oilseeds

The functional properties of oilseed proteins include the physical and chemical properties that influence the behaviour of proteins in food systems during processing, storage, cooking and consumption (Onsaard, 2012 & Yin *et al*., 2010). These properties depend on the protein's molecular weight size, charge distribution and three-dimensional structure (Joshi *et al*., 2012). The functional properties include water holding capacity, oil holding capacity, emulsifying properties, foaming properties and thermal properties. Extensive work has been done on the functional properties of hemp, flaxseed, peanuts, walnut, cowpea, Bambara groundnuts and sunflower protein concentrates (Malomo & Aluko, 2015; Tirgar *et al*., 2017; Yu *et al*., 2007; Mao & Hua, 2014; Mune *et al*., 2014; Mune & Sogi, 2016; Mao *et al*., 2018). Knowledge of these properties offers vital information for the food processing and manufacturing industry to improve on these food products. However, these properties can be affected by three factors known as intrinsic factors (anti-nutrients, hydrophobic/hydrophilic ratio, amino acids composition and sequence), extrinsic factors which includes processing treatment (pH, ionic strength, temperature, and extraction process) and application conditions (Shanthakumar *et al*.; 2022). The presence of anti-nutrients like phenolics may affect the functional properties by imparting certain sensory qualities such as causing a darker colour, bitter taste and astringency (Cai *et al*., 2021). The presence of anti-nutrients can limit the use of oilseed protein (e.g. rapeseeds) in food applications due to their resultant bitter taste in the protein and also interaction with the protein through the formation of complexes (Aider & Barbana, 2011; Khattab & Arntfield, 2009). A high pH, increased NaOH concentration, and elevated temperature may cause dissociation in the protein secondary structure that can then lead to an increase or decrease in functional properties. The increase in the isoelectric point of protein concentrates can result in denaturation of the protein structure which may lead to an increase in some functional properties of the protein. The different functional properties are discussed below.

2.5.1**. Water holding capacity**

Water holding capacity (WHC) is an enhanced ability of the protein concentrates or isolates to absorb or retain water. This enhanced ability can improve the water binding capacity, intensify flavour retention, improve mouthfeel and reduce the moisture of the food product (Shevkani *et al*., 2015a; Aider & Barbana, 2011). Water holding capacity can be influenced by the presence of tannins, carbohydrates and lipids in the protein concentrates (Mao & Hau, 2012). Other intrinsic factors affecting WHC of the food protein include amino acid composition, protein conformation, and surface polarity/hydrophobicity (Zhou *et al*., 2011 & Yu *et al*., 2007). The hempseed, rapeseed, walnut, and soybean protein concentrates have higher WHC and exceed the FAO recommended WHC range for commercial values for protein concentrates of about 1.9-2.20 ml/g protein (Malomo & Aluko, 2015; Jia *et al*., 2021; Mao & Hua, 2014; Mao *et al*., 2018). The high WHC of protein concentrate can be due to the greater ability of the protein concentrates to swell because they contain carbohydrates and crude fibre as major components, and these have an affinity for water molecules (Ettoumi & Chibane, 2015). The high WHC can also be caused by the hydrophilic parts of the protein, such as polar and charged side chains (Mao *et al*., 2018). The addition of salt to the protein may cause dehydration of the protein through the binding of the salt to the existing water, resulting in a decrease in WHC (Day *et al*., 2022). High WHC is vital in viscous food products such as soup, dough, and custard (Sreerama *et al*., 2012). In meat products, WHC helps retain meat juiciness, and enhances mouthfeel and flavour of the meat. In bakery products and cheese, WHC prolongs the products' shelf life (Gerzhova *et al.*, 2015).

2.5.2**. Oil/Fat holding Capacity**

Oil or fat holding capacity (OHC) in food products affects the retainment of flavour, influences consistency of the food product and enhances mouthfeel; however, OHC is highly affected by protein content and conformation (Ettoumi & Chibane, 2015; Khattab & Arntfield, 2009). Oil holding capacity (OHC) relies mainly on its capacity to physically entrap oil particles by a complex capillary-attraction process. This property is dependent on powder particle size and surface tension. An increase in OHC is shown to be positively correlated with the ability to form and stabilise emulsions. Most oilseeds such as bambara, cowpea, sesame and walnut have shown to have lower OHC; however, hemp, peanuts, rapeseed and sunflower protein concentrate have higher OHC (Adekele *et al*., 2018; Mune *et al*., 2014; Mao & Hau, 2014; Malomo & Aluko, 2015; Liu *et al*., 2019; Jia *et al*., 2021). High OHC can be due to a large proportion of hydrophobic groups (Leu, Ileu, Val and Phe) and nonpolar side chains of amino acids on the surface of protein concentrates (Day *et al*., 2022 & Kumar *et al*., 2022). The addition of salt to protein concentrates and an increase in pH during processing and extraction of protein concentrates can cause an increase in OHC through solubilisation of the protein structure. However, high OHC may not be desirable in certain foods such as doughnuts or pancakes as it may lead to 'greasiness' of the food products. This can be overcome by the addition of soy flour or protein to prevent excessive fat absorption during cooking (Hutton & Campbell, 1981). Higher OHC of protein concentrates or isolates is required in baked food products and the meat industry, as meat replacers or extenders (Onsaard, 2012).

2.5.3**. Foaming capacity and stability**

Food foams are a media where two immiscible substances such as liquid and gas, or air, are mixed with the help of a protein (surface agent). Foams can be associated with intrinsic molecular properties such as molecular size and shape, pH, flexibility, hydrophobicity and charge of the protein molecules (Gerzhova *et al*., 2015). The pH of the protein concentrates can lead to a weaker charge of the protein molecule surface, thereby increasing the protein flexibility and allowing the protein to spread at the interface. The foams are stabilised due to the viscoelastic film of hydrophobicity (Shevkani *et al*., 2019 & Gerzhova *et al*., 2015). High hydrophobicity absorbs more rapidly at the interface (Gerzhova *et al*., 2015). Flexible and loose protein molecules can unfold faster whilst a rigid, globular protein structure takes a longer time to absorb and lower the interfacial tension (Gerzhova *et al*., 2015). A protein must be soluble in the liquid phase and be capable of rapid migration to the interface to form a film around nascent gas bubbles; the protein can, therefore, perform well in foam (Obiegbuna *et al*., 2014).

Foaming capacity (FC) and foaming stability (FS) are important in food systems such as cakes, dessert, whipped toppings and ice cream mixes where aeration and overrun is required (Shevkani *et al*., 2015). The FC and FS depends upon their ability to move quickly to the air/liquid interface, and their solubility in the liquid phase so that they can rearrange themselves in the interfacial film (Malik & Saini, 2017). Canola and walnut protein concentrates have been shown to have a high foaming capacity with lower foaming stability, whilst other oilseeds such as peanut and Bambara groundnut have lower foaming capacity with higher stability (Gerzhova *et al*., 2015a; Mao & Hau, 2014; Liu *et al*., 2019 & Adekele *et al*., 2018). Lower foaming capacity and stability can be related to inadequate electrostatic repulsions, lesser solubility and excessive protein-protein interactions (Malomo *et al*., 2014). The addition of salt as sodium chloride results in protein denaturation of the disulfide bonds in the protein tertiary structure and solubilisation of the protein, facilitating multilayer formation at the interface. This can cause an increase in the foaming capacity and stability of the protein concentrates (Mao & Hau, 2014).

2.5.4**. Emulsifying properties**

An emulsion is a complex system that involves a multitude of chemical and physical phenomena that play different roles in the formation, stability and textural properties of a protein-fat water system. Emulsifying properties are mostly dependent on the physicochemical properties of the proteins (i.e. surface charge, hydrophobicity, solubility and size), production procedure and composition of protein isolates or concentrates (Benjamin *et al*., 2014). Emulsion capacity (EC) refers to the maximum quantity of oil that can be distributed within a solution of emulsifiers without the emulsion being separated or turning from an oil-water emulsion to a water-oil emulsion (McClements, 2010). The EC and Emulsion stability are related to the protein concentrate, pH and ionic strength. Plant protein isolates tend to have improved emulsifying properties compared to concentrates (Piornos *et al*., 2015).

2.6**. Concluding Remarks**

The protein composition of indigenous oilseeds depends on their genotype, growing conditions and oilseed type. The nutritional properties of indigenous seeds such as *Trichilia* seeds are not known. In addition, the protein body structure and protein composition in terms of seed microstructure, protein chemical structure and amino acids composition, as well as their functional properties with regards to foaming properties, emulsion activity, protein digestibility, water and oil holding capacities, are not known for *Trichilia* seeds. This knowledge is required to determine the potential use of *Trichilia* as a food source in rural communities of Sub-Saharan Africa, and as a food ingredient in the food industry.

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3. HYPOTHESES, OBJECTIVES AND STUDY DESIGN

3.1. Hypotheses

Hypothesis 1

The physical properties of *Trichilia* seeds will be similar to those of soybean seeds. Oilseeds, such as rapeseed, have higher sphericity and geometric mean diameter; however, castor oilseeds have higher porosity, lower sphericity and geometric mean diameter (Sedat *et al*., 2005; Unal *et al*., 2009 & Perea-Flores *et al*., 2011). *Trichilia* microstructure seeds will have spherical protein bodies with a network of spherical lipid bodies around them, similar to soybean protein bodies. *Trichilia* protein bodies will differ in the protein body variation and localisation to those of soybean protein bodies. Protein body microstructures of soybean, marama, almond, castor and peanut oilseeds have been found to consist of spherical protein bodies with a cluster of lipid bodies around them (Mosele *et al*., 2011a; Ellis *et al*., 2004; Perea-Flores *et al*., 2011 & Young *et al*., 2004a).

Hypothesis 2

The physicochemical and nutritional properties of *T. emetica* and *T. dregeana* seeds and their protein will be similar to soybean seeds and their respective protein. Oilseeds such as soybean, peanuts, sunflower, castor oilseeds and cotton seeds have a higher protein content (36%, 38%, 21% and 22%; respectively) and fat content (El-Shemy, 2011, Anjum *et al*., 2012; Ingale & Shrivastava, 2011; & Pereas-Flores *et al*., 2011). *T. emetica* and *T. dregeana* seeds will have the micronutrients Mg, Fe, Ca, Mn, Zn, Cu, P and K, which is similar to that found in soybean, peanut oilseeds (Jonnala, 2005), cottonseeds (Beyzi *et al*., 2019) and rapeseeds (He *et al*., 2013). The major amino acids in *T. emetica* and *T. dregeana* protein will be glutamine, aspartic acid, leucine, lysine and arginine, as in soy protein. Oilseeds such as sunflower, chia and flaxseed protein have been reported to contain high levels of methionine, arginine, lysine, leucine, aspartic acids and glutamine (Bautista *et al*., 1996; Coelho & Salas-Mellado, 2018, Nwachukwu & Aluko, 2018).

Hypothesis 3

Trichilia protein will have a similar chemical structure and functional properties to that of soy protein. The *Trichilia* protein will have more β-structure than the α-helix, similar to soy protein. Canola, sunflower, Bambara and cowpea protein have been reported to contain more β-conformation than α-helix (Gerzhova *et al*., 2015; Ishii *et al*., 2021; Mune & Sogi, 2016). The *T. emetica* and *T. dregeana* proteins will have stable emulsions, good foaming properties, gel formation, and water and fat holding capacities in comparison to the soybean protein. Peanut protein concentrates have been reported to have stable emulsions, good foaming properties; however, their water retention is poor (Yu *et al*., 2007). Walnut, canola, cowpea and bambara seed protein concentrates have also demonstrated good water and fat holding capacity (Mune & Sogi, 2016; Gerzhova *et al*., 2015; Mao & Hau, 2014) and will have stable gel formation, high water holding properties and emulsifying activity similar to soybean. High ionisable polar amino acids (glutamic acid and aspartic acid) bind too much water (Vioque *et al*., 2000).

3.2. Aim

To determine the physicochemical characteristics of *T. emetica* and *T. dregeana*, and to determine the nutritional quality and functional properties of their proteins to promote seed utilisation as a food ingredient, thereby improving food and nutrition security.

3.3. Specific Objectives

1. To determine the physical and nutritional properties (same as nutrient composition), gross- and micro-structure of *T. emetica* and *T. dregeana* seeds
2. To determine the nutrient and anti-nutrient composition of the flour and protein of *T. emetica* and *T. dregeana* seeds
3. To determine the chemical structure and functional properties of *T. emetica* and *T. dregeana* seed protein

3.4. Study design and the plant materials used

This section describes the experimental design of the methods used in this study. It covers the study design, extraction procedure and the plant materials used in this study.

3.4.1. **The study design**

The study design is shown in Figure 3.1. This design shows the analysis done on the indigenous seeds and also the extraction procedure of the protein.

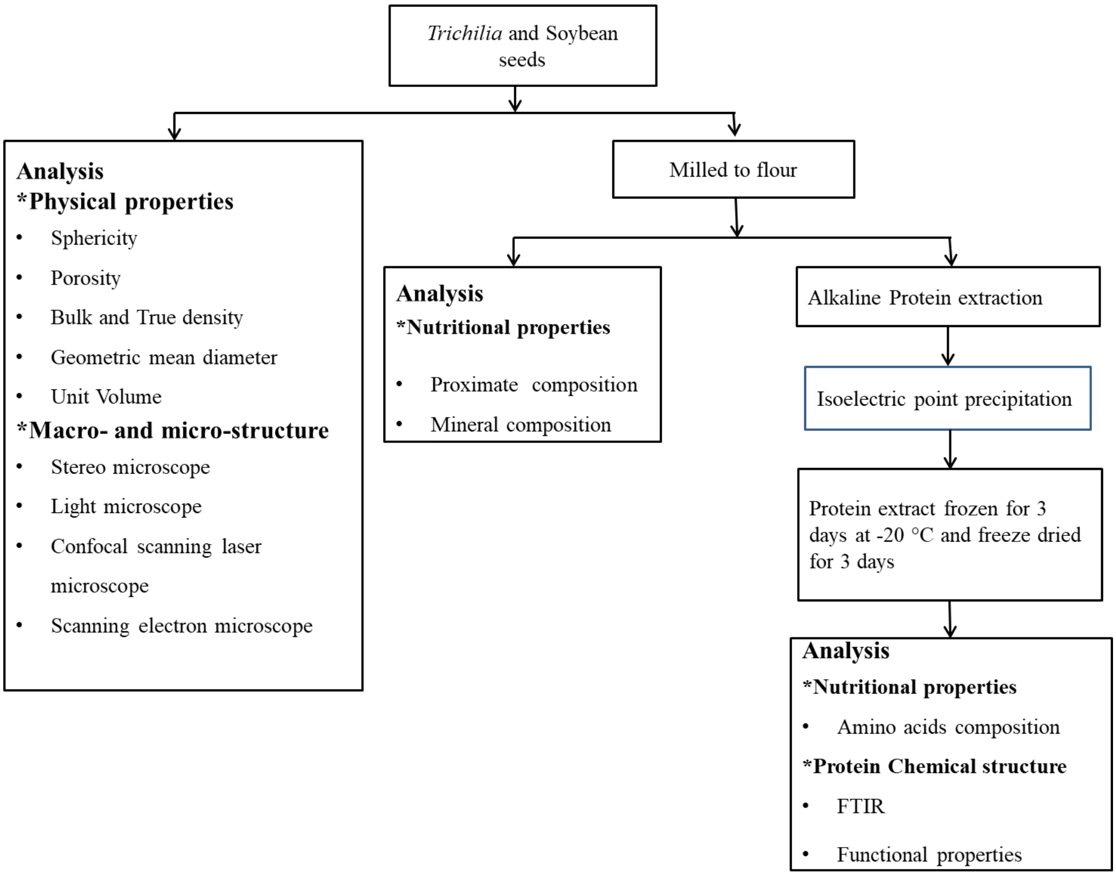

Fig 3.1: Research experimental design of *Trichilia* (*emetica* and *dregeana*) in comparison with soybean seed

FTIR: *Fourier*-transform infrared spectroscopy

3.4.2. **Plant materials used in this study**

Two indigenous *Trichilia* seeds (*Trichilia emetica* and *Trichilia dregeana*) used in this study were obtained from Agricultural Research Council-Tropical and Subtropical Crops, Mbombela, Mpumalanga province, South Africa (Fig 3.2). Mbombela received an average annual rainfall of about 934 mm. The Trichilia seeds need to be harvested when the fruit lobes capsules starts to open and not picked from the ground because the fruit quality would be deteriorated (Mathogone, 2014). A well-known oilseed (Soybean) was used as a reference oilseed was obtained from the Agricultural Research Council-Grain Crops (ARC-GC) campus in Potchefstroom, North West province, South Africa (Fig 3.2). This location received an average annual rainfall of about 660 mm.

Fig 3.2: The *Trichilia emetica* (A), *Trichilia dregeana* (B) and Soybean (C) seeds

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RESEARCH SECTION

This section is organised into three chapters based on the specific objectives of this study, as indicated below.

4. *Trichilia emetica* and *Trichilia dregeana* seeds macro- and microstructure, and their physical properties
5. Nutrient and anti-nutrient content of the flour and protein concentrates of *Trichilia emetica* and *Trichilia dregeana* seeds
6. Chemical structure and functional properties of *Trichilia emetica* and *Trichilia dregeana* seed protein

4. *TRICHILIA EMETICA* AND *TRICHILIA DREGEANA* SEEDS MACRO- AND MICROSTRUCTURE, AND THEIR PHYSICAL PROPERTIES[1]

ABSTRACT

Food and nutrition insecurity are prevalent in developing regions, especially in sub-Saharan Africa. Yet, there are several edible domesticated and wild plants that are grossly under-utilised as food sources. *Trichilia* seeds are under-utilised and under-researched indigenous seeds found in Africa and also in developing countries. This study determined and compared the physical properties, gross- (or macro-) and micro-structure of *Trichilia* seeds, which will help in understanding the potential value of oilseeds and promote their uses, thereby assisting in reducing food insecurity. *T. emetica* and *T. dregeana* seeds had higher bulk density (645.9 kg/m^3 and 433.6 kg/m^3), lower true density (875.8 kg/m^3 and 950.4 kg/m^3) and porosity (55.07% and 54.38%), respectively, than soybean. Geometric mean diameter (29.7 mm and 16.9 mm) and aspect ratio (1.72 and 1.85) of *T. emetica* and *T. dregeana* respectively were higher than soybean, while they had a lower sphericity (0.66 and 0.66) than soybean (0.99). LM, CLSM and SEM showed that *Trichilia* species had larger sized and more round-shaped protein bodies than soybean. The present research demonstrated that the physical properties and microstructure of *T. emetica* and *T. dregeana* seeds, which are comparable to existing commercial oilseeds, could have potential for commercial utilization, hence increasing the number of available food sources for food security.

Keywords: *Trichilia* seeds, physical properties, seed microstructure, seed macrostructure and Food security

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4.1. **Introduction**

Food insecurity is an ongoing challenge in various parts of the world, especially at the household level. Globally, food insecurity affects more than a quarter (26.4%) of the world's population and is associated with malnutrition and undernutrition (Cafiero *et al.*, 2018 & Huizar *et al.*, 2020). *T. emetica* and *T. dregeana* seeds represent an under-utilised and under-researched potentially valuable food source that may improve food security and could be comparable to sunflower, soybean, canola and peanut. Commercial utilisation of *Trichilia* species seeds could lead to improved food and nutrition security. Knowledge of seed gross structure, microstructure and physical properties would be vital for the efficient utilisation and commercialisation of seed protein as an alternative food source (Aguilera, 2005; Prada & Aguilera, 2007). The physical properties include hardness, geometric mean diameter, shape, size, surface area and density. Knowledge of these properties can facilitate the design of equipment for harvesting, handling, cleaning, sorting, dehulling, packaging, storage, transport and milling operations (Niveditha, 2013; Sadowska *et al*., 2013 & Wandkar *et al*., 2012). Seed microstructure is also important in understanding the food material functionality of the seed as it could affect the seed hardness (Aguilera, 2005; Prada & Aguilera, 2007).

To the best of our knowledge, there is no information on seed gross structure, microstructure and physical properties for *T. emetica* and *T. dregeana* seeds. Therefore, the present study investigated the gross structure, microstructure and physical characteristics of *T. emetica* and *T. dregeana* seeds. The results will facilitate the utilisation of the seeds for commercial and food security purposes.

4.2. **Materials and Methods**

4.2.1. **Materials**

Trichilia grains (*T. emetica*, *T. dregeana*) and soybean *(Glycine max (L.)* Merrill*)* grains were used. The *T. emetica* and *T. dregeana* grain-seeds were harvested (May 2018 to August 2018) at the Agricultural Research Council-Tropical and Subtropical Crops (ARC-TSC), Mbombela (-25.45127 S 30.96919 E), South Africa. The soybean grains, which served as a reference oilseed, were obtained from the Agricultural Research Council-Grain Crops (ARC-GC) campus in Potchefstroom (26.72866 S 27.07972 E), South Africa. The *T. emetica* and *T. dregeana* were distinguished based on their leaves, fruit-stem attachment and fruit morphology (Van Wyk *et al.*, 2000). The *T. emetica, T. dregeana* and soybean seeds were dried in an oven (AB 3000 Agridrier, Dryer for Africa, South Africa) at 50°C with a RH of 15% for 16 hours. The grains were then freeze dried (*Vir Tris* Bench Top Pro with Omnitronics, SP Scientific, St Louis, Pennsylvania) at -40°C at 500 mTorr for 4 days.

4.2.2 **Methods**

4.2.2.1 **Physical and functional properties of the seeds**

The average unit volume (V_1), bulk density (ρ_b) and porosity of *T. emetica*, *T. dregeana* and soybean seeds were determined by the fluid displacement method described by Pereas-Flores *et al.*, (2011). Sunflower oil (190 mL) and 30 seeds were added to a 500 mL graduated cylinder. The average unit volume (V_1) was determined by applying the following formula:

$$V_1 = \frac{oil\ displacement}{number\ of\ seed} \qquad [1]$$

Bulk density (ρ_b) was calculated as the ratio of mass to volume that was occupied by the seeds (n = 30). The true density (ρ) was determined by the benzene displacement method. A

50 mL graduated cylinder was filled with toluene (benzene) (20 mL) and seeds were added. The true density was calculated as the ratio of the mass of the sample to its true volume. Bulk and true densities were expressed as kgm^{-3}. The porosity (ε) of *T. emetica*, *T. dregeana* and soybean seeds were expressed in % and were calculated using the following formula:

$$\varepsilon = \left(1 - \frac{\rho b}{\rho_t}\right) \text{x } 100 \qquad [2]$$

The geometric mean diameter (GMD) (D_g) was calculated as the relationship between the length, width and thickness of the seed. The length (L), width (W) and thickness (T) of *T. emetica*, *T. dregeana* and soybean seeds were measured using a stereo microscope. A total of 60 seeds were selected by quartering, and on each individual seed the length, width and thickness were determined. The stereo microscopic analysis for dried *T. emetica, T. dregeana* and soybean whole seeds were done according to Pereas-Flores *et al*., (2011) with modification. An analytical sample (n>30 seeds) was obtained by the quartering method. The seeds were examined using a Zeiss Stereo Discovery. V20 microscope (Carl Zeiss, Germany) at 20X magnification. Geometric mean diameter was expressed in mm.

$$D_g = (LWT)^{1/3} \qquad [3]$$

The Aspect Ratio (AR) is a relationship between the minimum and maximum diameters. The value 1 for AR is related to equi-dimensional objects and tends to infinity for extended objects (Pereas-Flores *et al*., 2011). AR was determined using the following formula:

$$\text{AR} = \frac{\text{Max diameter}}{\text{Min diameter}} \qquad [4]$$

The sphericity ($\emptyset$) of *T. emetica*, *T. dregeana* and soybean seeds was calculated according to Sosa-Segura *et al*. (2014) and Pereas-Flores *et al*. (2011) using the following formula:

$$\emptyset = \frac{Dg}{L} \qquad [5]$$

4.2.2.2 **Light Microscopic (LM) analysis**

For LM, a single-edged razor blade was used to carefully remove the seed coat from each seed (n>30 seeds). Each seed was separated into two cotyledons and the cotyledons were cut into 1 mm^3 pieces. The cotyledon pieces were fixed using 0.15 M Sodium phosphate buffer, pH 7.2 containing 2.5% v/v glutaraldehyde/formaldehyde solution for 24 hours. The samples were then washed three times with 0.15 M sodium phosphate buffer for 15 minutes each. Samples were dehydrated using a graded series of ethanol (30%, 50%, 70%, 90% and 3x100%) for 15 minutes each. Resin embedding was done by using a graded series of LR White resin (SPI supplies) (20%, 40%, 60%, 80% and 100%) diluted in 100% ethanol, for 2 hours each, whilst samples were being rotated on an angled rotator. Resin embedding was used to preserve the tissue structure of the seed microstructure against mechanical and environmental damage. This was followed by four exchanges of fresh LR White resin, by leaving the samples at 4°C for 48 hours each. The resin was polymerised for 36 hours at 60°C. The polymerised samples were sectioned (1 µm) using a Reichert Jung Ultracut E ultra-microtome. Samples were stained with toluidine blue and were observed under a Zeiss Axio Imager.M_2 light microscope (Carl Zeiss, Germany) with the 20X, 0.45 NA and 40X, 0.75 NA objectives. The average size of the cells was measured from approximately 250 cells.

4.2.2.3 **Confocal Laser Scanning Microscopy (CLSM)**

Freeze dried seeds were cut into sections using a single-edged razor blade and placed in an oven at 60°C for 36 hours. Small tissue pieces (≤ 0.5 mm) were cut from the inner cotyledons by hand. The tissue pieces were stained with Nile Red (for lipid bodies) and Sulforhodamine 101 (for protein bodies) for 5 minutes. The stains were washed using 30% (v/v) glycerol solution. The stained sections were observed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss, Germany), using a 10x, 0.3 NA objective. Excitation laser wavelength for both dyes was 561 nm, and the emission spectra in the range of 565 to 720 nm. A total of 20 lipid and protein bodies were captured.

4.2.2.4 Scanning Electron Microscope (SEM) analysis

The freeze-dried samples were dispersed in liquid nitrogen for 2 minutes and were sectioned longitudinally and in cross-sections. The samples were placed in a Petri dish and stored in an oven at 60°C for 36 hours. Samples were coated with gold coating using EMITECH K550X (Quorum Technologies). The samples were observed under a Zeiss Ultra Plus Field Emission Scanning Electron Microscope (Carl Zeiss, Germany) at 500X, 1000X and 2000X magnification, with an accelerating voltage of 3kV and signal detected by a secondary electron detector. The average size of the cells was measured from at least 180 cells to provide statistical significance.

4.2.2.5 Image and statistical analysis

The ImageJ 1.43u freeware software (National Institute of Health, Bethesda, Maryland, USA) was used to analyse all the images obtained from the microscopy techniques. Images were binarised (black and white), and three dimensions (length, width and thickness) were measured.

Physical analysis data were analysed by one-way analysis of variance (ANOVA) using Statistica® version 8 (Statsoft Inc, Tulsa). Size distribution data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk test. The mean values of physical and structural analyses of *T. emetica*, *T. dregeana* and soybean seeds and flours were compared by the Fishers Least Significant Difference test (LSD) with a 95% confidence interval.

4.3. **Results and Discussion**

4.3.1. **The gross/external structure, physical and functional properties of *T. emetica, T. dregeana* in comparison with soybean seeds**

The shape, appearance and size of whole *T. emetica* and *T. dregeana* seeds compared with soybean seeds observed under the stereo microscope are shown in Fig 4.3.1. The *T. emetica* and *T. dregeana* seeds had a similar oblate shape and seed-coat colour (Fig 4.3.1B and 4.3.1A). Garnayak *et al*. (2008) and Deshpande *et al*. (1993) have observed that the spherical-shaped seeds had sphericity of above 0.8 and the sphericity of <0.70 was equivalent to sphere or oblate shape. Both *T. emetica* and *T. dregeana* seeds were black and almost completely enveloped by a bright red aril, while soybean seeds had a typical off-white to yellow seed-coat colour and were spherical in shape with a small hilum (Fig 4.3.1C).

The *T. emetica* seeds had an average size (Ferret's diameter) of 39.38 ±2.28 mm (aspect ratio: 1.72 ±0.33, n=30), while *T. dregeana* seeds had an average size of 24.95 ±1.41 mm with an aspect ratio of 1.85 ±0.00. The *T. emetica* and *T. dregeana* seeds were clearly larger than the soybean seeds, which had an average size of 13.94 ±1.24 mm (aspect ratio of 1.17 ± 0.178) (Fig 4.3.1). The aspect ratio (AR) of *Trichilia* spp indicated that the seeds were not a perfectly round shape, while the soybean seeds were more closely round in shape.

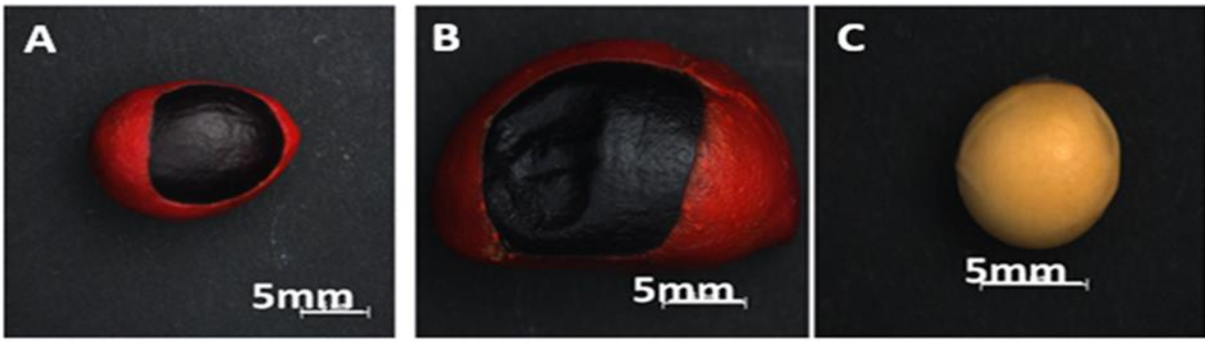

Fig 4.3.1: Stereo microscopy images showing the external seed structure of *T. dregeana* (A), *T. emetica* (B) and Soybean (Glycine max (*L.*) Merrill) (C) seeds

The sphericity of *T. emetica* and *T. dregeana* seeds were not significantly different ($p>0.05$). However, *T. emetica* and *T. dregeana* seeds had a significantly lower sphericity ($p\leq0.05$) than the soybean seeds (Table 4.3.1). The sphericity values for *T. emetica* and *T. dregeana* seeds are similar to the sphericity values for castor oilseeds (Pereas-Flores *et al.*, 2011), but lower than the sphericity values for rapeseeds (0.91- 0.93) (Sedat *et al.*, 2005). In 2011, Pereas-Flores *et al*. reported that castor oilseeds had a sphericity of 0.67 and that the shape of the castor oilseed could cause difficulty in rolling on surfaces, and promoted sliding instead. The present results indicate that *T. emetica* and *T. dregeana* seeds, like the castor oilseeds, could be more cumbersome to handle compared with soybean; however, adjustment of soybean-based systems or the design of a more appropriate system may be required.

The geometric mean diameter for soybean was significantly lower ($p<0.05$) than *T. emetica* (Table 4.3.1). Rapeseeds were previously reported to have a lower geometric average diameter (1.96- 2.11 mm) (Unal *et al*., 2009) than soybean (12.6 mm), *T. emetica* (29.7 mm) and *T. dregeana* (16.9 mm). The geometric mean diameter (GMD) is related to mass or energy transfer rates through the surface of the seeds (Niveditha, 2013). The GMD results of the current study suggest that *T. emetica* seeds have higher mass and heat transfer rates, which would facilitate more efficient milling, cooling, drying and heating operations compared with soybean seeds.

The physical properties of *T. emetica* and *T. dregeana* seeds in comparison with the soybean are shown in Table 4.3.1. The true densities of soybean, *T. emetica* and *T. dregeana* seeds were not significantly different ($p< 0.05$). True density above 1000 kgm^{-3} suggests that the seeds are heavier than water and this can be used to design cleaning or separation equipment (Unal *et al*., 2009). The bulk density of *T. emetica* seeds was significantly lower ($p<0.05$) than that of *T. dregeana* (Table 4.3.1). Both *T. dregeana* and *T. emetica* seeds had significantly lower bulk density ($p< 0.05$) compared with soybean (Table 4.3.1). Bulk density influences the storage of seeds (Unal *et al*., 2009), with higher bulk density preferred in the storage, packaging and transportation of food materials (Bhattachanya, 2011). Therefore, storage and packaging models used for soybean seeds may not be appropriate for the *Trichilia* spp. However, the results suggest that the lower bulk density of *T. emetica* and *T. dregeana* seeds may be advantageous in drying, particularly in tunnel and fluidized bed drying. The airflow in the seeds could occur more rapidly than in soybeans, which would imply shorter drying time and lower energy consumption relative to soybeans. Higher air flow rates and shorter drying times are important in minimizing physiological damage to seeds (Masken, 2000 & Zhang *et al*., 2006). Hence, under the same drying conditions (temperature, relative humidity and air speed) relatively more flavour compounds, colour and nutrients could be retained during the drying of the *Trichilia* spp compared with soybean seeds. This is further supported by the porosity results.

Table 4.3.1: Physical properties of *Trichilia* species and soybean

Physical properties	***T. emetica***	***T. dregeana***	**Soybean**
Sphericity	0.66 ± 0.15^{b}	0.66 ± 0.06^{b}	0.99 ± 0.07^{a}
True density (kg/m^3)	875.8 ± 94.4^{a}	950.4 ± 168.8^{a}	1026.1 ± 259.2^{a}
Bulk density (kg/m^3)	393.5 ± 31.1^{c}	433.6 ± 15.3^{b}	645.9 ± 46.2^{a}
Porosity (%)	55.07 ± 3.70^{a}	54.38 ± 1.68^{a}	37.05 ± 4.69^{b}
V1 (mm^3)**	7904.8 ± 212.1^{a}	6881.0 ± 126.0^{b}	6538.1 ± 67.9^{c}
GMD (mm)	29.7 ± 8.0^{a}	16.9 ± 5.4^{ab}	12.6 ± 4.0^{b}

*Mean ± SD is reported on dry basis. Means with different superscript letters in rows are significantly different ($p<0.05$)

**V_1 is the average unit volume and GMD is the geometric mean diameter

The *T. dregeana* had similar porosity ($p > 0.05$), with a significantly lower average unit volume ($p<0.05$) compared with *T. emetica*. The porosity of the *T. emetica* and *T. dregeana* seeds were significantly higher ($p<0.05$) than that of the soybean (Table 4.3.1). Porosity depends on the geometry and surface properties of the material (Unal *et al*., 2009). The ratio of the empty seed space to its total volume is frequently needed in airflow and heat flow studies (Mohsenin, 1986). This is used in the calculation of the rate of aeration and cooling, drying and heating, and the design of heat exchangers (Unal *et al*., 2009). The higher porosity values of these *Trichilia* spp seeds may be associated with irregular shape seeds and this suggests that the seeds have better aeration and water vapour diffusion during drying and storage when compared with soybeans. According to Adebowale *et al*. (2011), based on studies involving moisture reconstitution of seeds (10-30% moisture), higher moisture content was associated with higher porosity. On the other hand, the results of moisture content (<9.1% moisture) in the present study (See Chapter 5, Table 5.3.1) show that the *Trichillia* spp seeds had lower moisture content than that of soybeans, which contradicts the assertion that higher moisture content equates to higher porosity. The fact that the maximum moisture content in the present research (≤9.1%) was out of range of the values used by Adebowale *et al*., (2011) (10-30%), possibly also explains the difference due to the limited anticipated cohesion of seeds due to higher moisture content (>10%) which did not occur in the present research. It may be noted that the equation for porosity (Equation 2) includes a ratio of bulk density to true density. Therefore, in the present study, the influence of the lower bulk density of the *Trichilia* spp seeds (due to their unique external dimensions) compared with soybeans (Table 4.3.1) probably had a greater influence on the porosity more than the moisture content did.

4.3.2 Microstructure of *Trichilia* Species

Seed micro-structure helps in understanding the spatial arrangement of identifiable elements such as proteins, starch, fibres and lipids in food. Understanding the seed micro-structure can also improve the utilisation of the seeds for different purposes (Aguilera, 2005; Prada & Aguilera, 2007).

Thin sections (<0.5 mm thick) of resin-embedded *T. emetica*, *T. dregeana* and soybean seeds stained with toluidine blue were used to observe the interior tissue cell structure, as observed under light microscopy (Fig 4.3.2). In this study, *T. emetica* and *T. dregeana* seeds had ovoid-like parenchyma cells compared with soybean discoid cells (Fig 4.3.2). Furthermore, *Trichilia* seed cell size and shape differed from those of soybean. The *T. emetica* had a slightly pinkish-purple staining colour compared with the *T. dregeana* and soybean seeds. This may suggest that the *T. emetica* seed has more carboxylated polysaccharides compared with soybean seed (Mitra and Loque, 2014). However, further investigation for the presence or absence of carboxylated polysaccharides has to be done. The *T. emetica* had significantly fewer large cells (48 cells per micrograph) compared with *T. dregeana* (100 cells per micrograph), with larger protein bodies (107.35 ±93.56 µm) (refer to the black lines in Fig 4.3.2A and 4.3.2B). The *T. dregeana* had smaller cells (48.20 ±5.74 µm and aspect ratio of 1.54 ±0.20) compared with soybean (85 cells, 63.27±7.81 µm with aspect ratio of 1.84 ±0.35), and smaller sized protein bodies (56.03 ±16.90 µm) (Fig 4.3.2C).

T. dregeana had more protein bodies per micrograph (6 per micrograph at 100 µm) compared with *T. emetica* (average 1 per micrograph) (Fig 4.3.2A and 4.3.2B). The protein body size for *T. emetica* and *T. dregeana* seeds were larger than the range previously reported for other oilseeds, such as almond nuts with a protein body size of up to 12µm, and marama bean seeds of about 13 µm diameter (Young *et al*., 2004b & Mosele *et al*., 2011). *T. emetica* and *T. dregeana* had fewer and larger sized protein bodies compared with soybean (100 ± 9 per micrograph; protein bodies: 13.08±1.07 µm) (refer to the black lines in Fig 4.3.2C). This could be due to the lower protein content for the *Trichilia* spp in comparison with the same amount of soybean seeds protein content. The appearance of the purplish-blue-stained ovoid protein bodies was consistent with other oilseed parenchyma cells such as castor seeds (Pereas-Flores *et al*., 2011) and almond nuts (Ellis *et al*., 2004). In addition, Ellis *et al*., (2004) observed the presence of intracellular components (lipid and protein bodies) when almond nuts were stained with toluidine blue.

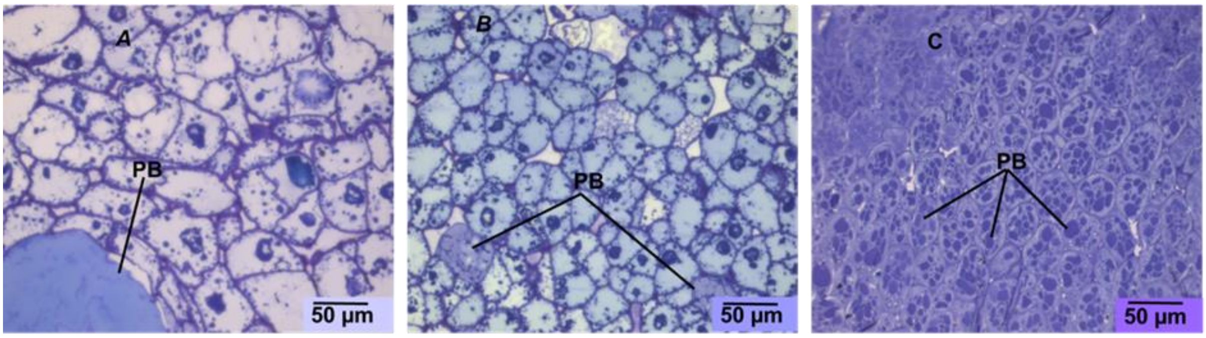

Fig 4.3.2: Light microscopy showing the interior tissue cell structure of ***Trichilia emetica* (A) and *Trichilia dregeana* (B)** compared with **Soybean (C)** seeds cells at 40X magnification. Protein bodies (PB) are stained blue with toluidine blue. Scale Bar: 50 µm

The microstructure of the seeds (*Trichilia* spp) was examined under confocal laser scanning microscopy (CLSM) to further confirm the different components in them. The CSLM results for *T. emetica* and *T. dregeana* showed that the seeds had more oval-shaped lipid bodies (size: 15.58 µm and 10.81 µm, respectively) compared with soybean discoid lipid bodies (10.41 µm) (Fig 4.3.3) as observed in BFLM and proximate analysis section (fat content). The shape of the lipid bodies of *Trichilia* spp and soybean seeds is similar to the shape of the lipid bodies of castor oilseeds (Pereas-Flores *et al*., 2011). However, the average sizes of peanut (1-3 µm) (Young *et al*., 2004a) and castor seeds (12.63 ±1.30 µm) (Pereas-Flores *et al*., 2011) are smaller than those of *T. dregeana*, *T. emetica* and soybean reported in the current study. These results could imply that methods used for the extraction of soybean oil can be adapted for extraction of the *Trichilia* spp oil.

The protein (ovoid) body of *T. emetica*, *T. dregeana* and soybean seeds differs in size (Fig 4.3.3). This was similar to the protein bodies observed under a bright field light microscope (Fig 4.3.2). *T. emetica* had more and larger sized protein bodies (12 per micrograph; 144.3 µm) compared with *T. dregeana* (8 per micrograph; protein bodies 38.8 µm) (Fig 4.3.3B and 4.3.3D). Soybean had the smallest protein bodies (29.2µm) when compared with *T. emetica* and *T. dregeana* (Fig 4.3.3F). These results imply that extraction of protein from the *Trichilia* spp could be simpler due to easy access to the larger protein bodies by the solvating agent. The variations in *Trichilia* spp protein body size and number may have contributed to the lower crude protein content of *Trichilia* spp and the high crude protein content of soybean (Amonsou *et al*., 2011). At higher magnification, smaller spherical globoid inclusion bodies were observed within the larger protein bodies of *Trichilia* spp as in soybean seeds (Fig 4.3.3G). The spherical globoid inclusions within the protein bodies of the *T. emetica* and *T. dregeana* seeds were similar to oilseeds such as peanuts (Young *et al*., 2004a), marama bean (Mosele *et al*., 2011a) and castor seeds (Pereas-Flores *et al*., 2011). This implies that similar methods for protein extraction for soybean seeds can be applied for *Trichilia* seed protein extraction.

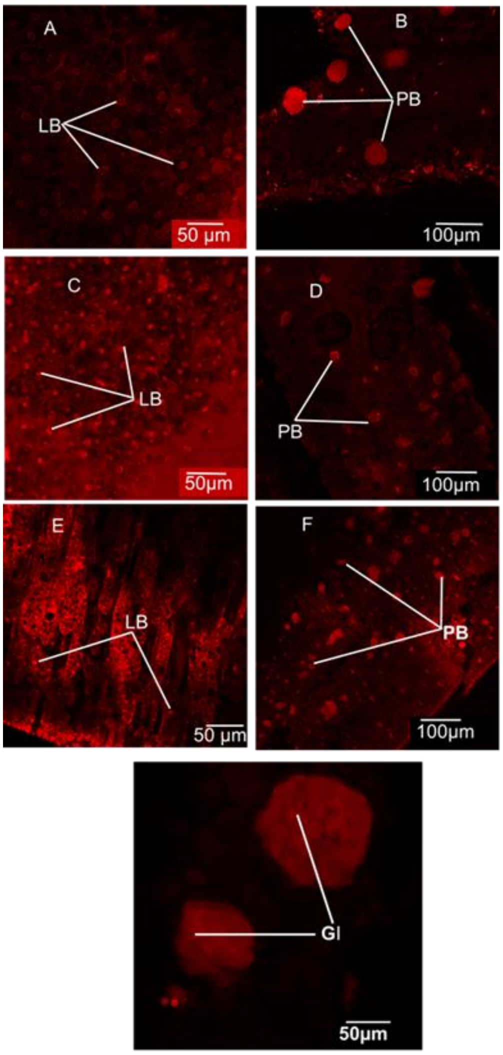

Fig 4.3.3: Confocal laser scanning microscopy showing the interior tissue cell structure of *T. emetica* (A-B) and *T. dregeana* (C-D) compared with Soybean (E-F) cells. The protein bodies contain globoids inclusions (**GI)**. The Nile red stained the lipid bodies (LB) and the sulfurhodamine 101 acid chloride stained the protein bodies (PB). **G** is the protein bodies with globoids inclusion at higher magnification

The results obtained under BFLM and CLSM were validated with SEM. The SEM showed that the parenchyma cells of *Trichilia* seeds contained protein bodies, which were embedded in a matrix of material that was reported for other oilseeds previously (Fig 4.3.4). The presence of lipid and protein bodies in the parenchyma cells of both *Trichilia* spp (*emetica* and *dregeana*) seeds agrees with previous observations made by Young *et al* (2004b) on almond nuts, and Wroniak *et al*. (2016) on rapeseed. However, the SEM results for soybean showed that there were no protein and lipid bodies at low and high magnification (refer to Fig 4.3.4C). This was probably due to the difference in response to the analysis methods for the soy sample, whereby the light microscopy and CSLM staining facilitated observations, and SEM coating hindered the soybean features.

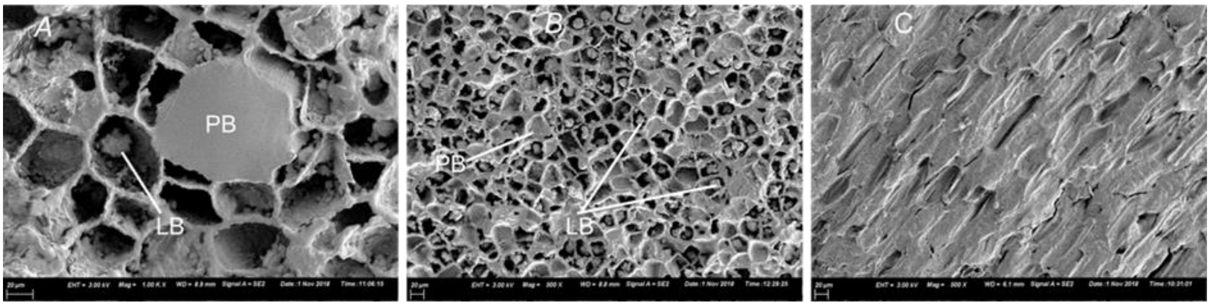

Fig 4.3.4: Scanning electron microscope (SEM) showing the interior tissue cell structure of *T. emetica* (A) and *T. dregeana* (B) compared with Soybean (C) seeds cells. LB: Lipid bodies and PB: Protein bodies. Scale Bar: 20µm

4.4. **Conclusion**

The present research successfully elucidates the gross structure, microstructure and physical characteristics of *T. emetica* and *T. dregeana* seeds. The physical characteristics of *Trichilia* spp indicate that modifications have to be made to existing technologies for drying, packaging and transportation purposes in order to handle the *Trichilia* spp seeds. The present research demonstrated the potential for commercial utilisation of *T. emetica* and *T. dregeana* seeds in the form of a butter, similar to peanut butter. This may increase the number of available food sources for food security, given they have comparable properties to existing commercial oil seeds.

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5. NUTRIENT AND ANTI-NUTRIENT CONTENT OF FLOUR AND PROTEIN CONCENTRATE OF *TRICHILIA EMETICA AND TRICHILIA DREGEANA* SEEDS[1, 2]

ABSTRACT

The issue of protein energy malnutrition (PEM) is of major concern in Africa. PEM is caused by protein and energy deficiency in a diet. Food insecurity is a global problem and is associated with malnutrition and undernutrition. *Trichilia* species seeds have been traditionally used as a complementary food in African countries to alleviate malnutrition (Komane *et al*., 2011). This study determined the proximate properties, macronutrients and micronutrients of *Trichilia* seeds and compared them with soybean. *T. emetica* and *T. dregeana* protein contents (25.6% and 17.3% w/w, respectively) were lower than soybean (45.4%), while fat contents (49% and 51.5% w/w, respectively) were higher for *Trichilia* than soybean (20.2% w/w). K (10930 mg/kg) and Ca (2970 mg/kg) where the main macro-minerals, while Fe (68.3 mg/ kg) and Zn (29.0 mg/ kg) were the main micro-minerals found in the *Trichilia* seeds. The amino acids of the oilseed proteins (*T. emetica* and *T. dregeana*) are higher than the recommended standard according to the Food and Agricultural Organisation. The research demonstrated that the nutritional characteristics of *T. dregeana* and *T. emetica* seeds and their protein concentrates could facilitate their commercial utilisation and application in the alleviation of nutrition insecurity.

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Keywords: *Trichilia emetica*, *Trichilia dregeana*, proximate composition, macro- and micro-nutrient, amino acids composition, protein content and fat content

5.1**. Introduction**

Protein energy malnutrition (PEM) has become an increasing problem in African countries, and other developing countries worldwide. African countries and developing countries use cereal-based foods such as maize as complementary foods, and this type of food does not meet the nutritional needs for infants and children (Dewey, 2013 & Suri *et al*., 2014). The cereal-based foods lack major essential amino acids such as Lys; however, they are a good source of carbohydrates (Temba *et al*., 2016). Commercial utilisation of *Trichilia* species seeds would lead to improved food and nutrition security. There are 260 species of *Trichilia* worldwide (Usman *et al*., 2018), they are commonly found in African countries, while *Trichilia* spp (*T. emetica* and *T. dregeana*) are also widely distributed throughout America. *Trichilia* plants are evergreen and grow in the wild, making them available throughout the year (South Africa National Biodiversity Institute [SANBI], 2004). Utilisation of *Trichilia* spp remains domestic and in African rural areas, *T. emetica* is used as a complementary food to alleviate malnutrition (Van Wyk *et al*., 2000 & Komane *et al*., 2011). The seeds can be soaked in water to produce a milky juice which is mixed with vegetables such as spinach or sweet-potatoes or squash to make delicious dishes (Komane *et al*., 2011 & SANBI, 2004). *Trichilia* oil is edible and is used as a preservative and also for cooking in rural communities (Komane *et al*., 2011).

To our knowledge, the information on the nutritional composition of *Trichilia* spp (*T. emetica* and *T. dregeana*) is very limited and the amino acid profile is unknown, hence requiring further investigation. Therefore, the present study investigated the nutrient and anti-nutrient composition of the flour and protein concentrate of *Trichilia emetica* and *Trichilia dregeana* seeds.

5.2. **Materials and Methods**

5.2.1**. Materials**

Trichilia seeds (*T. emetica* and *T. dregeana*) were harvested, distinguished and prepared as described in research section 4.1. Soybean (*Glycine max* (*L.*) Merrill) seeds were used as a reference, harvested and prepared as described in research section 4.1.

5.2.2. **Methods**

5.2.2.1. **Proximate composition of *Trichilia emetica and Trichilia dregeana* flour**

The macronutrient composition is a routine analysis that is used to provide classification of the seeds' components. This analysis determines water (moisture), crude fat (ether extract), crude protein and ash content.

The seed samples were milled into flour using a pilot-plant hammer mill (Drotsky S1, Alberton, Gauteng, South Africa) with a 0.8 mm sieve. The flours were stored in airtight containers at 4°C until analysed. The moisture and fat contents of the seed flours were determined using AOAC (Association of Official Analysis Chemists International) method No. 934.01 and AOAC method No. 920.39, respectively (AOAC, 2000). Crude ash content was determined by incinerating 2 g of the seed sample at 550°C in a muffle furnace, according to the AOAC method No. 942.05 (AOAC, 2010). Protein content (N X 6.25) was determined by the Dumas combustion analysis method, AACC (American Association Cereal Chemists International) method No. 46-30 (AACC, 2000) using a Dumatherm (DT, Gerhardt, Königswinter, Germany) instrument. All analyses were done in triplicate.

5.2.2.2 **Extraction of protein concentrates from *Trichilia seeds* and soybean**

The protein concentrates were prepared through isoelectric precipitation according to Boye *et al*., (2010) with modifications. The dried seeds of *T. emetica, T. dregeana* and soybean (1000 g) were dehulled manually to remove the seed-coat. Seed cotyledons of *T. emetica, T. dregeana* and soybean were milled to flour consistency using a Drotsky S1 miller with a 0.8 mm sieve. The flour was stored in an air-tight plastic container at 4°C until further analysis. The *T. emetica*, *T. dregeana* and soybean flour were defatted to constant weight using diethyl ether (1:5) at 25°C for 72 hours with constant stirring. The solvent was changed once every 24 hours for each flour, and this was repeated three times. The protein in the flour samples (*T. emetica*, *T. dregeana* and soybean) were solubilized using 2 N NaOH for 2 hours at 25°C with continuous shaking in an Incoshake© incubator (Labotec, South Africa) at 120 g (25°C). The slurry/mixture was filtered in cheese cloth folded six times and then samples were centrifuged at 2000 g for 5 minutes at 5°C. The protein in the supernatant's samples were precipitated by isoelectric precipitation using 2 M HCL at pH 4.25 (for soybean and *T. emetica*) and 4.35 (for *T. dregeana*) for 1 hour. The isoelectric points were estimated through weight yield centrifugation using pH's of 3.5, 4.0, 4.5, 5.0, 5.0 and 6.0 according to Chove *et al*., (2001) and Boye *et al*., (2010). The extract's precipitates were recovered by centrifugation at 3000 g for 10 minutes at 5°C and the supernatant was discarded. The sediment's precipitates were re-suspended in distilled water and were dialysed against distilled water for 48 hours at 25°C (with three changes of water) and centrifuged. The dialysed extracts were then frozen for 3 days, and then freeze dried for 3 days for further analysis, and the resulting powder stored in airtight containers at 5°C until further analysis. This extraction was done in triplicate for each seed type.

5.2.2.3**. Determination of amino acid composition of *Trichilia emetica* and *Trichilia dregeana* protein concentrates**

Amino acids were determined using Waters Acquity Ultra Performance Liquid Chromatograph (UPLC) AccQ-Tag system (Waters Corporation, Milford, Massachusetts, 01757, United States) with MassLynx software fitted with a Photodiode array (PDA) detector (Waters, Millipore Corp, Milford, MA) according to He *et al*., (2013) with modification. The principle of amino acids determination is based on reverse-phase chromatography with precolumn derivatisation following acid digestion. Samples were hydrolyzed with 6 M HCL at 110°C for 24 hours in a sealed tube prior to chromatographic analysis. The Cys and Met content were determined after performic acid oxidation, while Trp content was determined after alkaline hydrolysis (He *et al*., 2013). The digests were separated onto Waters Ultra Tag C_{18} column, USA (2.1 X 500 mm X 1.7 µm column) using a gradient of sodium citrate buffers (pH 3.85 and pH 10.85) at a flow rate of 0.70 ml/ minute.

5.2.2.4**. Mineral composition of *Trichilia* seeds and soybean seeds**

The mineral content of the dried *T. emetica*, *T. dregeana* and soybean seeds were determined using the Agri-Laboratory Association of Southern Africa (AgriLASA) method (2007). The seeds were oven dried at 60°C for 24 hours and milled using a Wiley mill to pass through a 1 mm sieve. The sample (0.5 g) was digested with 4 mL of 55% nitric acid and 2 mL of 70% perchloric acid using an aluminium digestion block (Labcon D60 model) for 2 hours at 100 °C. The samples were further heated at 180°C for 6 hours until decomposition of the organic substances. Deionized water was added to the cooled digested sample to make up to 25 mL.

The micro-minerals [iron (Fe), zinc (Zn), copper (Cu), manganese (Mn) and boron (B)] and macro-minerals [calcium (Ca), magnesium (Mg), phosphorus (P), sulphur (S) and potassium (K)] were determined by Atomic Absorption Flame Spectroscopy and Flame Emission

Spectroscopy (Varian AA200); with an Auto Analyser (Bran and Luebbe Auto Analyzer 3). The Ca, Cu, Fe, Mn, Mg and Zn were determined using AgriLASA method No. 6.5.1. The S, P, B and K were measured using AgriLASA methods No. 6.2.7.2; 6.3.1; 6.2.1.2 and 6.4.1, respectively. The analyses were carried out in triplicate.

5.2.2.5. **Statistical analysis**

Proximate, mineral analysis and amino acid profile data were analysed by one-way analysis of variance (ANOVA) using Statistica® version 8 (Statsoft Inc, Tulsa). Size distribution data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk test. The mean values of proximate and mineral analyses of *T. emetica*, *T. dregeana* and soybean flours were compared by the Fishers Least Significant Difference test (LSD) with a 95% confidence interval.

5.3**. Results and Discussion**

5.3.1 **Proximate composition of *T. emetica and T. dregeana* flour**

The composition results of *T. emetica*, *T. dregeana* and soybean seeds are shown in Table 5.3.1. The crude protein content of *T. emetica* was significantly higher ($p<0.05$) than that of *T. dregeana* but *Trichilia* spp seeds had significantly lower ($p<0.05$) protein content than soybean seeds (Table 5.3.1). The protein content for *T. dregeana* seeds obtained in the current study was lower than the previously reported protein content for canola seeds (23.6%) (Wanasundara, 2011). The protein content for *T. emetica* was within the range previously reported for canola seeds (23.6%) (Wanasundara, 2011) and sunflower seeds (20.4-40.0%), however at the lower range (Gonzalez-Peres & Vereijken, 2007). These results suggest that the two *Trichilia* spp are a comparable source of protein to common commercial oil seeds.

However, further studies need to be conducted to identify the amino acid profile of the proteins and anti-nutrients to ascertain their nutritional contribution and safety.

Table 5.3.1: Proximate composition of *T. emetica*, *T. dregeana* and Soybean flour

Proximate Analysis[a]	***T. emetica***	***T. dregeana***	**Soybean**
Crude Protein (%)	25.6 ±7.8[b]	17.3±3.0[b]	45.4 ±16.5[a]
Moisture Content (%)	5.7 ± 0.3[b]	4.9 ± 0.1[b]	9.1 ±0.8[a]
Ash (%)	3.8 ± 0.4[a]	3.4 ± 0.1[b]	3.8 ± 0.2[a]
Crude Fat (%)	48.6 ±3.4[a]	51.5 ±4.7[a]	20.2 ±4.7[b]

[*] Mean ± SD is reported on dry basis. Means with different superscript letters in rows are significantly different ($p<0.05$)
[a] Results are expressed in %

The crude fat content of *T. emetica* and *T. dregeana* were similar ($p>0.05$). The crude fat content of *T. emetica* and *T. dregeana* were in line with the crude fat content of other commercial sources of oil such as sunflower (30-36.8%) (Ingale & Shrivastava, 2011). Castor oilseeds on the other hand, were reported to have a crude fat content with a range of 33.8-56.2% which the *Trichilia* species was within that range (Pereas-Flores *et al*., 2011 & Vasco-Leal *et al*., 2018). In the current study, the crude fat content for the soybean was significantly lower ($p<0.05$) than the crude fat content of *T. emetica* and *T. dregeana* (Table 5.3.1). The high crude fat content of the two *Trichilia* spp seeds suggests that they can be a source of commercial fat. However, the fatty acid profile of the two seeds needs to be assessed for further identification of potential uses. In addition, the effect of agronomic conditions, extraction conditions and seed maturity on fat yield needs to be assessed. The ash content for *Trichilia* spp and soybean seeds was not significantly different ($p>0.05$) (Table 5.3.1). Soybean seeds had significantly higher ($p<0.05$) moisture content compared with the *Trichilia* spp seeds (Table 5.3.1). Although the moisture contents were significantly different ($p < 0.05$), they were all lower than 10%, which is the recommended level for long-term storage of seed products (Wilson & Downs, 2012).

5.3.2**. Amino acid profile of *Trichilia* protein concentrates compared with soybean protein concentrates**

The amino acid content of the protein concentrates was reported as a percentage of the protein content, which was 60.9, 54.0 and 69.4% w/w for *T. dregeana*, *T. emetica* and soybean, respectively (Table 5.3.2). The *T. emetica* and *T. dregeana* protein concentrates were rich in Phe, leu, Ile, val and lys with substantial amounts of Met and cysteine (Cys) (Table 5.3.2). The concentration of the essential amino acids of Phe, Leu, Ile, Val and Lys in *T. dregeana* was not significantly different ($p > 0.05$) to the concentration of the same amino acids in *T. emetica*. The results indicate that the *Trichilia* protein had comparable essential amino acid content to the soy protein; therefore, this protein could be used as an ingredient to improve the protein quality of foods that are deficient in essential amino acids.

The *Trichilia* protein concentrates had a significantly higher ($p < 0.05$) Ile and Val, and significantly lower ($p < 0.05$) Phe values, compared with soy protein. The Leu and Lys content were not significantly different ($p > 0.05$) for *Trichilia* protein. Lys concentration in *Trichilia* protein and soybean protein was not significantly different either ($p > 0.05$) (Table 5.3.2). These essential amino acid values mentioned are comparable with the amino acid profiles of oilseed protein concentrates of chia, sunflower and soybean protein previously reported (Coelho & Salas-Mellado, 2018; & Hughes *et al*., 2011). The concentration of the sulphur-containing amino acids Met and Cys, in *T. emetica* and *T. dregeana,* were not significantly different ($p > 0.05$). However, the soybean protein had a significantly higher ($p < 0.05$) Met and Cys concentration compared with the *Trichilia* protein (Table 5.3.2). The results are similar to the results previously reported for sunflower and flaxseed protein concentrates, in terms of

Val and Leu, but the Phe value for the *Trichilia* seed protein concentrates of the current study is higher than the Phe values for sunflower protein concentrates obtained in previous studies (Bautista *et al*., 1996; Nwachukwu & Aluko, 2018). Histidine values for *T. dregeana* and *T. emetica* were not significantly different (p>0.05); however, His concentration was significantly lower (p<0.05) in the *Trichilia* seed protein compared with the soybean protein. Threonine was found to be the limiting amino acid, with the lowest concentration in the protein concentrates of *Trichilia* compared with soybean protein. The Thr value for *Trichilia* protein was significantly lower (p<0.05) than the Thr value for soybean protein.

Hydrophobic amino acids (HAA) play an important role in antioxidant activities (Elias *et al*., 2005). The concentration of HAA in *T. dregeana* protein concentrate was not significantly different (p>0.05) from that in the *T. emetica* concentrate, however, soy protein concentrate had a significantly higher (p<0.05) HAA concentration compared with both *Trichilia* spp protein concentrates (Table 5.3.3). The *Trichilia* protein had lower HAA concentration than chia (3.80 g/100 g), flaxseed (3.73 g/100 g) and sunflower seeds (4.50 g/100 g) (Coelho & Salas-Mellado, 2018; Bautista *et al*., 1996; Nwachukwu & Aluko, 2018). The substantial concentration of HAA in the *Trichilia* seed protein concentrate suggests that the protein concentrate could be used as an antioxidant-rich ingredient in food systems.

Table 5.3.2: Amino acid profile of *T. dregeana* and *T. emetica* protein concentrate in comparison with soybean protein concentrates

Amino acid	***T. dregeana***	***T. emetica***	**Soybean**
Asp	15.24 ±0.16^{a}	14.82 ±1.11^{ab}	14.04 ±0.74^{b}
Glu	17.13 ±1.02^{b}	16.49 ±0.99^{b}	18.94 ±0.45^{a}
Ser	5.52 ±0.35^{ab}	5.34 ±0.03^{b}	6.02 ±0.17^{a}
Gly	6.04 ±0..44^{a}	5.85 ±0.11^{a}	6.19 ±0.24^{a}
His	3.62 ±0.23^{b}	3.35 ±0.21^{b}	4.35 ±0.01^{a}
Arg	9.53 ±0.41^{a}	4.46 ±0.21^{b}	9.20 ±0.00^{a}
Thr	2.93 ±0.22^{b}	3.16 ±0.16^{b}	3.94 ±0.04^{a}
Ala	5.27 ±0.21^{b}	5.72 ±0.64^{ab}	6.18 ±0.21^{a}
Pro	5.89 ±0.29^{ab}	5.90 ±0.20^{b}	6.32 ±0.18^{a}
Val	9.91 ±0.41^{a}	9.92 ±0.40^{a}	8.21 ±0.14^{b}
Tyr	3.17 ±0.16^{a}	4.03 ±1.52^{a}	3.59 ±0.03^{a}
Met	3.11 ±0.08^{b}	3.33 ±0.01^{b}	4.84 ±0.18^{a}
Cys	0.72 ±0.04^{a}	0.74 ±0.07^{a}	0.74 ±0.15^{a}
Ile	9.16 ±0.13^{a}	8.79 ±0.02^{b}	7.22 ±0.16^{c}
Leu	13.31 ±0.45^{a}	13.13 ±0.42^{a}	13.87 ±0.06^{a}
Phe	11.68 ±0.47^{b}	11.25 ±0.77^{b}	15.81 ±0.01^{a}
Lys	15.74 ±0.60^{a}	14.40 ±0.98^{a}	15.95 ±0.03^{a}
Trp	1.79 ±0.15^{b}	2.13 ±0.11^{a}	2.12 ±0.01^{a}
Protein content	60.9	54.0	69.4

*The amino acids content and protein content as dry basis were expressed in g 100 g^{-1} protein.

*Mean values of at least two replicates ± standard deviation (SD)

*Mean values within the same row with different superscript letters are significantly different ($p<0.05$)

Branched chain amino acids (BCAA) (Ile, Leu and Val) are important in the development of antihypertensive agents, they stimulate the building of muscle proteins and possibly reduce muscle breakdown, and they improve mental performance (Civitelli *et al*., 1992; Wu, 2009; Bifari & Nisoli, 2017). *T. dregeana* and *T. emetica* were not significantly different ($p>0.05$) with respect to BCAA concentration. The BCAA concentration in *Trichilia* seed protein concentrates was significantly lower ($p<0.05$) than in the soy protein concentrates (Table 5.3.3). The Ile and Leu values for *Trichilia* protein were significantly lower ($p<0.05$) than the corresponding values for soy protein concentrates. The *T. dregeana* and *T. emetica* BCAA results showed a similar trend with other oilseeds such as chia (14.9-15.3 g/100 g), sunflower (19.10 g/100 g) and flaxseed (15.4 g/100 g) (Coelho & Salas-Mellado, 2018; Bautista *et al*., 1996; Nwachukwu & Aluko, 2018).

The *Trichilia* protein concentrates contain substantial amounts of sulphur-containing amino acids (SCAA), which are the least limiting amino acids in other legumes and oilseed protein concentrates (Civitelli *et al*., 1992; Bifari & Nisoli, 2017). These amino acids contribute substantially to the maintenance and integrity of cellular systems. The *Trichilia* protein concentrates contain substantial amounts of SCAA. Results for sulphur-containing amino acids for *T. dregeana* and *T. emetica* were not significantly different ($p>0.05$); however, soy protein concentrates of SCAA were significantly higher ($p<0.05$) compared with *Trichilia* spp protein concentrates (Table 5.3.3). The current results for *T. dregeana* and *T. emetica* were lower than sunflower (3.75 g/100 g), chia (4.2-4.6 g/100 g) and flaxseed protein concentrates (2.38-3.7 g/100 g) (Bautista *et al*., 1996; Coelho & Salas-Mellado, 2018; Nwachukwu & Aluko, 2018).

Table 5.3.3: The chemical and nutritional classification of *Trichilia* in comparison with soybean amino acids

Classification	***T. dregeana***	***T. emetica***	**Soybean**
HAA*	63.01 ± 2.11^{b}	63.3 ±0.83^{b}	68.49 ±1.09^{a}
NCAA*	32.37 ±1.17^{a}	31.31 ±2.10^{a}	32.98 ±1.20^{a}
PCAA*	28.88 ±1.24^{a}	22.21 ±1.41^{b}	30.22 ±0.01^{a}
BCAA*	32.37 ±0.99^{a}	31.83 ±0.83^{a}	29.30 ±0.36^{b}
AAA*	17.44 ±1.61^{b}	17.40 ±0.64^{b}	21.52 ±0.03^{a}
SCAA*	3.83 ±0.03^{c}	4.06 ±0.08^{b}	5.57 ±0.33^{a}
E/T (%)**	52.77 ±0.81^{a}	53.42 ± 0.40^{a}	52.99 ±0.44^{a}
DIAAS (%)***	86.03 ±6.45^{b}	92.79 ±1.04^{b}	115.74 ±4.78^{a}

*The mean values of essential amino acid classification with standard deviation were expressed as mg/g crude protein. ***DIAAS (Digestible indispensable amino acids) = Lowest value of digestible indispensible AA reference ratio X100. **E/T is the proportion of essential amino acid (E) to the total amino acids (T). HAA is the hydrophobic amino acids, BCAA: branched chains amino acids, SCAA: sulphur-containing amino acids, NCAA: Negatively charged amino acids, PCAA: Positively charged amino acids and AAA: Aromatic amino acids. Mean values within the same row with different superscript letters are significantly different ($p<0.05$)

The aromatic amino acids (AAA) (Phe, Tyr and Trp) are required for biosynthesis in living cells (Sun *et al*., 2011 & Herman *et al*., 2010). The AAA for *T. dregeana* and *T. emetica* protein were not significantly different ($p>0.05$). However, soy protein concentrate had a significantly higher ($p<0.05$) AAA compared with *T. dregeana* and *T. emetica*. The AAA results for *Trichilia* protein were lower than the chia and sunflower protein concentrates (Coelho & Salas-Mellado, 2018 & Bautista *et al*., 1996).

Negatively charged amino acids (NCAA) enhance iron-reducing properties for the prevention of iron-induced lipid oxidation through the amino acid side chains' excess electrons (Nwachukwu & Aluko, 2018). These amino acids also help to improve skeletal muscle protein balance with an increase in liver glycogen synthesis (Vasconcelos, 2010). The NCAA for soybean, *T. dregeana* and *T. emetica* protein, which includes glutamic acids and aspartic acids, were not significantly different ($p>0.05$). The results for NCAA from this current study were lower compared with other oilseed protein concentrates such as chia (27.2-27.8 g/100 g), sunflower (26.4 g/100 g) and flaxseeds (30.8-35.1 g/100 g) (Coelho & Salas-Mellado, 2018; Bautista *et al*., 1996; Nwachukwu & Aluko, 2018). Positively charged amino acids (PCAA) such as Arg, Lys and His for *T. dregeana* were significantly higher ($p<0.05$) compared with *T. emetica*; however, the PCAA for soy protein was not significantly different ($p>0.05$) compared with *T. dregeana* protein. The PCAA for *T. emetica* protein was significantly lower ($p<0.05$) than the soy protein (Table 5.3.3). The *T. dregeana* contains twice as much Arg compared with *T. emetica*. However, the Lys content for *T. dregeana* and *T. emetica* was not significantly different ($p>0.05$) (See Table 5.3.2). The *T. emetica* protein Arg was significantly lower ($p<0.05$) than soy protein. Both *Trichilia* spp PCAA was lower than the flaxseed and chia PCAA (Bautista *et al*., 1996; Nwachukwu & Aluko, 2018).

Table 5.3.4: Essential amino acid score of *Trichilia* protein concentrates compared with the scores for soybean, chia, cunflower and flax protein concentrates

EAA	***T. dregeana* AAS**		***T. emetica* AAS**		**Soybean AAS**		**Chia AAS**		**Sunflower AAS**		**Flax AAS**		**Dietary FAO Recommendation**	
	Child	Adult	Child	Adult	Child	Adult	Child	Adult	Child	Adult	Child	Adult	Child	Adult
His	1.80±0.11^{b}	2.40 ± 0.15^{b}	1.68 ±0.11^{b}	2.23±0.14^{b}	2.18 ± 0.01^{a}	2.90±0.01^{a}	1.54-1.60	2.05-2.14	1.15	1.53	1.38	1.84	20	15
Ile	2.95±0.04^{a}	3.05 ± 0.04^{a}	2.83 ±0.01^{a}	2.93±0.01^{a}	2.33 ±0.05^{b}	2.41±0.05^{b}	1.08- 1.09	1.11- 1.12	1.44	1.48	1.34	1.39	31	30
Leu	2.11±0.07^{b}	2.26 ±0.07^{a}	2.08 ±0.07^{b}	2.22±0.07^{a}	2.20 ±0.01^{a}	2.35±0.01^{a}	1.14-1.17	1.22-1.25	1.35	1.44	0.97	1.04	63	59
Lys	3.03±0.11^{a}	3.50 ±0.13^{a}	2.77 ±0.19^{a}	3.20±0.22^{a}	3.07 ±0.01^{a}	3.54±0.01^{a}	1.15- 1.16	1.57-1.59	0.70	0.96	0.69	0.94	52	45
Met +Cys	1.37±0.01^{b}	1.74 ±0.01^{b}	1.45 ±0.03^{b}	1.85±0.04^{b}	1.99 ±0.12^{a}	2.54±0.15^{a}	1.49-1.66	1.90-2.11	1.34	1.70	0.85	1.08	28	22
Phe +Tyr	3.01±0.28^{b}	4.11 ±0.38^{b}	2.94 ±0.14^{b}	4.02±0.20^{b}	3.73 ±0.01^{a}	5.11±0.01^{a}	1.96-2.00	2.68-2.74	1.20	1.64	1.59	2.18	52	38
Thr	1.08±0.08^{b}	1.27 ±0.09^{b}	1.17 ±0.01^{b}	1.37±0.01^{b}	1.46 ±0.06^{a}	1.71±0.07^{a}	1.32-1.34	1.55-1.58	1.37	1.61	1.31	1.53	27	23
Val	2.36±0.10^{a}	2.54 ±0.11^{a}	2.36 ±0.10^{a}	2.54±0.10^{a}	1.95 ±0.03^{b}	2.10±0.04^{b}	1.04- 1.08	1.12-1.16	1.46	1.58	1.23	1.32	42	39
LEAA		THR		THR		THR	VAL	ILE		LYS		LYS		

*The mean essential amino acid (EAA) values were expressed as % w/w of crude protein on dwb.

**AAS (amino acid score) = mg amino acid in 1 g protein of test sample ⁄ mg amino acid in requirement pattern (FAO/WHO, 2007)

LEAA (limiting essential amino acids score) is the chemical score for the *Trichilia* protein and Soybean proteins. Chemical score = AAS X 100, truncated to 100% if >100.

*Mean values of at least two replicate with standard deviation

*Mean values within the same row with different superscript letters are significantly difference ($p<0.05$)

According to nutritional classification of amino acids, the *Trichilia* spp and soy protein concentrate amino acid results are classified into essential, non-essential and conditionally essential amino acids. Essential amino acids (EAA) are amino acids that must be obtained from the diet or supplements, and non-essential amino acids are amino acids that are synthesised by the human body. The EAA for *T. emetica* was significantly lower ($p<0.05$) than those of *T. dregeana*; however, soy protein concentrate was significantly higher ($p<0.05$) than *T. emetica* (Table 5.3.4). The total essential amino acid results for soybean, *T. emetica* and *T. dregeana* were below the values found in the chia, flax and sunflower protein concentrates (Bautista *et al.*, 1996; Coelho & Salas-Mellado, 2018; Nwachukwu & Aluko, 2018), and the FAO/WHO dietary recommendation for children. These essential amino acids help in calcium absorption, boost muscle growth and play an important role in collagen formation (Civitelli *et al*., 1992; Bifari & Nisoli, 2017).

The *Trichilia* species protein concentrates had lower amino acid scores for the essential amino acids (Thr, Lys, Met, Val, Ile, Leu, Trp and Phe+ Tyr) than the recommended FAO score for children (FAO/WHO, 2007) (Table 5.3.4). However, the *Trichilia* species amino acid scores for essential amino acids were similar to the soy protein scores. The *Trichilia* protein may have to be combined with other protein sources to improve its nutritional quality for children. The essential amino acids score for *Trichilia* species and soybean protein concentrates for adults was significantly higher ($p<0.05$) than the FAO requirements for adults (FAO/WHO, 2007) (Table 5.3.4). The results of this current study agreed with other oilseed protein concentrates, such as soybean, sunflower, chia and flaxseed (Hughes *et al*., 2011; Bautista *et al*., 1996; Coelho & Salas-Mellado, 2018; Nwachukwu & Aluko, 2018). The essential amino acids score (His, Ile, Leu, Val, Lys, Phe and Tyr) for the *Trichilia* species was higher than chia, sunflower and flaxseed protein concentrates for both children and adults. The essential amino acids score for *Trichilia* spp Met and Cys was within the range for chia; but higher than sunflower and flaxseed protein concentrates (Table 5.3.4). The Phe and Tyr amino acids score for flaxseed and sunflower was lower than the *Trichilia* spp (Bautista *et al*., 1996; Coelho & Salas-Mellado, 2018; Nwachukwu & Aluko, 2018). Threonine was the limiting amino acid for the *Trichilia* and soy protein (Table 5.3.4). Coelho & Salas-Mellado (2018) and Nwachukwu & Aluko (2018) found higher Thr values for flaxseed and chia protein than the *Trichilia* protein. Chia, sunflower and flaxseed are shown to be limited in Lys, especially for children, compared with the *Trichilia* protein (Table 5.3.4).

The proportion of essential amino acids (E) to total amino acids (T) for *T. dregeana*, *T. emetica* and soy protein were not significantly different ($p>0.05$) (Table 5.3.3). These nutritional results suggest that *Trichilia* protein had an equal proportion of amino acids pattern compared with soy protein and could be a better soy protein substitute. The digestible indispensable amino acids (DIAAS) for the *T. dregeana* and *T. emetica* were not significantly different ($p>0.05$); however, the *Trichilia* DIAAS was significantly lower ($p<0.05$) than soy protein. The DIAAS is for estimating the protein quality of human foods and allows for the calculation of amino acid quality of food proteins. The high DIAAS protein may contribute towards efficient body system functioning through the repair of body tissue, protein synthesis, and nutrient absorption; and can prevent muscle loss.

Another category of amino acids is the non-essential amino acids which can be redeemed as essential amino acids during illness or stress, resulting in them being called conditionally essential amino acids (Wu, 2009). Therefore, it is important that the NEAA profiles of novel dietary protein sources be characterized. Glutamic acid and aspartic acids are the major non-essential amino acids for *Trichilia* spp and soy protein (Table 5.3.5). The non-essential amino acids (NEAA) for *T. dregeana* were not significantly different from those of *T. emetica*

($p>0.05$) except for Arg and Trp. Soy protein concentrate had significantly higher ($p<0.05$) glutamic acid compared with *T. emetica* and *T. dregeana*. However, soy protein NEAA was comparable to those of *Trichilia* species proteins, to various extents (Table 5.3.5). The results for the non-essential amino acids followed the same trend as chia and sunflower protein in terms of Asp and Glu being the major NEAA (Coelho & Salas-Mellado, 2018; Nwachukwu & Aluko, 2018). However, other non-essential amino acids such as Glu, Ser, Gly, Arg, Ala & Pro for chia, sunflower and flaxseed were higher than *T. emetica* and *T. dregeana* non-essential amino acids (Bautista *et al*., 1996; Coelho & Salas-Mellado, 2018; Nwachukwu & Aluko, 2018).

Table 5.3.5: Non-essential amino acids of *Trichilia* and Soybean protein concentrates

NEAA*	***T. dregeana***	***T. emetica***	**Soybean**
Arg	9.53 ± 0.41^{a}	4.46 ± 0.21^{b}	9.20 ± 0.00^{a}
Ser	5.52 ± 0.35^{ab}	5.34 ± 0.03^{b}	6.02 ± 0.17^{a}
Gly	6.04 ± 0.44^{a}	5.85 ± 0.11^{a}	6.19 ± 0.24^{a}
Asp	15.24 ± 0.16^{a}	14.82 ± 1.11^{ab}	14.04 ± 0.74^{b}
Glu	17.13 ± 1.02^{b}	16.49 ± 0.99^{b}	18.94 ± 0.45^{a}
Ala	5.27 ± 0.21^{b}	5.72 ± 0.64^{ab}	6.18 ± 0.21^{a}
Pro	5.89 ± 0.29^{ab}	5.90 ± 0.20^{b}	6.32 ± 0.18^{a}
Trp	1.79 ± 0.15^{b}	2.13 ± 0.11^{a}	2.12 ± 0.01^{a}

*The mean values of the non-essential amino acids (NEAA) with standard deviation were expressed as mg/g crude protein. Mean values within the same row with different superscript letters are significantly different ($p<0.05$)

5.3.3. Mineral composition of *T. emetica* and *T. dregeana seeds*

The mineral composition of *T. emetica*, *T. dregeana* and soybean is shown in Table 5.3.6. Potassium (K) was the most prominent macro-mineral in the two *Trichilia* spp seeds. The K content was less in *Trichilia* spp seeds in comparison with soybean. The higher K content of *T. dregeana* suggests that it could be a better source of K for humans compared with *T. emetica*. The K helps in the maintenance of body fluids and electrolyte balance. It is involved in the proper functioning of heart muscles and also plays a role in carbohydrate metabolism and protein synthesis (Drewnowaki, 2010). The K content of *T. dregeana* and *T. emetica* seeds were higher than that previously reported for other oilseeds such as sunflower (0.067-0.075 mg$^{\cdot}$100 g^{-1}) (Gonzalez-Peres & Vereijken, 2007) and peanut oilseed (564- 614 mg$^{\cdot}$100 g^{-1}) (Jonnala *et al*., 2005). Cotton seeds and rapeseeds, however, have a higher K content (1110 mg$^{\cdot}$100g^{-1} and 1149-2232 mg$^{\cdot}$100g^{-1}; respectively) (He *et al*., 2013 & Beyzi *et al*., 2019) than the values obtained in the current study for *T. emetica*. The *T. dregeana* seeds provided 28.72% of the RDA for K for adult men and 26.4% for adult women, while *T. emetica* met lower values at 22.87% and 21.08% of RDA for men and women, respectively. On the other hand, the K contribution towards RDA requirements from soybean seeds was higher than the *Trichilia* spp seeds at 36.06% and 33.24% for men and women, respectively.

The Ca content in both *Trichilia* spp seeds makes Ca the second highest macro-mineral present. The Ca content between both *Trichilia* spp was comparable, but it was significantly higher ($p<0.05$) than soybeans in the current study. The *Trichilia* spp seeds met higher percentages of the Ca RDA compared with soybeans. *T. emetica* met 24.0- 29.0%, and *T. dregeana* met 25.0 – 30.0% of the RDA for Ca, for both women and men, whilst soybean provided 20.0 -25.0% of the RDA. *Trichilia* spp seeds were higher in Ca content than the previously reported oilseeds such as cottonseeds (145 mg$^{\cdot}$100 g^{-1}) and soybean (74-83 mg$^{\cdot}$100 g^{-1}) (He *et al*., 2013 & Biel *et al*., 2018). Oil-seed derived non-dairy milk products, such as soya milk, have to be supplemented with Ca due to their low Ca content (Chaiwanon *et al*., 2000). Non-dairy milk products derived from *T. dregeana* and *T. emetica* seeds could therefore require little to no supplementation at all with Ca, compared with soybean.

Table 5.3.6: Mineral composition of *T. emetica*, *T. dregeana* and Soybean flour

Elements^{a}	*T. emetica*	*T. dregeana*	Soybean
K	1075 ±58^{c}	1350 ±231^{b}	1695 ±173^{a}
P	259 ±23^{b}	272 ±191^{b}	463 ±23^{a}
S	171 ±12^{b}	142 ±6^{c}	367 ±69^{a}
Mg	185 ±23^{b}	142 ±81^{c}	233 ±17^{a}
Ca	285 ±58^{a}	300 ±11^{a}	245 ±58^{b}
Mn	2.50 ±0.00^{b}	1.80 ±2.00^{c}	3.83 ±4.16^{a}
Cu	1.00 ±0.00^{a}	1.13 ±0.58^{b}	1.07 ±0.58^{ab}
Fe	6.83 ±1.53^{b}	6.33 ±6.03^{b}	10.00 ±13.00^{a}
Zn	2.90 ±0.00^{b}	2.30 ±1.73^{c}	3.50 ±1.73^{a}
B	1.21 ±0.74^{b}	1.96 ±4.66^{b}	2.79 ±0.90^{a}

[*] Mean ± SD is reported on dry basis. Means with different superscript letters in rows are significantly different ($p<0.05$)

[a] Results are expressed in mg·100 g^{-1}

Fe and Zn were significantly lower ($p<0.05$) in *Trichilia* spp seeds in comparison with soybean (Table 5.3.6). The *T. dregeana* seeds met 79.1% and 23.4-35.2% of the RDA for Fe for men and women respectively, whilst *T. emetica* met about 85.4% and 25.3-37.9% of the RDA for Fe for men and women, respectively. Both *Trichilia* spp had lower amounts of Fe than soybean, which met 125.0% and 37.0-55.6% of the RDA for Fe for men and women, respectively. The *T. dregeana* provided 20.9% of the RDA for Zn for men and 28.8% of the RDA for Zn for women, compared with soybean which met 31.8% of the RDA for men, and 43.8% of the RDA for Zn intake for women. *T. emetica* contributed about 26.4% of the RDA for Zn for adult men, and 36.3% of the RDA for Zn for adult women. The *T. emetica* seeds had significantly higher ($p<0.05$) Mg, Mn and Zn compared with *T. dregeana*. However, the *Trichilia* spp seeds had significantly lower ($p<0.05$) Mg, Mn and Zn in comparison to soybean. Magnesium plays an important role in the function of smooth muscle and cell production, whilst Mn and Zn are required for the immune system (Drewnowaki, 2010). *T. dregeana* met 33.8-35.5% and 44.4- 45.8% of the RDA for Mg for men and women, respectively while *T. emetica* met a higher percentage of the Mg RDA at 44.0- 46.3% and 57.8- 59.7% for adult men and women, respectively. Both *Trichilia* seeds provided lower percentages of the Mg RDA compared with soybeans, which contributed 55.5- 58.3% and 72.8- 75.2% of the RDA for Mg for men and women, respectively. The *T. dregeana* seeds covered 100% of the RDA intake of Mn for women and 78.3% of the RDA intake of Mn for men. Soybeans exceeded the Mn recommended daily intake by providing about 165.5% for men and 212.8% for women. The *T. emetica* met about 108.7% and 138.9% of the RDA for Mn for adult men and women, respectively. These results could imply that, in general, the *T. emetica* seed is a better source of these minerals (Mg, Mn and Zn) compared with the *T. dregeana* seed.

The *T. emetica* and *T. dregeana* seeds had significantly lower ($p<0.05$) P, S and B contents compared with soybean (See Table 5.3.6). The *T. emetica* seeds met 47.09% of the RDA for P compared with 49.5% of the RDA for P met by *T. dregeana* for both adult men and women. Soybean met a significantly higher ($p<0.05$) percentage (84.2%) of P for both men and women. *Trichilia* spp had provided less of the recommended daily intake of P compared with cottonseeds and rapeseeds (Beyzi *et al*., 2019 & He *et al*., 2013). The *Trichilia* spp seeds met greater than 100% of the RDA of Cu and S. The *T. emetica* seeds met 125.6% of the Cu RDA, while *T. dregeana* met 111.1% of the Cu RDA for both men and women. Soybean met a similar percentage ($p>0.05$) of 118.9% of the Cu RDA. The sulphur RDA% met by *T. emetica* was 310.9% while that of *T. dregeana* was about 258.2% for both men and women. However, soybean met a significantly higher ($p<0.05$) percentage of the RDA for S than both *Trichilia* spp seeds.

The lower K, Mg and P of the *T. emetica* and *T. dregeana* seeds may be related to the lower crude protein content of the seeds. Protein body structures contain globoid inclusion bodies, which previously have been shown to contain mainly P, K and Mg (Madsen & Brinch-Pedersen, 2020 & Lott *et al*., 2017). The lower mineral composition of *T. emetica* and *T. dregeana* seeds compared with soybean is consistent with previous reports on other oilseeds. The presence of higher or comparable levels of Fe, S, Mg, P, K and Zn relative to other oilseeds apart from soybean indicates that the *T. emetica* and *T. dregeana* seeds may also serve as commercial oilseed food. The bioavailability of their minerals needs to be assessed in order to ascertain their potential nutritional contribution.

5.4**. Conclusion**

The protein, oil/fat and mineral content results for *Trichilia* seeds suggests that the seeds could be a source of oil that could be viable for commercial uses, such as cooking oil, in cosmetics and fragrances and for biodiesel production, but safety needs to be assessed first. The high Ca content of the indigenous seeds (*T. emetica* and *T. dregeana*) imply that they could be a source of non-dairy milk. The present research demonstrates the potential for commercial utilisation of *T. emetica* and *T. dregeana* seeds, thereby increasing the number of available food sources for food security, given they have comparable properties to existing commercial oil seeds. However, there is a need for further research to extensively assess the effect of agronomic conditions on their nutritional profile. These studies should include the bioavailability of minerals, fatty acid profiling and the amino acid composition of the *Trichilia* spp proteins. The essential amino acid content of the *Trichilia* seeds proteins was three to four times greater than the recommended FAO/WHO standards for adults. The amino acid profile can help in increasing the utilisation of the *Trichilia* protein.

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6. CHEMICAL STRUCTURE AND FUNCTIONAL PROPERTIES OF *TRICHILIA EMETICA* AND *TRICHILIA DREGEANA* SEED PROTEIN[1]

ABSTRACT

While *Trichilia* species are abundant in sub-Saharan Africa, their oilseeds are grossly underutilised as a food source, which seems mainly due to the scarcity of scientific knowledge on the oilseeds. *T. emetica* and *T. dregeana* contain an appreciable concentration of proteins, which may possess functional properties of significance in food processing and food quality. This study determined and compared the secondary structure and functional properties of the proteins in the *T. emetica* and *T. dregeana* seed protein concentrates. Results of the secondary structure analysis showed that the protein of the two *Trichilia* species (*T. emetica* and *T. dregeana*) had more β-conformation compared with the soybean protein. *T. emetica* protein concentrates showed higher water holding capacity (WHC), oil holding capacity (OHC), foaming capacity (FC) and foam stability (FS) compared with soybean protein and *T. dregeana* protein. The results suggest that *Trichilia* oilseed could be used as an ingredient in various food formulations due to its versatile functional properties.

Keywords: *Trichilia* species (*emetica* and *dregeana*), secondary structure, water holding capacity, oilseeds oil holding capacity, foaming capacity, globular structure and functional properties

6.1 Introduction

Trichilia seeds are indigenous seeds that are important in Sub-Saharan Africa. However, commercial production and potential further use of the seeds are still limited or unknown

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[2] Part of this work has been published: Tsomele, G.F., Meiring, B., Anyasi, T.A., Mlambo, V, Amonsou, E., Lepule, S.P., Siwela, M., & Wokadala, O.C. (2023). Influence of amino acids profile and secondary structure on nutritional and functional properties of *Trichilia emetica* and *Trichilia dregeana* protein concentrates. *International Journal of Food Science and Technology*. Doi: 10, 1111/ijfs.16425

(Van Wyk *et al*., 2000 & Orwa *et al*., 2009). The *T.emetica* and *T. dregeana* seeds have a high crude fat content (48.6% and 51.5%; respectively) and protein content (25.65% and 17.3%; respectively), with substantial concentrations of mineral nutrients (K, Mg, Ca, Fe and P), compared to other oilseeds such as soybean, peanuts, castor and sunflower seeds (See Section 5.1). In previous research, *Trichilia* protein bodies were shown to be similar to those of soybean in terms of shape and localisation within the cells (See Section 4.1). Thus, understanding the secondary structure of the *Trichilia* seed protein can help in the utilisation of protein concentrates in the food industry. The knowledge of protein secondary structure can help in locating the side chains, and the hydrophobic and hydrophilic groups of the protein surface. These can cause an electrostatic repulsion and ionic hydration that promotes the solubilisation of protein and causes an improvement of some functional properties such as gelation, emulsification and foaming when exposed to moderate pH values above or below the protein isoelectric point (Hettiarachchy *et al*., 2012; Day, 2013; Shevkani *et al*., 2015; Tang & Sun, 2011).

The secondary structure and major functional groups of the storage protein were previously determined using FTIR (Fourier-transform infrared spectroscopy) (Zhang *et al*., 2015), raman spectroscopy (Li *et al*., 2014), circular dichroism (CD) (Chandrapala *et al*., 2012), X-ray diffraction (XRD) (Jenkins *et al*., 2013), nuclear magnetic resonance (NMR) (Mao *et al*., 2014) and UV-spectroscopy (Barrios-Peralta *et al*., 2012). These methods provide valuable ways for studying conformational changes of proteins in solid, crystal and solution states with high-quality (absorption) spectra of about 1600-1700 cm^{-1}. The FTIR method may be limited by the type of protein material used and can also help in identifying the amide bands in the protein structure. The protein materials usually studied are protein extracts which include isolates, concentrates and meals; these extracts are composed of different individual proteins rather than purified fractions (Achouri *et al*., 2012). Raman spectroscopy is a convenient procedure and provides effective information about the molecular vibrations of proteins related to the secondary structure and microenvironment of the protein side chains. However, the use of Raman spectra is limited by strong biological fluorescence disturbance, which could hamper the collection of high-resolution spectra (Wang *et al*., 2017). While CD is used to determine the secondary structure, folding and binding properties of protein at lower protein concentrations, this procedure is time-consuming and provides low-resolution secondary structural information (Achouri *et al*., 2012) of the samples such as oilseeds.

Oilseeds are a valuable source of oil/fat and proteins; and extensive work has been done on soybean (Rao *et al*., 2002), chia (Coelho & Salas-Mellado, 2018), hemp (Wang *et al*., 2008), rapeseed (Gerzhova *et al*., 2015), peanut (Liu *et al*., 2019), sunflower (Ishii *et al*., 2021) and walnut (Mao & Hau, 2012) protein concentrates' secondary structure and functional properties (Zhang *et al*., 2013; Hadnadev *et al*., 2018). To the best of our knowledge, the secondary structure and functional properties of *Trichilia* protein concentrates are unknown. Hence, the objective of this current study was to determine the secondary structure and functional properties of two *Trichilia* species (*T. emetica* and *T. dregeana*) in comparison with soybean.

6.2. **Materials and Methods**

6.2.1. **Materials**

Trichilia seeds (*Trichilia emetica* and *Trichilia dregeana*) were harvested, distinguished and prepared as described in research section 4.1. Soybean (*Glycine max* (L.) Merrill) seeds were used as a reference, harvested and prepared as described in research section 4.1.

6.2.2. Methods

6.2.2.1. Extraction procedure of protein concentrates extraction for *Trichilia* species and soybean

The protein concentrates were prepared through isoelectric precipitation according to Boye *et al.*, (2010) with modifications. The *T. emetica*, *T. dregeana* and soybean protein concentrates were prepared as described in section 4.2.

6.2.2.2. *Fourier*-transform infrared spectroscopy (FTIR) of *Trichilia* and soybean protein concentrates

The FTIR analyses of *Trichilia* and soybean protein concentrate were performed according to Hadnadev *et al*. (2018) with modification using a Perkin Elmer FTIR Spectrometer (Spectrum Two, Version 10.5.4; Perkin Elmer Inc, Llantrisant, UK). The spectra were taken in the spectral range of 4000 cm^{-1} to 700 cm^{-1} with a 4.0 cm^{-1} resolution by an accumulation of 32 scans. The data were baseline corrected using the Perkin Elmer Spectra100 software. The second derivative of the spectral region 1700 cm^{-1} to 1600 cm^{-1} for Amide I Band was used to identify peak positions and to calculate their relative areas according to Sadat & Joye (2020) and Choi & Ma (2005) using Origin Pro-software (OriginLab Corporation). The Amide I peaks corresponding to different secondary structure elements (α-helix, β-turns, β-sheet and random coil) (Gallagher, 2009; Byler & Susi, 1986) was fitted to Lorentzian distributions with a resolution enhancement factor of 0.2 and the Amide I constitutive peak areas estimated. The peak area of each secondary structure was reported as a percentage of the total peak area.

6.2.2.3. Functional properties of *T. emetica, T. dregeana* and soybean protein concentrates

The functional properties of the *Trichilia* spp protein concentrate in comparison with soybean protein concentrate were determined in terms of water holding capacity (WHC), oil holding capacity (OHC), emulsion activity index (EAI), foaming capacity (FC) and foam stability (FS). The WHC and OHC were determined according to Jain *et al*., (2019) with modifications. For WHC, 1 g of a sample was dispersed in 10 ml distilled water and vortexed for 1 minute at high speed. The suspension was centrifuged at 2200 g for 30 minutes at 25°C and the WHC was calculated using the formula:

$WHC = (W_2 - W_1)/W_0 \, X100$

Where W_0 is the weight of the dry sample (g), W_1 is the weight of the tube with the dry sample (g) and W_2 is the weight of the tube with the sediment (g)

For OHC, 1 g of the sample was mixed with 10 ml vegetable oil or corn oil with vortexing every 1 minute for 10 minutes. The mixture was centrifuged (1600 g for 10 minutes) and free oil was decanted by venting the tube for 1 hour. The OHC was calculated using the formula:

$OHC = (W_2 - W_1)/W_0 \times 100$

Where, W_0 is the weight of the dry sample (g), W_1 is the weight of the tube with the dry sample (g) and W_2 is the weight of the tube with the sediment (g)

The EAI was determined according to Shevkani *et al*., (2015) with modifications. Samples of about 0.8 g were dispersed into 40 ml distilled water and homogenized (Polytron, PT 1300D, 1000 g) for 2 minutes. A vegetable oil (40 ml) was added to the suspension and further mixed using a homogenizer for 1 minute. The mixture was then centrifuged for 5 minutes at 1200 g. EAI was calculated using the formula:

$$EAI\ (\%) = \frac{\text{Height of emulsification layer}}{\text{Height of the content of the tube}} X100$$

The FC and FS were determined according to Shevkani *et al*. (2015). A 1 gram sample was dispersed into 100 ml distilled water to make a 1% (w/v) solution. The pH was adjusted using 0.1 N NaOH and the mixture was homogenized at 1000 g for 2 minutes. The volume of the foam was measured at 0 minutes, 15 minutes, 30 minutes and 60 minutes.

$$FC\ (\%) = \frac{\text{Vol after homogenization} - \text{Volume before homogenization}}{\text{Volume before homogenization}} X100$$

$$FS = \frac{\text{Vol at set time}}{\text{Initial vol of foam}}\ X100$$

6.2.2.4. **Statistical Analysis**

Secondary structure and functional properties analysis data were analysed by one-way analysis of variance (ANOVA) using Statistica® version 8 (Statsoft Inc, Tulsa). Analyses of the mean values of the secondary structure and functional properties of *T. emetica, T. dregeana* and soybean protein were compared by the Fishers Least Significant Difference test (LSD) with a 95% confidence interval.

6.3. **Results and Discussion**

6.3.1. ***Fourier*-transform infrared spectroscopy of *Trichilia emetica* and *Trichilia dregeana***

The FTIR spectra for *T. emetica*, *T. dregeana* and soybean protein concentrates is shown in Fig 6.3.1. A peak at lower wavenumber (1060 cm^{-1}) was observed for *Trichilia* and soybean protein concentrate on IR spectra (Fig 6.3.1). This peak is associated with intermolecular sheet interactions which support the oligomeric nature of the protein structure (Aider & Barbara, 2011). However, the peak for *T. emetica* protein concentrates intermolecular sheet interaction showed higher peak intensity compared with *T. dregeana* and soybean protein concentrates. The increase in peak intensity may be attributed to the higher bond polarity, the relative of two overlapped bonds and the repetition of the same functional group, resulting in larger and more intense peaks. The *Trichilia* protein concentrates (*T. emetica* and *T. dregeana*) intermolecular sheet interaction showed higher peak intensity compared with the soybean protein concentrates. The higher peak intensity of *Trichilia* protein concentrate intermolecular sheet interactions than that of soy protein concentrate may suggest that the *Trichilia* protein concentrates may have a more stable oligomeric nature of their protein than soybean does.

There are five other typical peaks for *Trichilia* and soybean protein concentrates' IR spectrum, which were observed. These peaks were attributed to Amide I, II, III, A and B bands which derived from bending and stretching vibrations of the molecular bonds (Sadat & Joye, 2020). Generally, the Amide I band (1631 cm^{-1}) showed an increase in peak intensity for *Trichilia* and soybean protein concentrates compared with Amide bands II, III, A and B (See Fig 6.3.1). This band is due to the C=O bonds stretching vibrations (Litvinov *et al*., 2012 & Riaz *et al*., 2018). The *T. emetica* protein concentrates showed an increase in peak intensity compared with soybean and *T. dregeana* protein concentrates. However, *T. dregeana* showed more increase in peak intensity than soy protein concentrates (Fig 6.3.1).

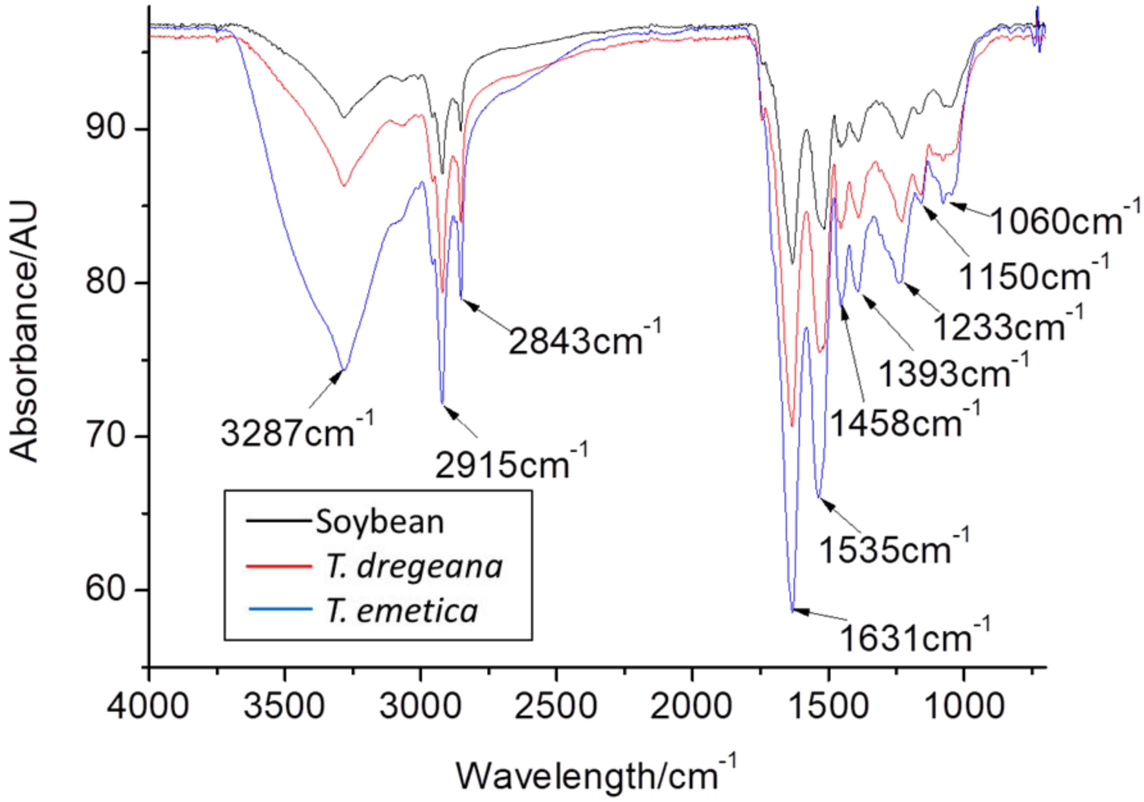

Fig 6.3.1: Illustration of Protein Amide bands found in *T. emetica* and *T. dregeana* compared with Soybean using FTIR. The different peaks in the IR spectra represent the absorptions of different Amide bands within each protein concentrate

Amide II (1488 cm^{-1} and 1535 cm^{-1}) observed in this study may be due to the bending vibrations of N-H bonds coupled with C-N stretching vibrations (Litvinov *et al*., 2012 & Riaz *et al*., 2018) in the *Trichilia* and soybean protein concentrates, Amide III (1233 cm^{-1} and 1393 cm^{-1}) for *Trichilia* and soybean protein may also be due to the stretching vibrations of C-N coupled with N-H bending vibrations with weak C-C stretching and C=O bending vibrations. The chia protein concentrates had previously been shown to have similar Amide II with the *Trichilia* and soybean protein (Coelho & Salas-Mellado, 2018). Amide A (3287 cm^{-1} for the current protein) reported in this study may be caused by the vibration of the O-H group with a carbonyl group of the peptide chain, and the Amide B (2843 and 2915 cm^{-1}) may be related to the stretching vibrations of CH_2 (Litvinov *et al*., 2012; Riaz *et al*., 2018).

The Amide band I for *T. emetica*, *T. dregeana* and soybean had a strong and broad peak (Fig 6.3.1). This increase in peak intensity may be attributed to a greater concentration of the amine groups for the *T. emetica*, *T. dregeana* and soybean protein concentrates. Bambara and cowpea protein concentrates had previously shown a high Amide I band of about 1633 cm^{-1} (Mune & Sogi, 2016) which concurred with the current results. The Amide II bands for chia (1549 -1551 cm^{-1}) (Coelho & Salas-Mellado, 2018), sunflower (1580 -1480 cm^{-1}) (Ishii *et al*., 2021), bambara (1550 cm^{-1}) and cowpea (1550 cm^{-1}) (Mune & Sogi, 2016) were found to be within range of the *Trichilia* and soybean protein concentrates. The presence of Amide B and II bands in the *Trichilia* and soybean proteins may be indicative that some of the peptide bonds between the H-bonds were not completely exchanged to deuterium. This may be due to the compact structure form of some native folded protein molecules (Clark *et al*., 1981 & Kavanagh *et al*., 2000). Both C=O and N-H bonds are involved in the H-bonding which takes place between the different elements of the secondary structure. Therefore, the locations of both the Amide I (C=O) and Amide II (N-H) bands are sensitive to the secondary structure content of a protein (Gallagher, 2009). However, due to the complex vibrations of multiple functional groups, the Amide II band is less useful for the protein structure prediction (Jackson & Mantsch, 1995).

The deconvoluted Amide I band IR spectra for the *Trichilia* spp and soybean protein concentrates with several bands were shown in Fig 6.3.2. The data revealed that the Amide I band for *T. emetica*, *T. dregeana* and soybean protein consisted of nine to eleven major component peaks which were all found in the IR spectra (Fig 6.3.2). The major component peaks of Amide I band for soybean and *Trichilia* (*emetica* and *dregeana*) were assigned as the side chain, antiparallel aggregates β-sheet, intra-molecular β-sheet, random coil, α-helix and β-turns (Fig 6.3.2) based on Achouri *et al.*, (2012). The β-sheet and α-helices provide strength and rigidity to the protein structure (Carbonaro *et al.*, 2012; Gropper *et al.*, 2009 & Shivu *et al.*, 2013). The intra-molecular β-sheet holds the 3-dimension tertiary structure of the protein together; inter-molecular β-sheet stabilises the secondary structure into a three-dimensional fold, while the side chain plays a crucial role in designing the three-dimensional conformation of the protein (Hettiarachchy *et al.*, 2012).

The α-helices in the deconvulated graph were approximately 1652 cm^{-1} and 1657 cm^{-1} for *T. dregeana*, and between 1652 cm^{-1} and 1658 cm^{-1} for *T. emetica*. Soy protein concentrates had similar α-helices of about 1652 cm^{-1} and 1660 cm^{-1} as the *Trichilia* species (Fig 6.3.3). The α-helices peak value for *Trichilia* spp and soy protein concentrates agreed with canola protein concentrates reported by Gerzhova *et al.* (2015); however, higher than the cowpea and Bambara protein α-helices of about 1648 cm^{-1} (Mune & Sogi, 2016). The unordered structure, or random coil, for *T. emetica* and *T. dregeana* was 1640 cm^{-1} meanwhile for soy protein concentrates it was about 1643 cm^{-1}. This random coil, or unordered structure, is less stable and lacks structural elements found in the primary structure of the protein (Carbonaro *et al.*, 2012). Canola protein concentrates had previously shown high random coil values compared with the current study (Gerzhova *et al.*, 2015). The high random coil could be associated with the presence of phenolic compounds in the protein concentrates.

The anti-parallel aggregates β-sheet is indicative of protein aggregation and this bond originates from intermolecular H-bonds or vibration of the carboxyl group of the amino acid's side chains (Carbonaro *et al.*, 2012; Gropper *et al.*, 2009 & Shivu *et al.*, 2013). The *T. emetica* and *T. dregeana* β-sheet (anti-parallel aggregates and intra-molecular) were about 1616 cm^{-1}, 1624 cm^{-1}, 1632 cm^{-1} and 1682 cm^{-1}, while the soy protein concentrate β-sheet was approximately 1618 cm^{-1}, 1626 cm^{-1}, 1634 cm^{-1} and 1682 cm^{-1} (Fig 6.3.2). The *Trichilia* spp β-sheet results are consistent with other oilseeds such as canola protein concentrate (Gerzhova *et al*., 2015a). The results of the present study agree with the soybean, sunflower, canola and walnut protein concentrates in terms of the location of the peaks for α-helices, random coil, β-turns and antiparallel aggregates β-sheet (Zhang *et al*., 2013; Day *et al*., 2022; Ishii *et al*., 2021; Gerzhova *et al*., 2015a; Mao & Hua, 2014). The presence of anti-parallel aggregates could imply that the protein was partially denatured during protein processing and the drying technique.

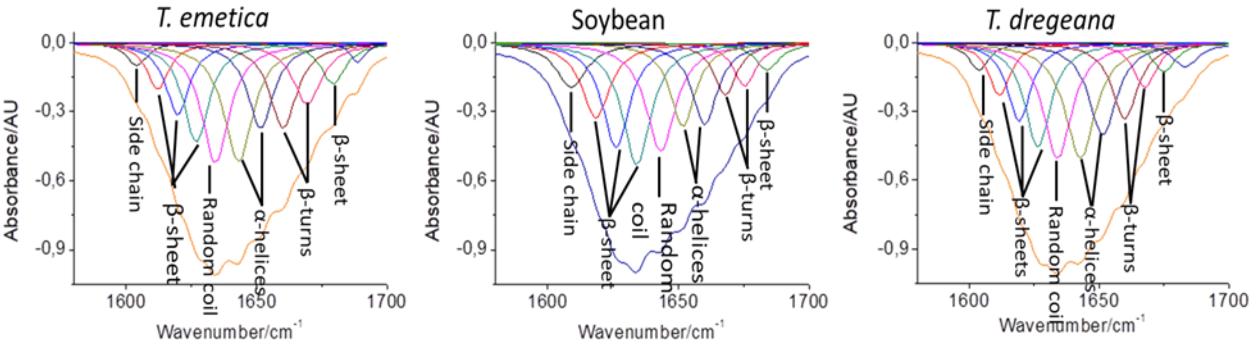

Fig 6.3.2: Deconvoluted *Trichilia* protein concentrates compared with soybean protein Amide I. These regions represent peak-fitting of the secondary derived curves in the Amide I region from the IR spectra.

The relative proportion of different *T. emetica*, *T. dregeana* and soy protein concentrates is shown in Fig 6.3.3. The secondary structure of proteins plays a vital role in understanding the nutritional quality of the protein and functional properties such as texture, protein bioavailability and digestibility (Zhou *et al*., 2008). This result suggests that the *Trichilia* species protein concentrates may be used in the food industry as an alternative food ingredient, since the results indicate similar IR spectra and deconvoluted Amide I components as soy protein concentrates do, and thus could lead to further utilisation of the *Trichilia* protein.

The secondary structure of *T. emetica* protein had a relatively greater proportion of the β-sheet than the secondary structure of *T. dregeana*. However, the *T. emetica* had no significantly different ($p>0.05$) proportion of the β-sheet in its secondary structure compared with the secondary structure of soy protein concentrates (Fig 6.3.3). The lower proportion of the β-sheet in the secondary structure of *T. dregeana* protein may suggest that the protein was partially unfolded during extraction. The unfolding of the protein could cause a decrease in the foaming capacity (FC) and an increase in emulsion activity index (EAI). Mune & Sogi (2016) previously reported that different cowpea and Bambara proteins dried using different methods caused partial unfolding that led to a decrease in β-sheet aggregates. The results of the relative proportion of the β-sheet in the secondary structure of *Trichilia* spp protein and soybean protein concur with what had been previously reported for cowpea (41-42%) (Mune & Sogi, 2016) and Bambara (40-42%) (Mune & Sogi, 2016). However, sunflower protein (30.7%) and walnut protein (11%) had a lower proportion of the β-sheet, and canola protein had a greater proportion of the β-sheet compared with the current results (Gerzhova *et al.*, 2016; Ishii *et al.*, 2021; Mao & Hau, 2014). The α-helices for the *Trichilia* protein were not significantly different ($p>0.05$) from those of soy protein (Fig 6.3.3). The proportion of α-helices in walnut protein (34.9%) was previously observed to be more than the current results for the *Trichilia* spp and soybean protein (Mao & Hau, 2014). However, canola protein and sunflower protein had a lower proportion of the α-helices compared with the *Trichilia* spp protein (Gerzhova *et al.*, 2015 & Ishii *et al.*, 2021).

The relative proportion of the β-turns in the secondary structure of *T. emetica* was significantly lower ($p<0.05$) than in the secondary structure of the *T. dregeana* protein. However, *T. dregeana* was significantly more ($p<0.05$) than the soy and *T. emetica* protein (Fig 6.3.3). Other oilseeds such as walnut (23.3%), sunflower (14.4-14.6%), cowpea (17-25%) and Bambara (18-24%) protein concentrates had more β-turns compared with the current results for *Trichilia* spp and soy (Mao & Hau, 2014; Ishii *et al*., 2021; Mune & Sogi, 2016). This may suggest that the *Trichilia* protein had a more aggregated protein structure than the other oilseeds (Cowpea, bambara and sunflower).

The random coil and side chains for *T. emetica* and *T. dregeana* were not significantly different ($p>0.05$) (Fig 6.3.3). However, soy protein concentrates had a significantly lower ($p<0.05$) random coil compared with the *Trichilia* spp. The side chain for soy protein was significantly higher ($p< 0.05$) than the *Trichilia* spp (Fig 6.3.3). Mao & Hua, (2014) reported walnut protein concentrates had a higher proportion of the α-helix and random coil (32%) which was different from the current observation for the *Trichilia* spp and soybean protein concentrates. Sunflower, canola, Bambara and cowpea protein concentrates were previously observed to have a greater β-sheet proportion (Ishii *et al*., 2021; Gerzhova *et al*., 2015; Mune & Sogi, 2016), which shows a similar trend to current results observed for the *Trichilia* spp and soy protein. The present results indicate that the *T. emetica* and *T. dregeana* proteins, as with most other oilseed protein concentrates (rapeseed, sunflower and soybean), had a higher proportion of β-conformations than the α-helix, with a more ordered secondary structure. The β-conformation is associated with the protein globular structure; this may imply that the *T. emetica* and *T. dregeana* have a globular structure which may cause a lower digestibility but improved functional properties such as water holding capacity and foaming capacity.

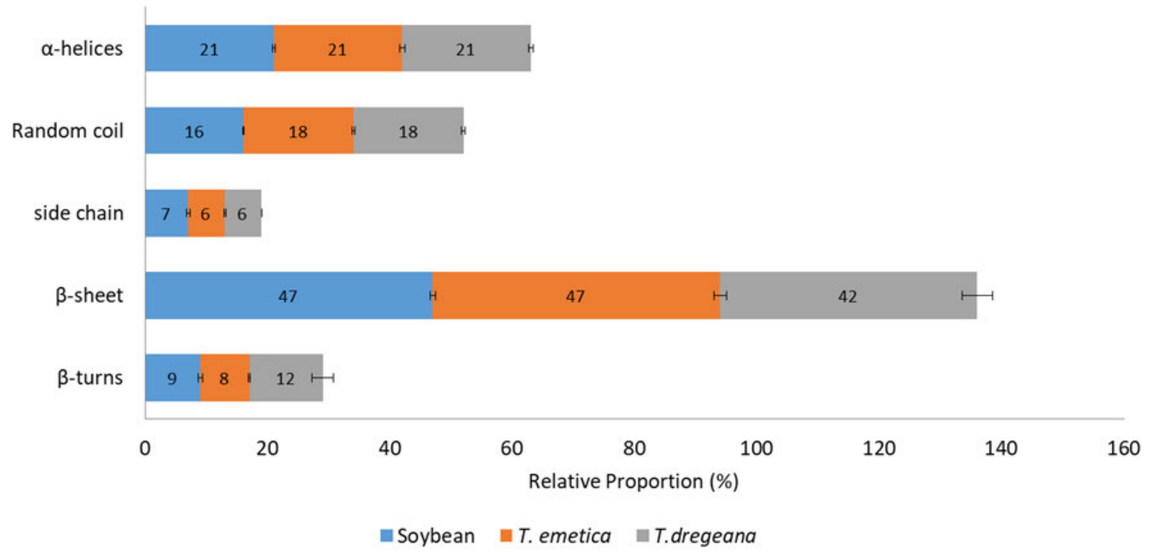

Fig 6.3.3: Relative proportion of different secondary structures in *T. emetica*, *T. dregeana* and Soybean protein concentrates from the Amide I IR Spectra. The combination/ addition of peak fitting derived areas for each secondary structure of FTIR Amide I (1600-1700 cm^{-1}) done according to Achouri *et al*., (2012) and Shevkani *et al*., (2019).

6.3.2. **Functional Properties of *T. emetica* and *T. dregeana* in comparison with soybean protein concentrates**

The water holding capacity (WHC), oil holding capacity (OHC), emulsion activity index (EAI) foaming capacity (FC) and foaming stability (FS) results for *T. emetica* and *T. dregeana* in comparison with soybean protein concentrates are shown in Table 6.3.1. High WHC and OHC contribute to flavour retention, desirable texture and mouthfeel, thereby enhancing the organoleptic acceptability of the food product (Hutton & Campbell, 1981). The WHC is essential in viscous foods such as soup, dough, baked foods and custard (Sreerama *et al*., 2012), while the high OHC is required in baked foods, meat replacers, emulsions (e.g. ice cream) and extenders in which the oil contributes to the texture of the finished food product (Shevkani *et al*., 2019).

Table 6.3.1: Functional properties of *T. emetica*, *T. dregeana* and soybean protein concentrates

Functional Properties	Soybean	*T. emetica*	*T. dregeana*
WHC (%)	800 ±50.00^{b}	921 ±48.02^{a}	450 ±0.00**^{c}
OHC (%)	466 ±28.87^{a}	200 ±0.00^{c}	250 ±0.00^{b}
EAI (%)	54.54 ±0.65^{a}	52.32 ±0.45^{b}	51.5 ±0.71^{b}
FC (%)	23.5 ± 2.12^{c}	90 ± 5.66^{a}	26 ± >0.00^{b}
FS (%): 15 mins	19 ± 1.41^{c}	80 ± 2.83^{a}	21.5 ± 0.71^{b}
FS (%): 30 mins	15 ± 1.41^{c}	75 ± 7.07^{a}	20 ± 0.00^{b}
FS (%): 60 mins	10 ± 0.00^{c}	75 ± 7.07^{a}	20 ± 0.00^{b}

*Mean values of at least two replicates with standard deviation
*Mean values with different superscript letters within the same row are significantly different ($p<0.05$)
**Standard deviations of 0.00 indicate >the detection limit

The *T. emetica* had a significantly higher ($p<0.05$) WHC compared with *T. dregeana,* followed by soy protein concentrates. This observation was in agreement with the HAA results of *T. emetica* (See Section 5.3.2). The WHC results for the current study agree with previous results reported for walnut, canola, cowpea and bambara groundnut protein (Mao & Hua, 2014; Gerzhova *et al*., 2015; Mune & Sogi, 2016). However, previous studies showed that hemp, soy and sesame protein concentrates had a higher WHC than the samples in the current study (Malomo & Aluko, 2015; Mao & Hua, 2014; Teh *et al*., 2014; Gerzhova *et al*., 2015; Adeleke *et al*., 2018 & Liu *et al*., 2019). The higher WHC of *T. emetica* protein may be due to the presence of exposed surface hydrophilic groups in the amino acid side chains. *T.*

emetica protein had a significantly lower ($p<0.05$) OHC compared with the *T. dregeana* protein. Soybean protein had a significantly higher ($p<0.05$) OHC compared with *T. emetica* and *T. dregeana* (Table 6.3.1). The higher OHC values for soy protein concentrates could be due to a higher proportion of hydrophobic groups and polar amino acids on the surface, which bind the aliphatic chains of the oil (Kumar *et al.*, 2022; Kaur & Singh, 2005; Shevkani *et al.*, 2019). The current results are in agreement with the amino acids profile results where the soybean protein had a higher proportion of hydrophobic amino acids such as Leu, Ileu, Pro, Gly, Val and Phe than the *Trichilia* species (See Section 5.3.2). Peanut, canola, sesame and chia protein had a higher OHC which is similar to the results reported for *T. dregeana* (Yu *et al.*, 2007; Gerzhova *et al.*, 2015; Escamilla-Silva *et al.*, 2003 & Coelho & Salas-Mellado, 2018).

The *T. emetica* and *T. dregeana* proteins had shown no significant difference ($p>0.05$) for EAI. However, soy protein concentrate had a significantly higher ($p<0.05$) EAI compared with *Trichilia* spp (Table 6.3.1). The high EAI may be related to the higher presence of hydrophobic amino acids in the soy protein concentrates (See Section 5.3.2). The current results for high EAI agree with other oilseeds such as soy protein concentrates and peanuts (Rao *et al*., 2002; Liu *et al*., 2019 & Yu *et al*., 2007).

Foaming capacity depends on the ability of the protein to diffuse to the interface and form a viscous film without excessive aggregation, while foaming stability is the time required to reduce the foam volume by 50 % (Shevkani *et al*., 2019). *T. emetica* had a significantly higher ($p<0.05$) foaming capacity (FC) and foaming stability (FS) compared with *T. dregeana*. Soy protein concentrates had significantly lower ($p<0.05$) FC and FS compared with the *Trichilia* spp (Table 6.3.1). The high FC for the *Trichilia* protein is not in agreement with the hydrophobic amino acids data; however, it does agree with the compact structure for the same *Trichilia* protein. Higher FC and FS for the *Trichilia* protein concentrates could be due to the balanced hydrophobic and hydrophilic side chains of the protein concentrates' amino acids. The results from this current study indicate that the *Trichilia* spp protein concentrates could be used as a food ingredient to improve foaming capacity requirements in products such as baked products and dairy products (e.g. egg-based ice cream).

6.4. **Conclusion**

The results from this study demonstrated the similarity in the chemical structure of *Trichilia* seed protein concentrates and that of soybean. The *Trichilia* seed protein results suggest that the two species used in this work have more β-conformations than α-helix, as found in soybean protein. The chemical structure of the protein concentrates can influence its functionality in food products. The *T. emetica* had a higher proportion of β-sheet and a lower proportion of β-turns, which results in higher water retention and foaming properties with lower oil retention. The knowledge of *Trichilia* protein structure can help food processors understand how to use the protein as a food ingredient during food processing and manufacturing. The *Trichilia* protein could be used in cakes, pizza dough, mousses and cappuccino foams.

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7. GENERAL DISCUSSION

The first part of this chapter describes the critical review of the methodology; it discusses how the different methods were used and applied. The second part discusses the criticisms and limitations of the major findings of this current study of *Trichilia* seeds.

7.1 Critique of the methodology

During the slide preparation of *Trichilia* seeds and soybean seeds for bright field light (BFLM) microscopy, the samples were fixed using 2.5% glutaraldehyde for 24 hours and post-fixed in osmium solution with subsequent dehydration in a graded series of ethanol according to Mosele *et al*. (2011), with modification. The fixation time, concentration, temperature and osmolality of the fixative agent and buffer may cause a loss of K and exchangeable Mg (Penttila *et al*., 1974; Lott & Buttrose, 1978). However, the loss of K and Mg is dependent on the glutaraldehyde concentration. Penttila *et al*. (1974) observed excessive loss of K at 5% and Mg at 3% glutaraldehyde concentrations. The lower concentration of glutaraldehyde used in this study may have helped in preserving the K in *Trichilia* protein bodies globoid inclusion. Spherical globoids are characterised by the high content of phytate and this could be lost through sample preparation whether by dissolving through fixation or torn from the sample during tissue sectioning (Madsen & Brinch-Pedersen, 2020). However, the *Trichilia* seeds showed the presence of the interior organelles within the protein body which were preserved during the fixation technique. The method used in this study is a standard method that has been used for peanuts, castor and marama (Young *et al*., 2004a; Perea-Flores *et al*., 2011 & Mosele *et al*., 2011).

In this current study, most of the *Trichilia* protein bodies appeared to be oval which is consistent with the reported protein bodies microstructure in oilseeds (Young *et al*., 2004; Lott & Buttrose, 1978; Pereas-Flores *et al*., 2011 & Mosele *et al*., 2011). However, some of the protein bodies in *Trichilia* and soybean were elongated in shape. This variation in protein bodies' shape and size could be due to the tissue sectioning with a razor blade during preparations and the fixation time. Vent *et al*. (2014) observed that a longer fixation time of up to 24 hours does not affect cell volume. The *Trichilia* protein bodies were larger and elongated than other oilseeds such as soybean (in the current study), peanut (Young *et al*., 2004a), almond (Young *et al*., 2004b), castor (Pereas-Flores *et al*., 2011) and marama (Mosele *et al*., 2011) due to the prolonged fixation time of 24 hours.

In this study, toluidine O was used to stain the cells and protein bodies which appear to be pinkish-purple cells and purplish protein bodies. The toluidine O is a polychromatic dye that can stain different elements of the cell wall in different colours (Mitra & Loque, 2014). However, other authors used Coomassie brilliant blue and methyl blue to stain the protein bodies but it does not stain other elements such as cell walls, compared to the Toluidine O (Perea-Flores *et al*., 2011 & Mosele *et al*., 2011). The purplish protein bodies were consistent with other oilseeds, which could mean that the Toluidine O has reacted with nucleic acid (Perea-Flores *et al*., 2011).

Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system with photodiode array (PDA) detector was used for separation and detection of individual amino acids in *Trichilia* protein. Acid hydrolysis is one of the important steps before chromatography. This hydrolysis must completely break down the peptide bonds in the protein and in the current study HCL has been used as a standard method to cleave *Trichilia* protein (Fig 7.1.1). Ghaly *et al*. (2013) found that acid hydrolysis could cause partial destruction or complete loss of certain amino acids. For example, asparagine and glutamine may be completely converted into aspartic and glutamic acids respectively; tryptophan and cysteine may be completely destroyed in acid hydrolysis; threonine and serine may be slowly destroyed and tyrosine may be partially lost while the methionine may be oxidised during acid hydrolysis.

However, in this current study, the glass vial was tightly closed with screw caps to limit the complete destruction of the amino acid, and argon or nitrogen gas was added to eliminate oxygen in order to prevent the methionine from undergoing oxidative degradation during hydrolysis. To accurately quantify the tryptophan, alkaline hydrolysis was done, while the methionine and cysteine were determined after performic acid hydrolysis according to Van Amburgh *et al*., (2015) & He *et al*., (2013).

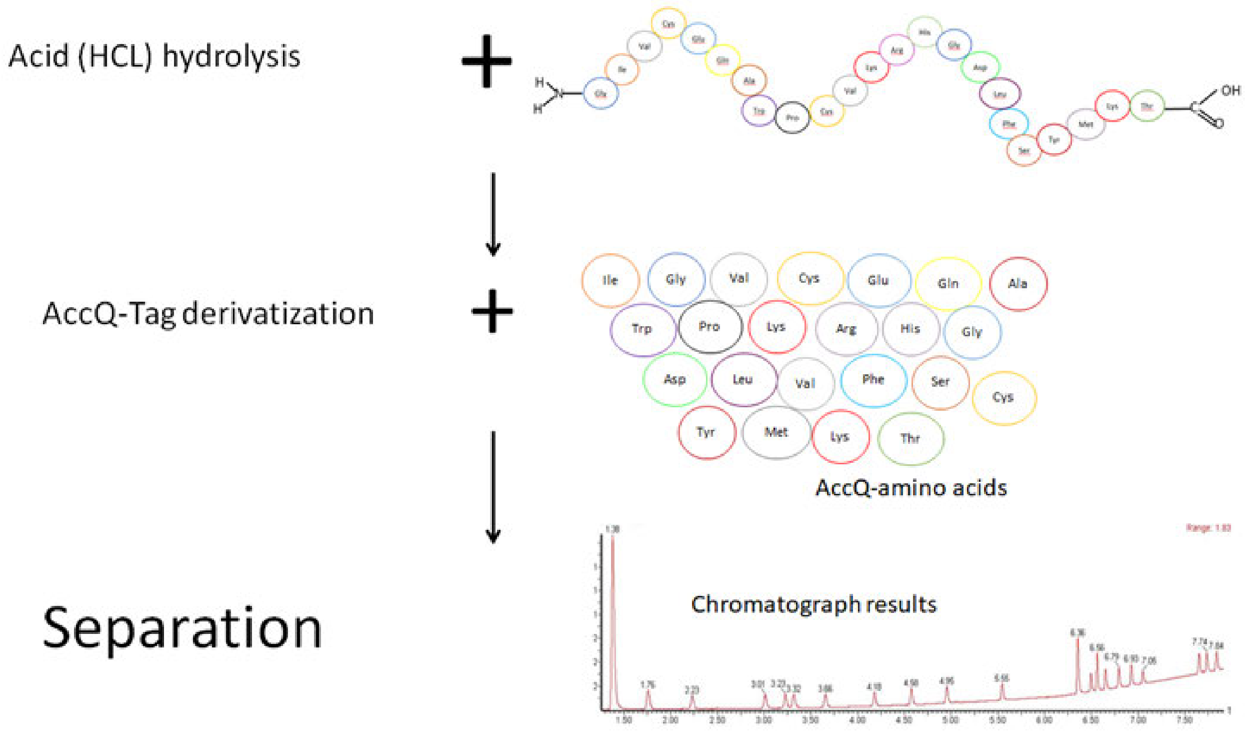

Fig 7.1.1: Illustration of determination of individual amino acids using acid hydrolysis combined with ultra-performance liquid chromatography

Physical localisation of protein bodies relative to the lipids has been found to influence protein extractability (Shand *et al*., 2007). The *Trichilia* and soy protein were extracted using alkaline solutions in combination with isoelectric point and precipitation techniques then freeze dried afterwards. The alkaline (sodium hydroxide) used may cause partial unfolding of the native protein structure. The isoelectric point of *Trichilia* protein was determined because it was unknown. The exposure of the protein to moderate values above or below the protein isoelectric point can cause the unfolding of the protein and/or activation of the buried sulfhydryl group (Wang *et al*., 2017). This unfolding of the protein could lead to lower water and oil holding capacity as well as increasing protein digestibility by exposing more amino acid residues (Perreault *et al*., 2017). However, in this study the objective was to determine the native secondary structure and functional properties of the *Trichilia* protein for use as a food ingredient, not to improve the functional properties of the protein, hence the isoelectric point was determined before. Owusu-apenten (2005) has found that acid precipitation may cause partial denaturation of the protein. In this current study, the *Trichilia* protein was precipitated with acid which could confirm the higher hydrophobicity of the protein, leading to better water retention and oil holding with greater foaming capacity and stability experienced in this study (See Section 5.2.2 and 6.3.2).

The *Trichilia* and soy protein were also freeze-dried after precipitation and dialysis during the protein extraction (See section 5.2.2). Environmental factors, such as the method of protein extraction (e.g. purification and drying techniques) could affect the functional properties of the protein (Yu *et al*., 2007 & Damandoran, 1997). Other authors illustrated the effect of freeze-drying, drum drying and spray drying techniques during protein extraction of peanut protein concentrates, and soy protein concentrates. They also explained the effects of freeze-drying on the functional properties such as WHC, OHC, EAI, FC and FS (Ahmed & Schmidt, 1979; Yu *et al*., 2007; Mune & Sogi, 2016). These authors found that the spray-dried protein sample had better water holding capcaity, oil holding capacity, foaming capacity and stability than the freeze-dried protein sample. This was associated with the exposure of more hydrophobic amino acid sites due to the temperature and duration used for the drying method. Therefore, other methods for drying the *Trichilia* protein concentrates could be explored to produce a competitive commercial protein concentrate product with better functional properties.

The secondary structure of *Trichilia* and soy protein concentrates was done using *Fourier*-transform infrared spectroscopy (FTIR). However, there are other available techniques to characterise the secondary structure of the protein such as circular dichroism (CD), raman spectroscopy, fluorescence spectroscopy, X-ray diffraction (XRD) and UV-spectroscopy (Wang *et al*., 2017). The FTIR methods study the protein conformation from solid, crystal and solution states with high quality spectra (Achouri *et al*., 2012). The method used in this current study has an easy sample preparation technique compared to the other methods which are time-consuming, such as UV, CD and fluorescence. The FTIR technique also allows the characterisation of the impure sample, such as the *Trichilia* protein concentrates used in this study, in addition to isolates and meals, compared to other methods which require a purified sample (Achouri *et al*., 2012 & Wang *et al*., 2017). The infrared spectrum arises from the absorption of energy by chemical bonds which leads to the stretching and bending motions. During *Trichilia* protein sample preparation for FTIR, the sample was not diluted or mixed with water, nor any other liquid, to avoid the stretching and bending vibrations from the water $^{-}$OH group which could be mistaken for any amide band in the IR spectra. However, other authors diluted the samples in D_2O-phosphates buffer which ensures complete hydrogen-deuterium exchange (Achouri *et al*., 2012). The FTIR method has been identified as a powerful technique for the chemical and structural characterisation of the protein (Carbonaro & Nucara, 2010).

7.2 Discussion of results

7.2.1 Critique of the findings

As described earlier (Chapter 4.3; Table 4.3.1 and Fig 4.3.1 to 4.3.4), this study shows that the *Trichilia* seeds had higher unit volume and porosity with lower sphericity, bulk, and true density. These findings indicate that larger-sized seeds such as *Trichilia* seeds had lower physical properties such as sphericity, true density and bulk density. The *Trichilia* seeds had larger protein bodies compared with soybean seeds which were smaller when observed under BFLM, CSLM and SEM. These findings agreed with Sadowska *et al* (2013) observation that larger-sized seeds affect the sphericity, bulk density and true density; hence the seeds under SEM show larger starch content and protein bodies with more interpleural space between the components.

Protein-energy malnutrition (PEM) has been an increasing problem in developing countries and African countries. Africa and other developing continents use cereal-based foods such as sorghum, cassava, millet, wheat, rice and maize as complementary foods and these types of food do not meet the specific nutritional needs of children (Tripathi *et al*., 2012; Dewey, 2013 & Suri *et al*., 2014). The *Trichilia* seeds were found to contain a substantial amount of protein (17% and 26%) in this current study, more so than other staple foods such as maize (9.2%), cassava (0.95-6.42%), sorghum (10.4%), wheat (11.6%) and brown rice (7.9%) (Shewry *et al*., 1995; Ceballos *et al*., 2006 & Kulamarva *et al*., 2010). Protein is vital in maintaining strong immune systems, contributing to cell growth, repair, and maintenance; it acts as enzymes and hormones, and helps maintain fluid and acid-base balance in the human body (Thompson *et al*., 2008). Therefore, the *Trichilia* seeds may be used as a complementary food, or a food source, in order to minimize the rate of nutritional complications such as protein energy malnutrition in adults and children, and to improve the livelihood of individuals from rural communities of sub-Saharan Africa.

The findings of this study indicate that the *Trichilia* protein contains high essential amino acids such as lysine, with a substantial amount of tryptophan (Chapter 5; Table 5.3.2 and Table 5.3.4). Cereal-based foods such as maize, sorghum, Bambara groundnut, cassava, wheat and rice lack major essential amino acids such as lysine; however, they are a good source of carbohydrates and fibre (Temba *et al*, 2016 & Oluwole & Taiwo, 2009). The *Trichilia* protein can be used in rural communities in Sub-Saharan African countries as a food ingredient to address the deficiency of essential amino acids in cereal-based, tubers (cassava), sorghum, wheat and rice staple foods. *Trichilia* spp are evergreen plants that are not affected by drought and are less costly, unlike maize, sorghum and soybean which are expensive and susceptible to drought, which could result in food energy shortages and ultimately malnutrition. In addition to lysine, the *Trichilia* protein also contains high levels of leucine which serves as a nutrient signal for initiating the process of muscle protein synthesis (Hertzler *et al*., 2020).

The most prevalent micronutrient deficiencies in Sub-Saharan African countries are related to iron, zinc, vitamin A and iodine (Hassen *et al*., 2020). These micronutrients act as immunomodulators and determine an individual's resistance to infections (Mitra *et al*., 2022). Generally, the main sources of micronutrients are from animal-based foods and vegetables. However, *Trichilia dregeana* and *Trichilia emetica* has been found to contain a significantly high level of Fe (6.33 and 6.83 mg/ 100 g; respectively) which was above the recommended daily intake according to the FAO standards, compared to other staple foods such as maize, brown rice, wheat and sorghum (Kulamarva *et al*., 2010 & Gibson *et al*., 2010). The *Trichilia* seeds contain a substantial amount of zinc which was within the same range as the zinc found in sorghum (1.2 mg/ 100g- 2.3 mg/ 100g) (Keyata *et al*, 2021).

Other major macronutrients found in *Trichilia* seeds were Ca, P, Mg and K. The presence of these micronutrients agreed with the globoid inclusion observed in the *Trichilia* microstructure under CLSM. Madsen and Brinch-Pedersen (2020) found that the globoid is the storage site for phosphorus, potassium, magnesium and calcium. This indicated that these micronutrients may have originated from the globoid site of the *Trichilia* protein body. Due to the high levels of calcium present, consumption of *Trichilia* seeds as a food source could help in the reduction of osteoporosis prevalent in elderly individuals; potassium can regulate heartbeat and ensure the proper functioning of the muscles (Basson, 2012). Garcia *et al*. (1997) found that soy protein could interact with other components such as minerals, ascorbic acids, and antioxidants such as phytic acid and fibre. In this current finding, the soluble fibre for *Trichilia* seeds was higher (between 33.2% and 57.1%). However, the soluble fibre results were not reported in the proximate composition results section. This may be due to the soluble fibre results not being consistent when repeatedly tested. High fibre content may contribute to the slow digestibility of the seeds; which could keep the consumer full for a longer period of time, and may contribute to the lowering of the glycaemic index (Devi *et al*., 2014). The phytic acid and fibre could also cause a decrease in the bioavailability of minerals and proteins. Therefore, further studies could be done to determine the bioavailability of the *Trichilia* protein and minerals.

Conformational (secondary structure) and functional properties of plant proteins are closely related to various intrinsic factors (e.g. amino acids composition and sequence, hydrophobicity and protein structure - secondary, tertiary and quaternary), and external factors (e.g. pH, salt concentration and temperature), or interaction with other food constituents (Wang *et al*., 2017 & Yu *et al*., 2007). Generally, protein structure tends to assume a globular shape when the protein contains a large number of hydrophobic residues (Wang *et al*., 2017). In this current study, the *Trichilia* protein was found to have more β-conformation, with a lower proportion of α-helix as seen in soy protein (See section 6.3.1). This was in agreement with the high HAA (hydrophobic amino acids) results in *Trichilia* protein (See section 5.3.2) which means the *Trichilia* protein possesses a globular shaped protein. *Trichilia* protein globular structure could affect the functional properties of the protein in the food matrix. In this study, the major findings for the *Trichilia* protein functionality were high water holding capacity, foaming capacity and stability (Section 6.3.2). The protein functionality of food could impart the food quality (such as flavour, mouthfeel, enhanced organoleptic acceptability and texture) and its properties, such as food processing properties. Therefore, the high water-holding capacity of the *Trichilia* protein could improve the juiciness and enhance the flavour of the meat in the meat industry. In addition, the high water retention of the protein could be used in soup, dough making, custard and baked food industries. The high foaming capacity and stability results of *Trichilia* protein could be useful in air/liquid interfaces such as in dairy products (ice cream), whipped topping and baked goodies. This foaming capacity can be stabilised through binding water or air with the *Trichilia* protein at the air/liquid interface. The high fat content of the *Trichilia* seeds could help in friction reduction during food processing in the food manufacturing industry. The investigation and current report on the functional properties of *Trichilia* were based on crude protein in the seeds. This is a limitation in that pure protein from the seed was not obtained for the investigation during protein extraction.

7.2.2 Principal Component Analysis showing the relationship between the amino acid content, secondary structure and functional properties of *Trichilia seeds* and soybean protein

A correlation based PCA was done to investigate the relationship between protein chemical structure, amino acids and functional properties of *Trichilia emetica*, *Trichilia dregeana* and soybean protein (Fig 7.2.1). The first two PC score plots explained 92.5% of the total variation which implied that this component was effective in defining the chemical structure of the protein. The PC1 explained 56% of the total variation. Random coil and BCAA had large (>0.8) positive loading on PC1 while OHC, SCAA, HAA, AAA and side chains had larger (-0.8) negative loading on PC1 (Fig 7.2.1). This implied that the component measured intergrated the chemical structure, amino acids profile and functional properties of the protein concentrates.

The clustering of variables on a correlation PCA plot indicates positive correletaion between the variables. The OHC had a positive correlation with the side chain structure, SCAA, AAA and the HAA (left-side of PC1, Fig 7.2.1). This indicated that the protein structure hydrophobic amino acid side chains could bind with the oil. In this current study, the PCA results agreed with the soy protein amino acids profile which was found to have significantly higher ($p<0.05$) HAA, SCAA and side chains, hence the proximity of soy to the cluster on the PCA plot (Fig 7.2.1).

The EAI was closely related with the AAA, HCAA and SCAA (Fig 7.2.1). The EAI was previously observed to be positively correlated with surface hydrophobicity (Shevkani *et al*., 2015). Thus, surface hydrophobicity would be due to increased exposure of hydrophobic amino acids during the partial unfolding of the protein concentrates (Shevkani *et al*., 2015;

Mune & Sogi, 2016). As has been described before soy protein had higher EAI with an increase in β-sheet and AAA compared with *Trichilia* protein.

The OHC had a positive correlation with the side chain of the protein structure, SCAA and the HAA on the left-side of PC1 (Fig 7.2.1). This indicated that the HAA and SCAA side chains were probably anchored in the hydrophobic oil phase and facilitated the binding of the oil. This agreed with the soy protein amino acid content (Table 5.3.3) and secondary structure (Fig 6.3.1 and Fig 6.3.2) which showed significantly higher ($p>0.05$) HAA, SCAA and side chains, respectively, compared to the *Trichilia* spp proteins (Fig 7.2.1).

The PCA showed that the random coil and BCAA were positively related (Fig 7.2.1). This could imply that the random coil segments were mostly made up of branched amino acids. The random coil and BCAA were opposite to EAI and OHC on the PC1 plots (Fig 7.2.1). This indicated that the random coil and BCAA were negatively correlated with EAI and OHC. The BCAA containing random coil segments probably lead to increased segmental flexibility (Mune & Sogi, 2016), which limited interphase dispersion of the protein at the expense of intra-phase dispersion.

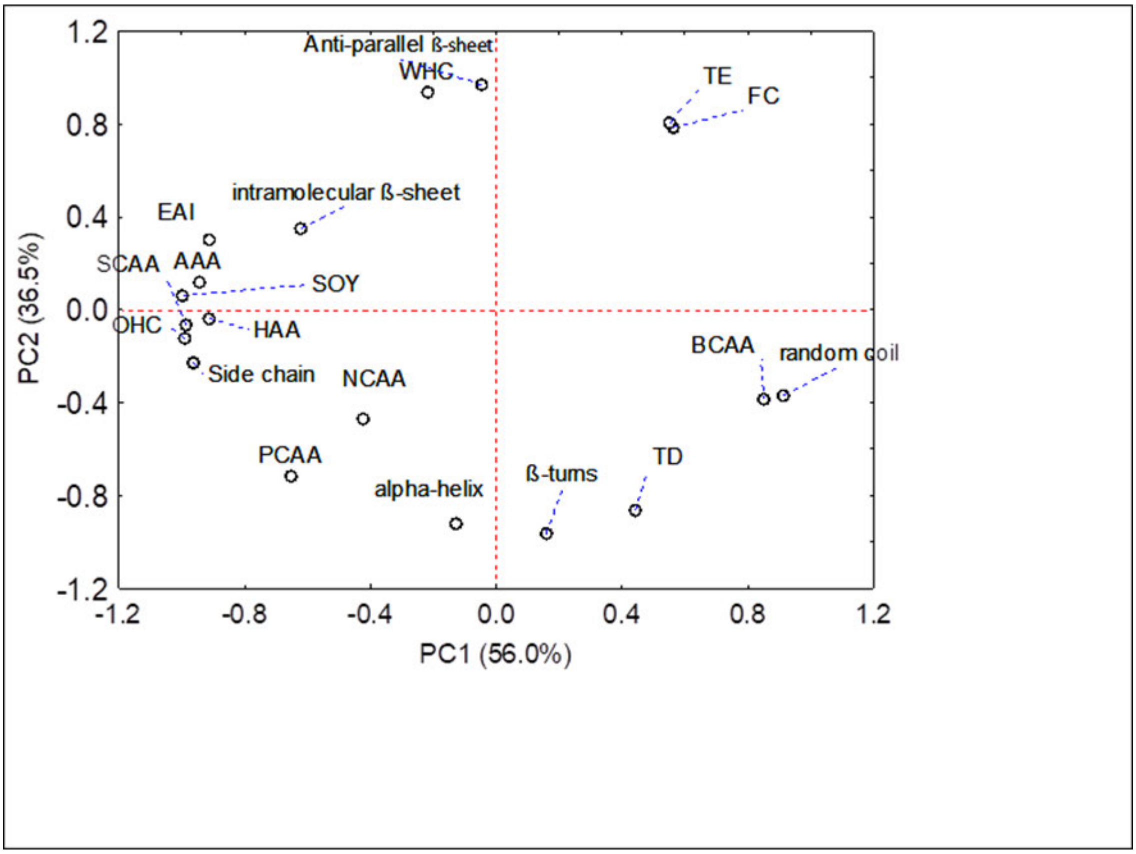

Fig 7.2.1: Principal component analysis plot describing the relationship between the amino acid classification composition, protein chemical structure and functional properties of *Trichilia emetica*, *Trichilia dregeana* and Soybean protein. AAA: aromatic amino acids; BCAA: branched amino acids; EAI: emulsion activity index; FC: foaming capacity; HAA: hydrophobic amino acids; NCAA: negatively charged amino acids; OHC: oil holding capacity; PCAA: positively charged amino acids; SCAA: sulphur-containing amino acids; SOY: Soybean; TD: *Trichilia dregeana*; TE: *Trichilia emetica*; WHC: water holding capacity

Water holding capacity (WHC) was positively correlated with anti-parallel β-sheet aggregates (Fig 7.2.1). This implied that the protein structure had decreased in partial unfolding which lead to a decrease in flexibility and buried hydrophobic amino acids. However, negatively related to the α-helix and β-turns on the far end opposite of PC2 (Fig 7.2.1). Shevkani *et al*. (2015) had previously observed that the WHC was positively related to the protein aggregates structure of the protein.

Foaming capacity (FC) was diagonally opposite to the NCAA and PCAA on the far opposite end of PC2. This implies that FC may be negatively related to the NCAA and PCAA. The

NCAA and PCAA probably contribute to the low structural integrity of films formed. The random coils were closely correlated with BCAA, which implied that the branched amino acids probably contributed to the unorganised structure of the random coils. Mune & Sogi (2016) observed a negative correlation between the unordered structure (random coil) and FC. An increase in random coils possibly causes a decrease in FC due to the effect of segmental flexibility of the protein, which probably led to decreased ability to form durable foams. The samples which include soy, *T. dregeana* (TD) and *T. emetica* (TE) were widely separated. This could imply that the samples were different in protein chemical structure arrangement, amino acids sequencing and composition, hence the different protein functionality. *T. emetica* was closely related to the FC whilst *T. dregeana* was related to the β-turns, and soy with the side chains and intra-molecular β-sheet.

Variables modeling power values closer to one are regarded as more relevant to the model. The variables power values of the chemical structure, amino acids and functional properties decreased in the order: FC (0.941)> anti-parallel β-sheets aggregates (0.937)> WHC (0.922)> EAI (0.920)> AAA (0.905)> intra-molecular β-sheet (0.505). The EAI, FC and WHC were closely related to the AAA and anti-parallel β-sheets aggregates. The PCA indicated that the EAI, FC and WHC increased with the increase in AAA and more anti-parallel β-sheets aggregates; however, an increase in intra-molecular β-sheets could lead to a decrease in EAI, FC and WHC.

8. Conclusion and Recommendations

The physical properties of *T. emetica* and *T. dregeana* are different from the soybean. The processing techniques suitable for soybean, such as milling machines, sorting and drying, will need to be modified to suit the processing of *Trichilia* seeds. The protein body microstructure of *T. emetica* and *T. dregeana* was similar to soybean in shape. However, the *Trichilia* protein bodies are large with spherical globoid inclusion. The *Trichilia* seed protein bodies are located outside the parenchyma cells, unlike in soybean.

The proximate composition and mineral composition of *T. emetica* and *T. dregeana* are comparable to soybean. However, the oil/fat for *Trichilia* seeds is higher than soybean. The protein composition of *Trichilia* is equivalent to soybean. These seeds are richer in Ca and K than soybean seeds. *Trichilia* proteins are richer in Lys, Leu, Glu and Pro, with a substantial amount of sulphur-containing amino acids as found in soybean protein.

The chemical structure of *Trichilia* protein is similar to soybean protein structure. However, the *Trichilia* protein has more β-conformations comparable to soybean protein; the *Trichilia* protein, especially *T. emetica,* has a higher water holding capacity than the soybean protein. *Trichilia* protein also has a greater foaming capacity, with more stable foams, than soybean protein.

Further studies must be done to assess the physical safety of the *Trichilia* seed oils for edible purposes, cosmetics and biodiesel. Even though the seeds are soaked before use to remove the seedcoat, the presence of phytates and trypsin needs to be determined, especially for processing purposes of the *Trichilia* seeds (milling, sorting and drying) if *Trichilia* butter needs to be produced for human consumption. Knowledge of the amount of anti-nutrients needs to be known so that the manufacturers will understand the safety of the seeds when milled with or without the seedcoat, and whether they can or cannot cause hazardous effects on the milled product. Further studies on anti-nutrients, like phenolic content, must be done on the seed cotyledons to determine the bioavailability of the protein, as the phenolic compounds and phytic acids could bind to the proteins and minerals. Further work must also be conducted on the purification of *Trichilia* protein concentrates into protein isolates, thereby improving our understanding of the functional properties; and further research also needs to elucidate the major storage protein, protein digestibility and molecular weight of the *Trichilia* protein.

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