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Edible Oil from Tiger nut (*Cyperus esculentus* L.): Mechanical Pressing and Aqueous Enzymatic Extraction Methods

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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List of Publications

The following papers have been published:

Ezeh, O., Gordon, M. H. & Niranjan, K. (2014). Tiger nut oil (*Cyperus esculentus* L.): A review of its composition and physico-chemical properties. *European Journal of Lipid Science and Technology*, **116**, 783-794.

Ezeh, O., Gordon, M. H. & Niranjan, K. (2016). Enhancing the recovery of tiger nut (*Cyperus esculentus)* oil by mechanical pressing: Moisture content, particle size, high pressure and enzymatic pre-treatment effects. *Food Chemistry*, **194**, 354-361.

The following paper is currently in submission:

Ezeh, O., Niranjan, K. & Gordon, M. H. Characteristics of enzyme aided pressed and aqueous enzymatic extracted edible oil from tiger nut (*Cyperus esculentus* L.) and oligosaccharides in residual meals in the Journal of the American Oil Chemists' Society.

Abstract

The tiger nut tuber of the *Cyperus esculentus* L. plant is an unusual storage system with similar amounts of starch and lipid. The extraction of its oil employing both mechanical pressing and aqueous enzymatic extraction (AEE) methods was investigated and an examination of the resulting products was carried out. The effects of particle size and moisture content of the tuber on the yield of tiger nut oil with pressing were initially studied. Smaller particles were found to enhance oil yields while a range of moisture content was observed to favour higher oil yields. When samples were first subjected to high pressures up to 700 MPa before pressing at 38 MPa there was no increase in the oil yields. Ground samples incubated with a mixture of α- Amylase, Alcalase, and Viscozyme (a mixture of cell wall degrading enzyme) as a pre-treatment, increased oil yield by pressing and 90% of oil was recovered as a result.

When aqueous enzymatic extraction was carried out on ground samples, the use of α-Amylase, Alcalase, and Celluclast independently improved extraction oil yields compared to oil extraction without enzymes by 34.5, 23.4 and 14.7% respectively. A mixture of the three enzymes further augmented the oil yield and different operational factors were individually studied for their effects on the process. These include time, total mixed enzyme concentration, linear agitation speed, and solid-liquid ratio. The largest oil yields were obtained with a solid-liquid ratio of 1:6, mixed enzyme concentration of 1% (*w*/*w*) and 6 h incubation time although the longer time allowed for the formation of an emulsion. Using stationary samples during incubation surprisingly gave the highest oil yields, and this was observed to be as a result of gravity separation occurring during agitation. Furthermore, the use of high pressure processing up to 300 MPa as a pre-treatment enhanced oil yields but additional pressure increments had a detrimental effect.

The quality of oils recovered from both mechanical and aqueous enzymatic extraction based on the percentage free fatty acid (% FFA) and peroxide values (PV) all reflected the good stabilities of the oils with the highest % FFA of 1.8 and PV of 1.7. The fatty acid profiles of all oils also remained unchanged. The level of tocopherols in oils were enhanced with both enzyme aided pressing (EAP) and high pressure processing before AEE. Analysis on the residual meals revealed DP 3 and DP 4 oligosaccharides present in EAP samples but these would require further assessment on their identity and quality.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AEE	Aqueous Enzymatic Extraction
AEP	Aqueous Extraction Processes
AV	Acid Value
^{o}C	Degree Celsius
DP	Degree of Polymerisation
DSC	Differential Scanning Calorimetry
EAP	Enzyme aided pressing
FA	Fatty Acid
FFA	Free Fatty Acid
FOS	Fructo-oligosaccharide
G	Gram
GAE	Gallic Acid Equivalent
GC	Gas Chromatography
h	hour
HPP	High Pressure Processing
HPP-AEE	High Pressure Processing-Aqueous Enzymatic Extraction
HPAEC-PAD	High Performance Anion Exchange Chromatography-Pulsed Amperometric Detector
HPLC-RI	High Performance Liquid Chromatography-Refractive Index
HPLC-UV	High Performance Liquid Chromatography-Ultra Violent
kV	Kilovolts
µg	Microgram

mA	Milliampere
mg	Milligram
ml	Millilitre
mM	Millimole
min	Minutes
M	Molarity
MUFA	Monounsaturated Fatty Acid
MPa	Mega Pascal
N	Normality
OAV	Odour Activity Values
PME	Pectin Methyl Esterase
PUFA	Polyunsaturated Fatty Acid
PV	Peroxide Value
rpm	Revolutions per minute
RTNO	Roasted Tiger Nut Oil
s	Seconds
SEM	Scanning Electron Microscopy
S/L	Solid-Liquid Ratio
TCA	Trichloroacetic acid
TNO	Tiger nut oil
TPC	Total Polyphenolic Content

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Chapter 1

INTRODUCTION AND OBJECTIVES

1.1 Introduction

Despite its misleading name, tiger nut is actually a tuber. *Cyperus esculentus* L. var. *sativus* is a perennial herb with rhizome producing tubers. These tubers are naturally called tiger nuts. Other names by which they are identified include chufa (Spanish), and earth almond. The ancient Egyptians recognised the importance of this crop, and cultivated it for culinary and medicinal purposes (Negbi, 1992). Defelice (2002) reports that the tubers, are thought to be the third most ancient domesticated foodstuff of ancient Egypt, after emmer wheat and barley, since the fifth millennium BC. Today, they are cultivated in Spain, Australia, Africa, North, and South America and in small amounts in China. They can be eaten raw, roasted or ground to make beverages. Popular in Europe as carp bait, the application of tiger nuts is varied, from attracting game to its use in cosmetic industries (Tortajada Forner, 2010).

Tiger nut is a fast growing plant taking an average of three months till maturity and is often mistaken for weeds in some areas. The weedy or wild type, although closely related to tiger nut is different and known as yellow nutsedge (Gerald and Bonny, 1976; Dyer, 2006). Tiger nut is preferably grown in well-drained sandy or loamy soils and its production increases as the ambient temperature increases (Bamishaiye and Bamishaiye, 2011) . It is an easy plant to grow and does not necessarily need additional fertilisers (Cochran, 2011) .

Tiger nut oil (TNO) shares a similar fatty acid (FA) profile with olive oil (Coşkuner *et al.*, 2002; Arafat *et al.*, 2009; Sánchez-Zapata *et al.*, 2012) having oleic acid as its most abundant fatty acid. In recent years, the significance of plant oils for health has been recognised especially their effects on heart health. The World Health Organisation (WHO, 2003) recommends oleic acid to make up daily total fat intake after adequate polyunsaturated fatty

acids intake. New and emerging research has identified dietary unsaturated fatty acids as playing a role in affecting an individual's risk of developing other diseases including diabetes, asthma and cancer (Lunn and Theobald, 2006). TNO contains these beneficial fatty acids as well as vitamin E and phenolic compounds that contribute to its stability. Its phytosterol content, particularly stigmasterol and campesterol, is higher than is present in olive oil, thereby differentiating it from olive oil (Sánchez-Zapata *et al.*, 2012). Its properties have been exploited in a number of studies including its suitability as biodiesel (Barminas *et al.*, 2001; Qu *et al.*, 2007; Xueshe *et al.*, 2008).

Presently, tiger nut oil is sold as cold pressed oil. For the purpose of research, TNO is often extracted with the use of organic solvents or a laboratory press (Ali Rehab and El Anany, 2012; Yeboah *et al.*, 2012). Although the most efficient recovery is attainable using solvent extraction, over 95% as reported by Rosenthal *et al.* (1996), there are concerns regarding the sustained availability of petroleum based solvents, including the contribution of these solvents to volatile organic compounds emissions. In the bid to find more environmentally friendly alternatives, some techniques have been re-visited such as mechanical pressing, whilst some have been developed using non-organic extraction systems. In addition, pre-treatment effects are often included in such studies to improve the efficacy of extraction. Some of these include employing enzymes, ultrasonic waves and high hydrostatic pressure treatment. Enzymes are used to degrade cellular wall components such as cellulose and pectin, to facilitate oil release from the cells. They are commonly used with aqueous extraction processes where they contribute to increasing oil extraction yields from oil seeds. Almonds, hemp seed, soybeans, corn germ, and horse radish seeds are some materials that have benefitted from the use of enzymes in aqueous oil extraction (Mat Yusoff *et al.*, 2015). In spite of the promise of aqueous enzymatic oil extraction, the formation of emulsions limits the viability of the process as extracted oils are entrapped in these emulsions. Only a few studies have implemented enzymatic treatments prior to mechanical oil extraction despite

the potential benefits it may offer. With mechanical presses, there is no difficulty of de-emulsification that arises with aqueous extraction and this eliminates an additional processing step.

The high quality oil obtained from using mechanical processes is one of the reasons why these are still in existence especially as there is an increasing niche market for novel oils. In some rural areas, it also remains the sole method of oil extraction but it still is an inefficient process. Depending on the equipment used, researchers have conducted studies to increase oil recoveries and optimise the process by varying operational variables like temperature, applied pressure and time.

Irrespective of the oil extraction method adopted, sample preparation is quite critical. Particle size reduction, thermal conditioning, extrusion, and flaking are some steps that can be carried out on the samples. Typically a combination of two or more steps is often undertaken. Generally these treatments lead to increases in oil yields because they tend to either soften and/or destroy cellular structure thus aiding extraction.

There is a paucity of research on extraction of oil from tiger nut. Major growers and the entire value chain of the tuber can benefit from more research, particularly with its ease of growth and the oil's myriad uses ranging from salad oil to biodiesel (Barminas *et al.*, 2001; Abdulkarim *et al.*, 2007). Furthermore, TNO could be utilised to improve the diets of consumers in impoverished areas or replace more expensive imported olive oil. The potential increase in interest in the tuber was identified as far back as 1964 when the storage characteristics and stability of the oil under various storage conditions of two varieties from Ghana and Nigeria were studied (Martin, 1964). This interest needs to be rekindled and fuelled with more research on TNO: extraction methods of the oil, its stability, composition and applications. Therefore, this project was developed with the following aims and objectives.

1.2 Aim and Objectives

The overall aim of this project was to evaluate two methods of tiger nut oil extraction and investigate how oil yields can be maximised, taking into account the qualities of oil and residual meals. To achieve this aim, the following objectives were established:

- Investigating the effects of physical properties of tiger nuts (particle size), high pressure pre-treatment and enzymatic pre-treatment on oil recovery using a mechanical press.
- Investigating aqueous enzymatic oil extraction and the effect of experimental factors on oil yield. Parameters include enzyme concentration, time of incubation or hydrolysis, water-solid ratio, enzyme-substrate ratio and agitation speed. In addition, the effect of high pressure processing on aqueous enzymatic oil extraction was examined.
- Characterisation of extracted oils using mechanical press and aqueous oil extraction for quality attributes.
- Evaluation of soluble sugars in residual meals.

Chapter 2

LITERATURE REVIEW

Part of this chapter is based on the review article "Tiger nut oil (*Cyperus esculentus* L.): A review of its composition and physico-chemical properties" published in the European Journal of Lipid Science and Technology (Ezeh *et al.*, 2014).

In the first part of this chapter, the *Cyperus esculentus* L. plant will be introduced and described, followed by a review of the composition and properties of its oil. In the later part, existing oil extraction methods and pre-treatments will be reviewed including the novel technology of high pressure processing.

2.1 Tiger nut tuber (*Cyperus esculentus* L.)

Cyperaceae or sedges are a family of monocotyledonous angiosperms found worldwide both in tropical and temperate regions. They make up the seventh largest angiosperm and third largest monocotyledonous family. Only 10% are used by humans and mostly in the tropics such as in Thailand and Southern India where they are cultivated for matting and for basketry (Simpson *et al.*, 2011). To the untrained eye, they appear similar to grasses (Gramineae family). Some characteristics are given in Table 2.1 to assist in distinguishing between grasses and sedges.

Cyperus L., a large genus belonging to the Cyperaceae family comprises of more than 500 species and *Cyperus esculentus* is one of these. It is a perennial plant that develops rhizome producing tubers. The plants are on average 24-90 cm tall, depending on the origin. Stems of the plant are triangular, about 2 mm wide, with leaves reaching up to 30 cm long and 8 mm wide. Characteristically, they have approximately 4 bracts, about 20 cm long and 5 mm wide subtending the inflorescence (Lowe and Stanfield, 1974). Depending on the variety, they may or may not flower. Wild varieties are noticeably observed to flower while flowers are rarely found in cultivated varieties (Figure 2.1) (Dyer, 2006).The cultivated variety is commonly known as chufa, which is the Spanish name for tiger nuts and in this review they will be interchangeably used. Although this variety has been cultivated as far back as 2400 B.C. in ancient Egypt (Vries, 1991), it is still being grown in different parts of the world but the paucity of information on it may be due to the confusion between the cultivated and wild types. Most research papers focus on the wild variety, yellow nutsedge, because of its notoriety as a weed. Distinctions exist between these two types though. Chufa grows best when temperatures are above 20 ^{o}C, more specifically when temperatures alternate between day and night temperatures of ca. 30 ^{o}C and 20 ^{o}C respectively (Dyer, 2006). It is not as tolerant as the weedy varieties and would not survive frosty weather conditions. Chufa also

tends to have more fat, starch and sugar with less fibre compared to their weedy cousins (Vries, 1991; Defelice, 2002). A typical composition of chufa is 29.5% starch, 30% lipid, 5% protein, 47% carbohydrates, 3.75% moisture and 6.5% fibre (Arafat *et al.*, 2009). These are values for already dried tubers which are the form it is commonly found for preservation purposes.

The growth of chufa is encouraged by moist sandy soils with a pH range of 5.0 - 7.5. If grown in a rich soil or soil with an average fertility rate, it often does not require additional fertiliser (Pascual *et al.*, 2000), and hence decreasing costs of raw materials needed for its cultivation. However, a recent study by Dyer (2006), suggested that addition of Schultz plant food (a common brand of plant food) can lead to an increase in tuber production and tuber mass. In the same study, chufa's production was found to be highly sensitive to time of sowing and temperature. Although it was concluded that chufa was not affected by soil type, the effect of competition with wild nutsedge was prominent when sandy soil was used. When other types of soil were used, competition did not pose a threat to tuber production. Sandy soils have been reported to support growth of the *Cyperus esculentus* in general and the wild nutsedge being a more vigorous variety may have reduced the production of cultivated chufa. Plant size was also correlated to tuber production with larger plants having more tubers. On average, a tiger nut plant can give rise to up to 50 tubers, and have tuber yields of 17,000 kg per hectare (Pascual *et al.*, 2000).

Cultivated tiger nuts are used for food purposes mainly including, but not limited to, raw consumption, making ice-cream, production of gluten free flour, milk-type extract (horchata de chufa), and edible oil. The range of uses has grown increasingly due to an increased interest in the plant. The tuber has been described as having a characteristic taste almost resembling a hazelnut (Vries, 1991). About 19% of chufa is dietary fibre and this has been investigated as a potential source of fibre in food (Linssen *et al.*, 1989).

Figure 2.1: Wild and cultivated tiger nuts. Left is wild and right is cultivated (Vries, 1991)

Table 2.1: Differences between sedges and grasses (Lowe and Stanfield, 1974)

	Sedges	Grasses
Stems	Usually solid, triangular; some hollow, tubular.	Hollow, tubular (solid at nodes); solid.
Leaves	In 3 (or more) rows, except Coleochloa in 2 rows; sometimes apparently leafless, with bladeless sheaths.	In 2 rows; never leafless.
Leaf Sheath	Entire, i.e. a closed tube.	A tube split down 1 side or margins connate.
Ligule	Usually absent.	Usually present.

2.1.1 Varieties of the Tiger nut Tuber

There are two approaches in describing the varieties of the tiger nut tuber. One refers to its botanical classification while the other is based on its colour. Botanically, eight varieties were distinguished by Kukenthal in 1936, only four wild varieties *(leptostachyus, esculentus, hermanii, and macrostachyu*s) and one cultivated variety, *sativus* are now acknowledged (Pascual *et al.*, 2000).The cultivated variety was found to have a higher fat and sugar content (Defelice, 2002). In the colour based classification, four varieties have been reported to exist; red, brown, black, and yellow. There is a tendency that the brown and yellow refer to the same variety as the tubers appear brown when dried and unwashed. Once they have been soaked and cleaned, they become lighter in colour and appear to take on a yellow hue. They are commonly found in Africa and Spain. In literature, the red variety has only been analysed in Cameroon while the black has been found in Cameroon and Ghana (Ejoh *et al.*, 2006; Abano and Amoah, 2011). According to Ejoh *et al.* (2006), no significant variation exists between the lipid content of the black and brown varieties but more between the tubers obtained from different areas in Cameroon. The yellow variety is often preferred over other varieties due to its attractive colour, bigger size and fleshier body. However, it has a lower fat content and higher protein content (Abano and Amoah, 2011).

2.1.2 Tiger nut Oil Composition

As mentioned previously, tiger nut oil has a similar fatty acid composition to olive oil. This means that it predominantly consists of oleic acid with values ranging from 65.5%-76.1% of the oil content (Muhammad *et al.*, 2011; Yeboah *et al.*, 2012) compared to values for olive oil from 56% to 85% (Visioli and Galli, 1998; Fomuso and Akoh, 2002). Other major fatty acids in TNO are palmitic acid, linoleic acid, and stearic acid. The colour, variety and geographical location in which the tubers are grown, and the harvest season have an impact on the relative proportion of fatty acids present in its oil (Mosquera *et al.*, 1996). The percentage of

oleic acid in TNO from Egypt, Ghana, Nigeria, the East Mediterranean region, and Turkey were 69.5, 65.6, 76.1, 72.7 and 68.9-73.3% respectively as shown in Table 2.2.

The variation in FA composition is distinct in that some fatty acids such as α-linolenic are present in small amounts in TNO from certain regions but was not detected in samples from other regions like Ghana. This may be in part due to climatic or environmental factors as well as the variety cultivated, which is not usually specified. In addition, the methods of analysis employed may vary from author to author. For example, Kim *et al.* (2007) employed GC for FA analysis while Yeboah *et al.* (2012) used GC-MS. GC-MS has the advantage of identifying compounds using both retention time and mass spectrum so it offers more accuracy. The total saturated FA content is low with a minimum of 15.4% in TNO from East Mediterranean to a maximum of 22.3% from Ghana.

Table 2.2: Percentage fatty acid composition of TNO from different areas

Fatty Acid	Egypt[a]	Ghana[b]	Nigeria[c]	East Mediterranean[d]	Turkey[e]	South Korea[f]	China[g]
Myristic	0.80	ND	1.7	-	ND	NR	NR
Palmitic	14.50	16.32	10.4	14.80	12.55-14.12	15.4	14.99
Palmitoleic	1.50	ND	-	-	ND	0.2	NR
Stearic	3.40	5.33	0.3	-	1.80-3.35	2.2	2.56
Oleic	69.50	65.55	76.1	72.7	68.92-73.29	65.5	69.32
Linoleic	8.80	12.13	11.8	11.4	9.96-15.46	16.2	13.11
α-linolenic	0.40	ND	0.6	0.5	0.14-0.69	0.5	0.00
Arachidonic	0.20	0.68	6.1	0.6	ND	NR	NR

a; (Arafat *et al.*, 2009), b; (Yeboah *et al.*, 2012), c; (Muhammad *et al.*, 2011), d; (Ozcan *et al.*, 2010), e; (Coşkuner *et al.*, 2002), f; (Kim *et al.*, 2007), g; (Zhang *et al.*, 1996)

ND: not detected; NR: not reported

The low concentrations of PUFAs in TNO are of course favourable. Vitamin E homologues (tocopherols) naturally present in TNO contribute to increasing the oil's shelf life due to their antioxidant properties (Ali Rehab and El Anany, 2012). Only one study has provided information on the tocopherol content of TNO. The tocopherol content consists mainly of α-tocopherol (86.7 μg/g) and β-tocopherol (33.4 μg/g) making up a total of 120.1 μg/g (Yeboah *et al.*, 2012). Jing *et al.* (2012) claimed that *in vitro* studies into the antioxidant capacity of TNO indicated that its radical scavenging ability was equal to that of vitamin C. However, this work ignored the fact that lipids are not soluble in water, and ignored the fact that the active antioxidants were minor components in the oil. Hydroxyl radicals are the most active and toxic free radicals (Jing *et al.*, 2012) and thus hydroxyl scavenging ability was used as an indicator of antioxidant activity although this work ignored the lack of selectivity of hydroxyl radicals.

Other bioactive compounds found in TNO are phytosterols and polyphenols. The most abundant phytosterol class (about 90%) in TNO was identified to be 4-desmethyl sterol and its dominant component was β-sitosterol at 50.37% (Yeboah *et al.*, 2012). Other 4-desmethyl sterols present were stigmasterol, campesterol, δ^5-avenasterol, and δ^7-avenasterol. Trace concentrations of 4, 4-dimethyl sterols were also found (5.60%). These amounted to a total 4-desmethyl sterol content of 986.49 μg/g oil in TNO. Oderinde and Tairu (1992) analysis on TNO sterols confirmed β-sitosterol as the major sterol but with a much lower quantity of total sterols (100.02 μg/g oil) with only 0.1% cholesterol. This variation might be a result of the variety of the tuber. Temperature and planting location have been found to have an effect on sterol and tocopherol contents in soybeans where an increase in growing temperature leads to elevated phytosterol levels (Vlahakis and Hazebroek, 2000).

Recently, the concentration of polyphenols in TNO was quantified and given as 16.5 mg Gallic Acid Equivalent (GAE) per 100 g oil (Ali Rehab and El Anany, 2012). No study has been performed to identify all the phenolic compounds in TNO. Parker *et al.* (2000) used

several concentrations of sodium hydroxide to extract esterified phenolic acids of cell wall material from the skin and peeled tiger nut tubers. The 13 compounds detected are listed in Table 2.3. The most abundant acid in the peeled tubers was ferulic acid released using 0.1 M NaOH while *p*-coumaric acid dominated in the skin and required a stronger alkali (2 M NaOH) to be released (Parker *et al.*, 2000). It should be expected that the concentration of these compounds will be reduced to a certain degree during oil extraction, depending on the production process employed and the materials used. Pumpkin seed oil was found, for example, to produce oils with a higher antioxidant capacity when a polar solvent was used during its extraction (Fruhwirth and Hermetter, 2007).

Table 2.3: Total Esterified Phenolic Acids (mg/g) of cell wall material from skin and peeled tubers of Chufa (Parker *et al.*, 2000)

Phenolic Acid	0.1M NaOH		1M NaOH		2M NaOH	
	Skin	Tuber	Skin	Tuber	Skin	Tuber
monomeric components						
p-hydroxybenzoic acid	6.0	0.0	6.3	0.0	6.7	7.0
vanillic acid	25.3	8.0	17.7	5.8	18.6	3.0
p-hydroxybenzaldehyde	134.0	15.9	39.3	8.2	26.9	4.0
vanillin	68.7	34.0	62.5	24.2	48.5	15.5
p-trans-coumaric acid	3239.0	635.0	6801.0	1416.0	4228.0	479.0
trans-ferulic acid	2025.0	2284.0	1095.0	536.0	404.0	106.0
p-cis-coumaric acid	85.8	0.0	244.0	37.8	121.0	0.0
cis-ferulic acid	142.7	218.0	53.8	27.0	18.0	3.5
dimeric components						
8-8′ AT diferulic acid	0.0	127.0	0.0	240.0	0.0	0.0
8-5′ diferulic acid	22.3	99.0	0.0	0.0	33.3	0.0
5-5′ diferulic acid	180.4	262.0	49.6	32.1	0.0	5.0
8-*O*-4′ diferulic acid	384.1	507.0	94.5	41.1	27.8	6.8
8-5′-(B) diferulic acid	178.2	199.0	0.0	0.0	0.0	0.0

2.1.3 Physico-Chemical Properties

The oils have been characterised by iodine, saponification, acid, and peroxide values. Iodine value (IV) is a measure of degree of unsaturation of fats or oils. A higher IV simply indicates a higher degree of unsaturation. For TNO, a wide range of IV has been reported (76.60-142.35) (Power and Chestnut, 1923; Ejoh *et al.*, 2006). Ejoh *et al.* (2006) discovered no significant variation between the IVs of the black and brown varieties obtained from the same area in Cameroon. Variation did exist when compared to the tubers from other locations within the same country. From the reported IVs for TNO, it can be considered a non-drying oil or semi-drying oil but most authors describe it as non-drying oil (Ejoh *et al.*, 2006; Muhammad *et al.*, 2011; Ali Rehab and El Anany, 2012). Oils are classified as drying or non-drying oils depending on their iodine values. Typically, oils with iodine value above 140 are classified as drying oils, while oils with IV of less than 125 are non-drying (Koleske, 1995). Oils with values intermediate values are semi-drying oils. Its iodine value also indicates that it is liquid at room temperature, thus making it suitable for soap manufacture and vegetable based ice cream (Barminas *et al.*, 2001).

The concentration of free fatty acids is quantified by acid value (AV) given in mg KOH/g oil. A high acid value indicates an oil with a reduced quality (Bioriginal, c2004) and thus AV is considered an important indicator of the quality of vegetable oils (Kardash and Tur'yan, 2005). For TNO, most of its reported acid values are quite low; 0.03-1.38 mg KOH/g oil (Coşkuner *et al.*, 2002; Ejoh *et al.*, 2006; Arafat *et al.*, 2009; Ekeanyanwu and Ononogbu, 2010; Ozcan *et al.*, 2010; Muhammad *et al.*, 2011; Yeboah *et al.*, 2012). Tiger nuts grown in USA were reported to have a high AV of 15.7, but this indicates poor handling and processing of the nuts (Baughman and Jamieson, 1923). Typically, accepted AVs should be less than 4 mg KOH/ g oil (Bioriginal, c2004). Baughman and Jamieson (1923) suggested that this unusually high value might be a result of a very active fat splitting enzyme in the tuber. The

enzyme was observed to be present in small amounts as it slowly hydrolysed amygdalin (Power and Chestnut, 1923). Amygdalin, a naturally occurring glycoside in food plants forms an ester bond with fatty acids (Vemula *et al.*, 2006). Thus the presence of the enzyme may have slowly led to hydrolytic rancidity due to free fatty acids released.

Other high values reported in the literature were from studies done in Egypt, comparing the composition of germinated and ungerminated tubers. Oil from germinated and ungerminated tubers had AVs of 5.97 and 9.07 respectively (Elsaidy *et al.*, 1986). In Nigeria, TNO was exposed to sunlight over a 9 weeks period and its hydrolytic stability was monitored via its acid value (Ezebor *et al.*, 2006). The change in AV was 6.06 and its initial value was quite high at 8.48. There was no record as to how old the tiger nuts were before the study was conducted, it may mean that hydrolytic rancidity had already begun to take place in the tuber before the study commenced as indicated by the high AV.

The presence of amygdalin also indicates the presence of cyanogenic compounds (Mierina *et al.*, 2011) which is in agreement with the finding that the raw tubers contain cyanogenic glycosides. Preliminary examinations on the acute toxicity of tiger nut extract, revealed that it was not toxic to mice at the administered concentrations (Sánchez-Zapata *et al.*, 2012).

Peroxide value (PV) acts as an indicator of an oil's freshness and quality. Peroxides are primary reaction products formed when unsaturated fatty acids undergo oxidation. Following from that, the higher the PV, the more oxidised the oil has become, and this can be manifested by oxidative rancidity. Typical values of TNO's PV range from 0.30-6.9 mEq/kg (Choo *et al.*, 2007; Arafat *et al.*, 2009) which fall well below the acceptable value of 10 mEq/kg for fresh oils (Bioriginal, c2004). These indicate that TNO has good keeping capacity being able to withstand long time storage without undergoing oxidative peroxidation (Muhammad *et al.*, 2011).

The characteristic flavour of roasted tiger nut oil (RTNO) was recently evaluated and the contributing compounds responsible were identified (Lasekan, 2012). Roasting tiger nut tubers is common and has been shown to reduce anti-nutrients such as tannins and oxalates (Chukwuma *et al.*, 2010). The flavour was described as dominated by chocolate and butterscotch-like character, with an additional coffee-like note. There were also less intense peanut-like and nutty-sweet flavours present. Lasekan (2012) identified 75 volatile and four non-volatile compounds and chose to calculate the odour activity values (OAV) of 13 (shown in Table 2.4) out of the 75 compounds. This was based on their high flavour dilution factor (the relative sensory impact of the volatiles) and perception by at least 6 assessors. OAVs were calculated using the concentrations of the odorants and their nasal odour threshold values in bland sunflower oil. The OAVs revealed that vanillin and to a lesser extent 5-ethylfurfural and 2, 3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one contributed intensely to the overall aroma of RTNO (Lasekan, 2012).

Aromatised foods have been on the rise in the past decade, mainly due to an increase in industrially produced food and with that, aroma losses occur during production and storage (Almonds *et al.*, 2009). RTNO can thus serve as a source for natural aroma substances and flavourings. Additionally, its flavour profile can be used to evaluate its quality and detect any oil adulteration along with an analysis of its oxidation products by GC as is recommended for olive oil (Frankel, 2010).

Table 2.4: Key odorants of RTNO (Lasekan, 2012)

Odorant	Concentration (mg/L)	Flavour dilution factor	OAV	Odour description
Vanillin	5.76	128	58	Chocolate, vanilla
5-Ethylfurfural	0.30	32	15	Caramel, spicy
2,3- Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one	2.20	32	14	Caramel-like
Phenyl acetaldehyde	0.47	32	4	Honey-like
Ethanone,1-(4-hydroxy-3-methoxy phenyl)	0.43	16	2	Faint vanilla
5-Hydroxymethylfurfural	2.09	32	<1	Chamomile flower
Octanal	0.47	16	<1	Citrus-like
para-Hydroxybenzaldehyde	1.36	16	<1	Bitter almond
(Z)-14-Methyl-8-hexadecenal	0.14	16	<1	Sweet
2,5-Dimethylcyclohexanol	0.23	16	<1	Musky
Ethyl hexadecanoate	4.35	16	<1	Soapy, fatty
n-Propyl 9,12-octadecadienoate	3.35	16	<1	Fatty
(Z)-4-Octadecenoic acid	1.64	16	<1	Fatty

2.2 Oil Extraction Operations

2.2.1 Terminology and Equations

Some common forms through which data are expressed are noted below. Most often oil yield is quoted as a measure of extraction efficiency. Oil yield is defined as follows:

$$Oil\ yield\ (\%) = \frac{Mass\ of\ oil\ extracted\ (g)}{Total\ mass\ of\ starting\ material(g)} \times 100 \quad \textbf{(}\text{Equation 2.1)}$$

The moisture content needs to be maintained to keep the oil yield calculations consistent. Of the oil extracted, not all of it can be recovered, hence the notion of oil recovery. This requires the measurement of the total oil in the sample.

$$Oil\ recovery\ (\%) = \frac{Mass\ of\ oil\ extracted\ (g)}{Total\ mass\ of\ oil\ in\ starting\ material\ (g)} \times 100 \quad \textbf{(}\text{Equation 2.2}\textbf{)}$$

Generally the higher the oil recovery, the more efficient the process is.

2.2.2 **Mechanical Pressing**

Traditionally, oil has been isolated from oleaginous materials by mechanical means. The stamper and wedge press depicted in Figure 2.2 was in use before the 18th century. Ground oleaginous materials were put into bags and placed on a plank between wedges supported by logs. The wedges were pressed to squeeze the oil out which was collected into a trough underneath the plank (Khan and Hanna, 1983). In the beginning of the 18th century, the hydraulic press was invented for oil extraction. The oil seeds were covered with filter cloths, loaded into perforated, horizontal boxes below a head block and above a ram. An upward hydraulic motion pressed the boxes together and oil passed through the filter cloths. The oil and filter cloths had to be removed manually (Shahidi, 2005). There were enhancements to the technology both in Europe and America as the capacities of these presses were increased. The residual oil content of the pressed cakes typically varied from 5-7% (Zai-Chun, 1989).

Despite the high quality of oil produced using the hydraulic press, it remained an inefficient process with high labour requirements. This batch wise operation was then replaced by the continuous screw press. The original screw press consisted of a horizontal screw of increasing diameter and a vertical feeder. The screw is enclosed in a cylindrical drainage cage that acts as a filter, preventing the release of fines in the pressed oil (Hoffmann, 1989). Over the years, the efficiency and capacities of screw presses also improved due to machine improvements and residual oil content of the cake of 3 to 9% is common (Board, 2002).

Mechanical presses are used either for a pre-press or a full press operation. Full press operations are not as common as the pre-press or the widely used solvent extraction process. Some reasons for which they still exist include the niche market for natural and/or novel oils

produced without heat and chemicals and the high value protein by-product which is preferred over solvent extracted meals. They are also more reliable and used in smaller scale industries especially in hot tropical countries (Shahidi, 2005). During a pre-press process, the oil content in the oilseed is reduced to about 20% before undergoing further solvent washing to extract residual oil. This is done with high oil bearing materials such as canola and flaxseed. Typical oil recovery of a pre-press process goes up to 70% while a full press system can attain recovery of up to 90% (Boeck, 2011).

In a pressing operation, the pressure exerted by the mechanical force reduces the volume of the oil bodies or sacs to release oil. At the same time, the bodies are narrowed, sheared and sealed by the increasing pressure and thus it becomes a self-defeating process limiting the amount of oil that can be extracted from the oilseed. Hence the efficiency of the mechanical oil press rarely exceeds 90% (Khan and Hanna, 1983).

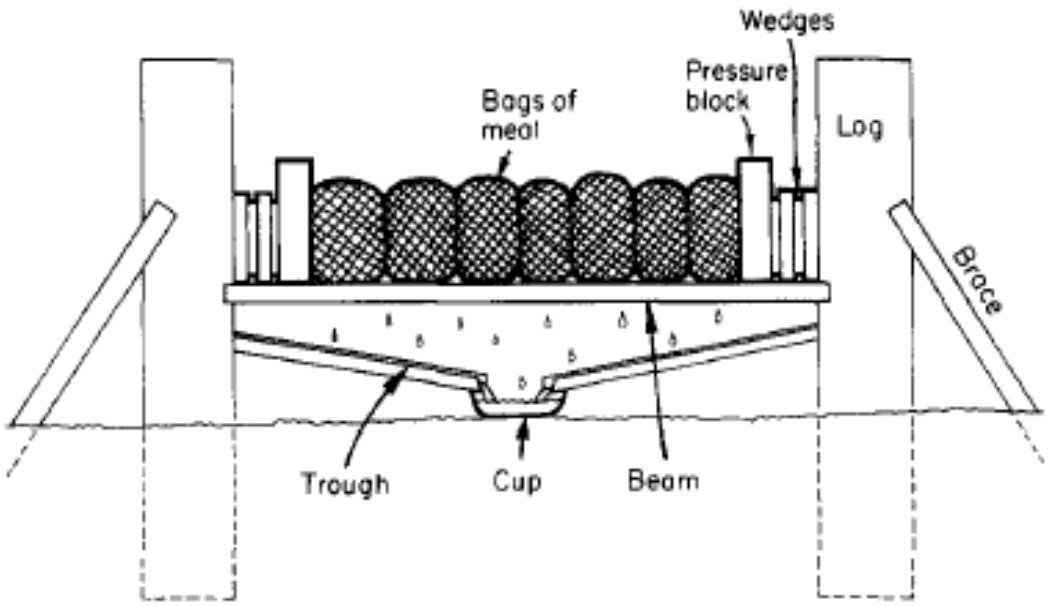


Figure 2.2: Stamper and wedge press (Khan and Hanna, 1983)

2.2.3 **Solvent Extraction**

The total oil unrecoverable by mechanical pressing increases as the oil content of the oleaginous material decreases (Board, 2002). This problem is solved by employing the highly efficient solvent extraction that leaves a residual oil content of less than 1%. It is most suitable for oilseeds with about 20% oil (OCCA, 1993). In solvent extraction, non-polar solvent percolates the solid matrix of a prepared sample, dissolving and extracting components miscible in it. The oil is desolventised in steam evaporators and the recovered solvent is recycled. Commercially, *n*-hexane is commonly the solvent of choice as it can be easily evaporated, and it leaves no odour or taste. The quality of oil and protein rich meal produced is high because the temperature can be controlled whereas in mechanical pressing, frictional heat can elevate the temperature of the oil (OCCA, 1993). The vapour pressure of hexane restricts operating temperature to 50-55 ^{o}C. However, there are a number of disadvantages accompanying solvent extraction processes. It poses safety concerns such as risk of fires and explosions (n-hexane is flammable) as well as a higher risk of dust explosion (Wan and Wakelyn, 1997). In some cases, toxic organic residues remain in the extracted oil. Toxicological and environmental concerns including occasional scarcities of hexane instigated the search for replacement solvents (Johnson and Lusas, 1983).

The petroleum fraction referred to as hexane often varies in the range of 45-90% n-hexane. The other major components consist of 2-, 3- methyl- pentane, methyl cyclopentane and cyclohexane. Studies have shown that pure n-hexane extracts oil at a slower rate than its mixture, but it extracts less free fatty acids and colour pigments. Different petroleum distillate fractions have also been investigated for their viability as potential solvents. Commercial grades of *n*-pentane, isohexane, *n*-hexane, isopentane, and *n*-pentane; technical grades of neohexane, diisopropyl, 2-methyl pentane, cyclohexane, *n*-heptane, 3-methyl pentane, methyl cyclohexane; and pure grades of isopentane, *n*-pentane, cyclohexane,

n-heptane, and methyl cyclohexane were compared by Ayers and Dooley (1948). It was shown that commercial hydrocarbons resulted in higher oil yields and the yields increased as the boiling point of the hydrocarbons increased. The best candidate was methyl pentane, possibly because of its high concentration of commercial hexane (Johnson and Lusas, 1983). Isopropyl alcohol (IPA), n-heptane, n-propanol and ethanol were also as effective as n-hexane in the extraction of soybean oil (Gandhi *et al.*, 2003) The drawback with using alcohols is the higher energy required for desolventisation. The load on the refinery may increase if sugars and other components are extracted due to the polar nature of alcohols (Erickson, 1990). On the other hand, with a mixture of alcohols and n-hexane, lower quantities of the organic solvent are then used and there is an increase in safety from using higher flash point solvents such as IPA. The anti-nutritional factors of the meal are similar to hexane extracted meal and so the nutritional profile is not significantly changed.

Acetone, butanone, furfuryl alcohol and fufural are ketones and aldehydes that have been of interest in oil extraction. The only reported commercialisation of an acetone based plant is the Vaccarino Process where acetone is used to extract cottonseed oil. It is claimed to produce good quality oil with low refining losses. Gossypol and aflatoxins in cottonseed were also successfully removed from the meals as the aim of the study was to simultaneously extract oil and reduce these anti-nutrients in the meals (Hron *et al.*, 1982; Kuk *et al.*, 2005). In another work, when varying concentrations of hexane and acetone for the extraction of cottonseed oil, no significant differences were observed in the oil extraction yields, only in the quality of the meals (Kuk *et al.*, 2005). The colour intensity of the extracted oils however deepened, as acetone concentration increased which were attributed to gossypol in the oil.

In spite of the potential viability of these substitutes, hexane continues to remain the chosen solvent for a variety of reasons: there is a higher long-term price for the substitutes,

modification to existing plant equipment will be expensive, there is a lack of sufficient data on the safety and toxicity of the substitutes and there are reliable supplies of hexane (Erickson, 1990).

2.2.4 **Aqueous Processes**

Water is one universal solvent that has been extensively researched into with regards to oil extraction. In aqueous extraction processes (AEP), the comminuted sample is shaken with distilled water in a centrifuge and left to incubate at a desired temperature for a specific time (Lee, 2012). Water dissolves soluble cellular materials and allows oil to be released into the bulk liquid. The cream emulsion formed can be broken down using a centrifuge and then separating the different phases. Unlike solvent extraction where hexane is miscible with the oil and releasing the oil simply requires hexane evaporation, 10-15% of the extracted oils in AEP gets trapped in the emulsion phase thus affecting the yield (Campbell and Glatz, 2009). The focus of most research on AEP is on releasing the trapped oils from the emulsions. Campbell and Glatz (2009) proposed that since soy protein bodies occupy most of the intercellular volume, they could act as a physical barrier to oil release. At certain protein/oil ratios, the insoluble protein may also bind the oil by physical entrapment. The factors that affect oil and protein extraction include pH of the dispersion, water- to- solid ratio, time, agitation speed and temperature. In a rice bran oil AEP study, the most influential factor that influenced oil extractability was the pH of the dispersion medium (Hanmoungjai *et al.*, 2000). Oil extractability is linked closely with protein solubility which in turn is affected by pH depending on its isoelectric value (p*I*). The p*I* value of each protein differs and thus the optimal pH of each AEP will be different as well as the outcome on the oil yield at each p*I*. For soybeans, the yields of soy protein and oil have been reported to be lower at the p*I* value of the proteins while in the case of peanuts, a better isolation of the oil is achieved at the isoelectric point of peanut protein than in more alkaline conditions (Rhee *et al.*, 1972;

Campbell and Glatz, 2009). In most cases, the further away the pH is from isoelectric point, the better the protein solubility and hence protein and oil extraction becomes easier (Rosenthal *et al.*, 1996).

Recently, Tabtabaei and Diosady (2013) developed two and three stages AEP for oil and protein extraction from dehulled yellow mustard flour. In the two stages process, the incubation was carried out at pH 4.8, centrifuged into three fractions: skim, solid residue and emulsion. The solid fraction was incubated further at pH 11 and the same procedure repeated. In the three stage process, the emulsion from the first and second incubation were combined and incubated at pH 11. The extra stage was adopted to destabilise the emulsion produced in the second stage and enhance the protein concentration in the skim fraction. It was discovered that although this aim was achieved, it also increased oil dispersion in the skim fraction decreasing the protein quality. This was the case for the two and three stages process. A previous investigation had been carried out at only pH 11 for a two stages process and that was decided to be the best compromise for both oil and protein extraction. There was no significant difference in the oil yields between the adopted two stages at pH 11 and two stages process at pH 4.8 and 11.

Cater *et al.* (1974) were able to recover 93% and 91% of oil and protein respectively from copra (coconut) using an aqueous process. Copra oil extraction differs from other oil seeds as the milk is first expressed followed by oil and/or protein extraction. A simplified flow diagram of the process developed by Cater *et al.* (1974) is shown in Figure 2.3. Hanmoungjai *et al.* (2000) observed that the quality parameters of AEP rice bran oil, commercial samples and hexane extracted oil were comparable with the exception of the higher peroxide value of aqueous extracted oil. Hexane extracted oil appeared darker due to the presence of carotene and chlorophyll pigments. It also had significantly higher free fatty acid content. The AEP oil may thus require less bleaching treatment.

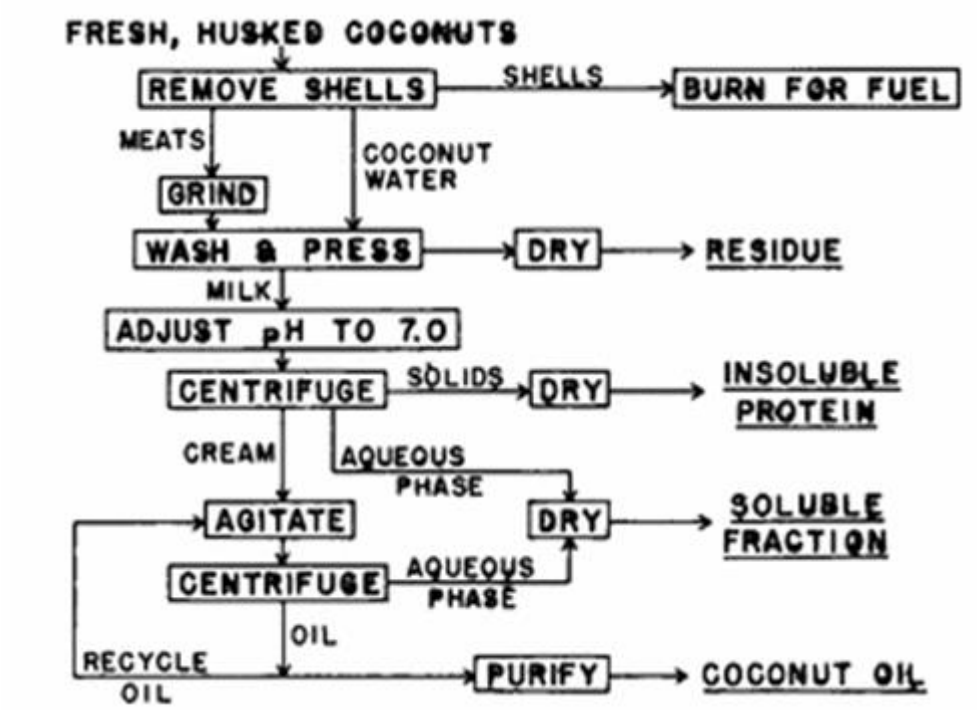


Figure 2.3: Simplified flow sheet for copra oil and protein extraction (Cater et al., 1974)

2.2.5 **Raw Material Preparation**

Prior to any oil extraction, the oleaginous material needs to undergo certain preparatory steps to optimise the extraction process. These vary depending on the structure and composition of the material. Commonly, this would comprise of cleaning, drying, removal of husks, shells or seed coats, and crushing. Rolling may be carried out to increase the surface area to improve the extraction (Höfer, 2009). Dehulling ensures that fines in expressed oil are reduced as total solids are minimised.

Pre-treatments are either mechanical or thermal and are essential as they prepare and break or weaken the seed cell wall to facilitate oil release. Size reduction either by flaking, crushing or grinding produces different types of surfaces and shortened pathways for oil to travel during extraction. Thermal treatments like cooking are necessary to inactivate undesired anti-nutritional factors such as trypsin inhibitor in soybeans. In addition, they provide moisture conditioning of the seed, increase plasticity, destroy thermo-labile toxic

components and reduce oil viscosity. Conditioning of the seed is quite important as moisture content is known to be a major factor in controlling oil expression. Depending on the oilseed specie, its effects are different. Conventionally, an increase in moisture content decreases oil yields during mechanical pressing. This has been observed for rapeseed, flaked cooked cuphea, crambe and flaxseed (Savoire *et al.*, 2013). There appears to be a range of moisture content values for which oil yield increases after which it declines. Walnuts and peanuts have been observed to display such a trend. Oil yield from walnuts processing increased from 61%- 83.5% when the moisture content increased from 2.4% to 7% (Sivakumaran, 1985; Martínez *et al.*, 2008). Moreau *et al.* (2005) reported an interesting outcome as significantly greater oil yields were achieved over a wide range of moisture contents (1.1 - 7.3%) when extracting corn germ oil.

Some equipment such as the expander/extruder bypass the need for dehulling, pre-heating or pre-moistening. When used for soybeans, the resulting soy meal subsequently becomes urease and trypsin –inhibitor free (Riaz, 2000). The effect of extrusion has been shown to enhance oil extraction yields. Bargale *et al.* (1999) increased soybean oil recovery prior to mechanical compression using an extrusion treatment. A maximum recovery of 90.6% was obtained after 20 minutes of pressing. Microwave radiation is another thermal pre-treatment that has been observed to improve hazelnut oil extraction (Uquiche *et al.*, 2008). The microwave treated oils exhibited higher oxidative stability compared to untreated oils. Peanut oil recovery was also found to increase after receiving microwave treatment prior to pressing (Ramesh *et al.*, 1995), although the oil was found to have higher free fatty acid content and peroxide value.

2.3 Enzymatic treatments

2.3.1 **Solvent Extraction**

The effects of enzymes on the extraction of oil have been studied for more than 30 years. Fullbrook (1983) investigated the production of protein isolate from waste seeds of melon fruit using proteolytic enzymes. It was discovered during the study that extra oil was released from the crude isolate. The principle was then adopted and tried for soybeans and rapeseeds. Using enzymes from *Aspergillus* in the presence of a solvent resulted in a higher recovery for soybean oil, up to 90% but not rapeseed oil. As the cell structures of the materials were not considered in the choice of enzymes, the difference in oil yield is not a surprise. In a different research involving canola, different types of carbohydrases were employed to enhance oil yields and reduce extraction times. The treatment was carried out on flaked canola prior to hexane extraction and it was discovered that a mixture of enzymes (cellulase, β-glucanase, hemicellulase, pectinase, cellobiase, arabanase, xylanase, α-galactosidase, and protease) resulted in the highest oil extraction yield (Sosulski *et al.*, 1988). A similar result was observed for oil extraction from soybeans where Driselase, an enzyme preparation with multi activity, was used (Bargale *et al.*, 2000). The majority of soybean is protein so it came as no surprise that the effects of proteases were higher than that of cellulases. Pectinase did not show any effect. The optimum pH and temperature range for Driselase was 3-5 and 40-55 ^{o}C respectively while for the mixed enzyme used for canola oil extraction these were 5.65 and 50 ^{o}C with an incubation time of 12 h (Sosulski *et al.*, 1988).

The action of the enzymes allows more access for the solvent to the oil bodies by degrading the cellular structure of the seeds. The material should also be finely ground to optimise the oil extraction process as there is then an increase in the surface area for the enzymes to act upon. Using solvent extraction for soybeans, oil was more readily extracted from flakes than from grits (Bargale *et al.*, 2000).

Incubation times seem to vary between oleaginous materials. In the enzymatic extraction of avocado oil, an optimal time of 1 h was deemed sufficient (Buenrostro and López-Munguia, 1986) which is in contrast with an optimal time of 12 h used in canola oil extraction. However, it should be highlighted that the enzymes used for the studies differed in their concentration and their activity type which may have been reflected in the optimal incubation times.

2.3.2 **Aqueous Extraction**

Aqueous enzymatic extraction (AEE) is simply a variation of the aqueous extraction process with the additional step of incubation with enzymes. The incubation step occurs either during the aqueous extraction or before, but using enzymes during AEP is mostly common. Inactivation of the enzymes follows by raising the temperature. A general flow diagram of the process is shown in Figure 2.4. With AEE, oil recoveries up to 97% have been achieved (Russin *et al.*, 2011). This effect is a result of hydrolysis of the proteins that form lipid membrane bodies and/or hydrolysis of the polysaccharides forming the cell wall of oilseed (Rosenthal *et al.*, 2001). The limitation of the process still lies in the stability of the emulsion that forms afterwards but the advantage associated with it is the simultaneous recovery of both oil and high quality protein (Sant'Anna *et al.*, 2003). The reason for this occurrence is based on the observation that oil bodies in the cytoplasm are surrounded by proteinaceous membranes (Rosenthal *et al.*, 2001). Amongst different types of enzymes, proteases and cellulases are reported to lead to higher incremental effects compared to hemicellulase and pectinase. For *Jatropha curcas* seeds, the best oil yield (86%) was obtained using an alkaline protease, Alcalase (Winkler *et al.*, 1997).

Olsen (1988) reported similar results with rapeseed (95%), coconut (95%) and flaxseed (72%). A combination of cellulase, α-amylase, polygalacturonase and protease gave higher oil yields than when the same mixture without protease was used in the extraction of coconut oil (Man

et al., 1996). In contrast with coconuts, cellulases had a greater effect on the oil recovery of sesame oil compared to both trypsin and papain, both having proteolytic activity (Hou *et al.*, 2013). Information on the components of the structural cell wall of the oilseed is paramount in the correct selection of enzymes.

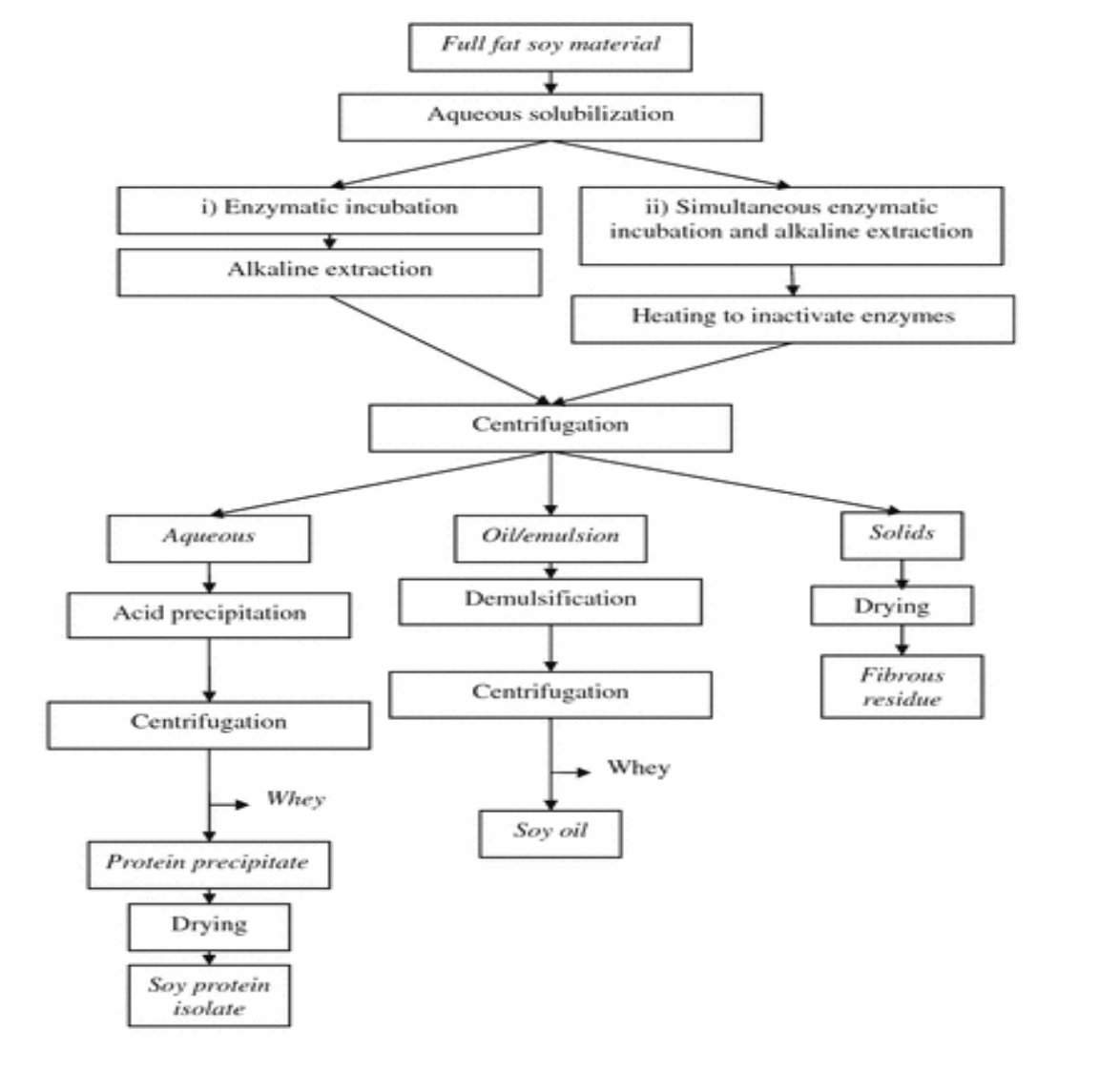


Figure 2.4: Process flow for the enzyme assisted extraction of oil from soybeans using *i)* enzymatic pre-treatment followed by aqueous extraction and *ii)* simultaneous enzymatic treatment and alkaline solubilization (Russin *et al.*, 2011)

The parameters that generally affect the overall yield of oil are the same mentioned previously for aqueous processing with the inclusion of substrate concentration, number of extraction stages, enzyme concentration and enzyme type (Campbell *et al.*, 2011). The

optimum pH for the enzymes incubation should preferably be away from the isoelectric point of the oilseed proteins. Soybean whose cytoplasm volume is occupied mostly by protein bodies had a decrease in oil extractability after the action of cellulase (Rosenthal *et al.*, 2001). Campbell *et al.* (2011) proposed that this occurred because the cellulase treatment was done at pH 5 causing the proteins to precipitate. In another study, soybean oil extraction yield was increased from 92% to 98% using a mixture of cellulase, hemicellulase and pectinase at pH 5 with 0.5 M NaCl and 0.4 M sucrose as the extraction medium. The high salt concentration of the medium contributed to the formation of a supernatant rich in solubilized protein. This reflects the importance of extraction conditions (Campbell *et al.*, 2011). However the opposite applies to peanut whose isoelectric point (pH 4) facilitates oil recovery with Protizyme, a mixture of acid, neutral and alkaline proteases (Sharma *et al.*, 2002). Though Jiang *et al.* (2010) did find out that Alcalase (pH 8.5) gives a much higher oil yield than Protizyme with peanuts. Safflower oil extraction yields are maximised at pH 5 and 6 after employing commercial enzymes Celluclast and Alcalase respectively (Gibbins *et al.*, 2012). The isoelectric point of safflower protein also happens to be 5 (Betschart, 1978). It is uncertain if this is only a coincidence.

There seems to be no understanding as to why certain commercial enzymes of similar activity produce higher yields than others. Also, it is unknown if there is any relationship between the isoelectric point of oilseed proteins and the enzymatic optimum pH.

The substrate concentration also affects the extraction yield although it does not get addressed in most papers. Avocado oil extraction had its best yields at 20% w/w (Buenrostro and López-Munguia, 1986), coconut oil at 25% w/w (Barrios V.A., 1990) and *Jatropha curcas* seeds 46% w/w (Winkler *et al.*, 1997). Particle size is especially critical in the outcome of the oil yield. Rosenthal *et al.* (1998) demonstrated that yields are proportional to the inverse of

the particle size. Oil yield was reported to increase from 22 - 65% when the particle size went from 1200 to 100 μm.

Oil quality is not affected by AEE. Man *et al.* (1996) evaluated the effect on free fatty acid, saponification, p-anisidine, iodine values and colour of coconut oil and found there was no significant difference between the treated samples and the control (without the enzymes). They also met the quality criteria proposed by the International Standard for the Asian and Pacific Community. Likewise, no significant variation was found between oils extracted with and without enzymes in aqueous processing (Latif *et al.*, 2011). The free fatty acid content and colour substances in the AEE oil were lower than that of hexane extracted oils as the conditions in AEE tend to be gentler. Colour pigments also are less soluble in water than hexane. In addition, the AEE oil was observed to contain significantly higher tocopherol content compared to AEP oil.

2.3.3 **Mechanical Pressing**

A few studies have been carried out on enzymatic pre-treatments prior to mechanical expression of oil from oilseeds compared to extraction using aqueous processing. The effect of enzymatic pre-treatment was first carried out on the mechanical pressing of olives by Santos Antunes (1978). Subsequently Smith *et al.* (1993) studied the effect of enzymatic hydrolysis on soybean oil expression. In their study, a mixed activity enzyme from *Aspergillus fumigatus* was reported to be more effective than mixed enzymes from other micro-organisms. The moisture content of the soybeans was first adjusted by adding the required quantity of water before adding the enzyme solution. These were left in the refrigerator to equilibrate. After equilibration, they were incubated at 45 ^{o}C for a desired time after which the hydrolysed sample was dried to inactivate the enzyme and to re-adjust the moisture level to be suitable for mechanical expelling. A number of factors were investigated to determine their effects on the oil extractability. Upon using response surface modelling to

optimise the extraction conditions, the optimal parameters were determined to be: incubation time of 13. 24 h, pressure of 75.0 MPa, moisture content of 9.36%, wet basis (w.b) during pressing, pressing time of 5.36 min, moisture content during hydrolysis of 23.00% w.b and enzyme concentration of 11.84% v/wt. of sample. The enzyme treatment did enhance the oil recovery by 11.7% on a total oil extractable basis.

The enzymes in mechanical extraction act by only hydrolysing the cell wall possibly because of the low water medium used during hydrolysis. Also, there is an added protective effect on the enzyme stability and reactivity. The formation of polysaccharide-protein colloids in aqueous enzyme systems on the other hand presents a different challenge for the enzymes. Concha *et al.* (2004) infer that because of this, the influence of variables such as temperature, enzyme concentration, reaction times and agitation is not necessarily the same as that for aqueous enzymatic extraction. The flow chart shown in Figure 2.5 illustrates a general flowchart followed by different authors on enzyme aided pressing.

In the study by Concha *et al.* (2004) on rose-hip oil extraction, only carbohydrases were used in the form of two different mixtures composed of three different commercial enzymes. The enzymes were Cellubrix (cellulase and hemicellulase activities), Olivex (pectinase, cellulase and hemicellulase activities) and Finizym (betaglucanase, cellulase and hemicellulase activities) while the mixtures were Cellubrix-Olivex (CO) and Cellubrix-Finizym (CF). The impact of pectinase (CO) was being investigated and it was confirmed that there were no significant differences in the oil extraction yields using both mixtures, thus indicating that pectinolytic activity is not necessary to enhance the action of hemicellulase and cellulase. This was attributed to the low content of pectin. The cell wall consisted mainly of cellulose and hemicellulose.

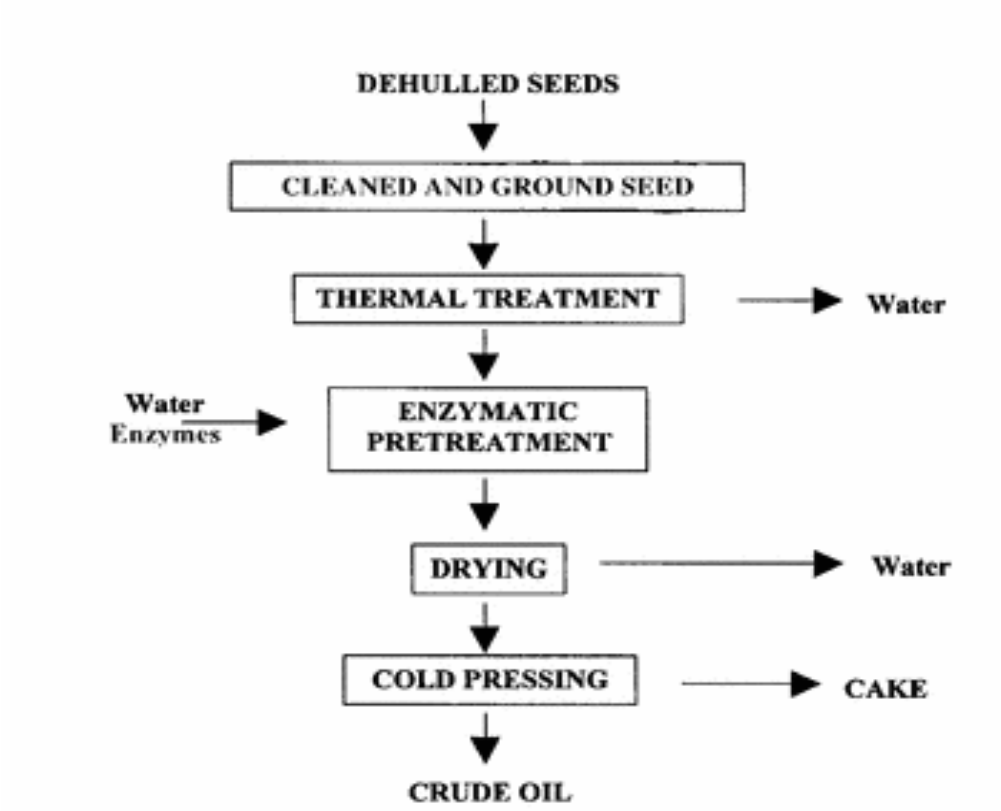


Figure 2.5: Enzyme aided process for oil expression (Concha *et al.*, 2004)

A contrasting study on Chilean hazelnut revealed that pectinase did enhance the action of other carbohydrases (Zúñiga *et al.*, 2003). Both studies used the same incubation time of 6 h and CO enzyme mixture. Szydłowska-Czerniak *et al.* (2010) reported higher oil yield when pectinases were used for rapeseed oil extraction, confirming that pectin was prevalent in the cell structure of rapeseed. In the hazel nut study, Zúñiga *et al.* (2003) suggested that pectinase was beneficial as the pectin content in the outer cell wall structure inhibited the degradation of the inner cellular components. This was also reflected in the crude fibre content of the meal where the percentage of pectin was reduced.

A comparison of the crude fibre content between a control sample and enzyme treated ones in another rosehip oil extraction study showed no significant difference between the two (Concha *et al.*, 2006). This was not explained by the authors. These studies reflect the need to

investigate first the structural components of the cells of any oilseed before oil extraction as each oilseed differs in its structural components.

The optimal incubation temperature for hydrolysis appears to fall within the same range for different oilseeds. Hazel nut, evening primrose oil, tomato seeds and pomegranate seeds had optimal temperatures of 40 ^{o}C while rose hip was 45 ^{o}C (Zúñiga *et al.*, 2003; Concha *et al.*, 2004; Collao *et al.*, 2007; Mirzaakhmedov *et al.*, 2011). A similar observation was seen in the extraction of borage oil where the yield increased from 35 ^{o}C to 45 ^{o}C but declined above 45 ^{o}C (Soto *et al.*, 2007). Moisture content of the samples is an important factor affecting oil extraction yield. Optimal moisture content for rose-hip and canola was identified to be 30% (Concha *et al.*, 2004; Soto *et al.*, 2007). This differs from that of soybean (23.0%), hazelnut (40%) and borage (20%) (Smith *et al.*, 1993; Zúñiga *et al.*, 2003; Soto *et al.*, 2007). Soto *et al.* (2007) suggested that this difference may be due to better water absorption by crushed borage and thus a higher water activity enhancing the enzyme activity which depends more on binding water rather than the total water in the system.

Generally, enzymatic treatments do not have negative impacts on the quality of the expressed oils. The fatty acid profiles do not significantly change. This trend has been observed by different authors (Soto *et al.*, 2007; Latif and Anwar, 2009). The iodine number and unsaponifiable matter did not differ significantly for enzymatic extracted hemp oils. On the other hand, the levels of tocopherols were higher and the oxidative stability and sensory scores were improved (Latif and Anwar, 2009).

2.4 High Pressure Processing

The technology of high pressure processing (HPP) involves subjecting materials to pressures up to 1000 MPa with a fluid (often water) as the pressure transmitting medium. This can last from a few seconds to several minutes. The pressure is applied equally in all directions and one of its advantages is that the pressure is uniform and almost instantaneous throughout the food (Torres and Velazquez, 2005). It was first tested on bacteria in the 1890s and years later it was applied to different food items such as vegetables and fruits (Avure Technologies, 2015). Commercially, it is employed as a non-thermal food preservation tool. The high pressures exerted on food can destroy spoilage inducing microorganisms whilst retaining the food's nutritional and sensory components.

So far the use of HPP has been restricted to food preservation. However, there is growing research on alternative applications. The pressures used during HPP mainly act on non-covalent bonds such as hydrogen and hydrophobic bonds (Hendrickx and Knorr, 2012) so there is a wide array of potential effects on food systems. Examples of some research on alternative uses of HPP include high pressure shifted freezing and thawing, minimising anti-nutritional factors, and acceleration of cheese ripening process (Ahmed *et al.*, 2009) to name a few.

On the subject of oil extraction, research into the application of HPP is limited. Uhm and Yoon (2011) reported on the higher extraction rate and yield achieved with HPP pre-treatment on solvent oil extraction from soybean. HPP treated samples were observed to contain more pores than untreated ones and this may have been responsible for the increased extraction rate. In the extraction of grapeseed oil using supercritical CO_2, increasing the pressure above 300 MPa decreased the oil yield (Magalhães *et al.*, 2008) but similar to Uhm and Yoon (2011) the speed of extraction was faster. The particle size used for solvent extraction (0.4 mm) was smaller compared to the supercritical CO_2 (1.3 mm) which

may affect the yields as this would impact diffusion of oil out of the oilseeds. In addition, the time for HPP treatment was longer for the soybean study. These two types of oil extraction (solvent and supercritical fluid CO_2) rely on a similar mechanism of oil extraction (diffusion of oil out of the oilseed solid matrix into the bulk fluid) and as such, the comparable effect of increased kinetics is not surprising. Work done by Jung and Mahfuz (2009) on AEP of soybean oil show that oil yield is not enhanced by HPP. The speed of extraction was not studied so no comparison can be made. The effect of HPP would differ not only across different extraction methods but also with different oleaginous materials, especially as their compositions vary. This necessitates research on different materials to understand the role HPP plays.

2.5 Oil Processing By-Products

In a world conscious of minimising waste, different approaches are considered in oil extraction processes. The meals that remain after oil is extracted tend to be energy dense and depending on the crop, are high in protein. For this reason, they are used for animal feed and in some cases fish feed. Common oilseeds that fall under this are soybean and rapeseed or canola meals. Despite their popularity as animal feed, they contain anti-nutrients that can limit protein digestibility (Meng and Slominski, 2005) and in some cases cause liver damage in poultry (Gattinger *et al.*, 1990). In order to increase the variety of the use of oilseed meals, research has been conducted to provide more safe options. Gattinger *et al.* (1990) showed that canola meal can be used as a substrate for the production of xylanase enzyme and it performed better than commercially manufactured cellulose.

Another viable option is the use of oilseed meals as a source of energy for bioethanol production. Currently, farm lands primarily used for food are being converted to produce biofuels and studies have shown that this act intensifies global warming (Searchinger *et al.*, 2008). By harnessing by-products especially those rich in starch and sugars, the demand on

using good cropland for energy crops could decrease. To efficiently utilise oilseed meals, an analysis of their chemical constituents should be mandatory to permit proper appropriation. In some cases, the properties of different components require research to evaluate their quality.

2.6 Conclusion

Cyperus esculentus L. can be both a cultivated and a wild variety. For food purposes, the cultivated variety is used and is thus the focus of this review. Also known as chufa or tiger nut, its oil consists mainly of unsaturated fatty acids, predominantly oleic acid. Also present are some bioactive compounds such as Vitamin E homologues, phytosterols, and polyphenols. The low level of polyunsaturated fatty acid, combined with the presence of Vitamin E and polyphenols, contribute to the stability of the oil. Quality indices parameters such as its acid and peroxide values give an indication of the oil's freshness and these have been found to be low, thus concluding that it has a good keeping capacity.

Existing oil extraction methods include mechanical pressing, solvent extraction and aqueous extraction processes (AEP). The most commonly used is the solvent extraction process but this is plagued with safety concerns and toxic organic residues remaining in the extracted oil. However, this method gives the highest achievable oil extraction recoveries. Mechanical pressing, being the earliest technique, produces high quality oil but it is less efficient alongside with aqueous extraction processes. With all these methods, pre-treatments exist which have been investigated in the attempt to increase oil yields. Enzymatic treatments are one of those and have been used substantially with AEP. In most cases, they do contribute to increasing oil yields, especially with AEP but there can be problems with the stable emulsions that form during the process. There is limited information on enzyme treatment with pressing with only a few cases on hazel nut and soybean oil extraction.

Another potential tool that has yet to be fully examined in depth as a pre-treatment is the use of high pressure processing. Although it is an established means of non-thermal pasteurisation, it has been applied in a few solvent extraction processes where it has increased extraction rates. As a result, it is worthwhile investigating its effects on different types of oil extraction methods.

Notwithstanding the oil extraction process used, making use of all products and by-products is necessary to minimise process waste. The oilseed meal, a by-product is commonly used as animal feed because it can be rich in protein. But, this cannot be said for all oilseeds. There have been suggested alternative uses of oilseed meals including the production of bioethanol and valuable food ingredients such as oligosaccharides. Recommending a suitable use of a by-product would thus require analysis and evaluation of the components of the oilseed meal. In light of this, three hypotheses were established and investigated in the following chapters:

a) The mechanical extraction of tiger nut oil can be enhanced using enzymes and HPP treatments.

b) Aqueous enzymatic extraction of tiger nut oil can be improved by using a combination of enzymes and HPP treatments

c) Enzymes and HPP as pre-treatments do not affect quality of oils extracted using mechanical pressing and aqueous extraction.

Chapter 3

MECHANICAL PRESSING OF TIGER NUT (*CYPERUS ESCULENTUS* L.) OIL

This chapter is based on a paper titled "Enhancing the recovery of tiger nut (*Cyperus esculentus* L.*)* oil by mechanical pressing: moisture content, particle size, high pressure and enzymatic pre-treatment effects" that has been accepted for publication in Food Chemistry (Ezeh *et al.*, 2016).

In this aspect of the study, the first hypothesis was assessed: The mechanical extraction of tiger nut oil can be enhanced using enzymes and HPP treatments. Different factors that may affect mechanical pressing oil yield were looked at as well as the effects of different pre-treatments such as high pressure processing.

Abstract

Tiger nut (*Cyperus esculentus* L.) tuber contains oil that is high in monounsaturated fatty acids, and this oil makes up about 23% of the tuber. The study aimed at evaluating the impact of several factors and enzymatic pre-treatment on the recovery of pressed tiger nut oil. Smaller particles with a moisture content range of 6.9-8% were more favourable for pressing. High pressure pre-treatment did not increase oil recovery but enzymatic treatment did. The highest yield obtained by enzymatic treatment prior to mechanical extraction was 33% on a dry defatted basis, which represents a recovery of 90% of the oil. Tiger nut oil consists mainly of oleic acid; its acid and peroxide values reflect the high stability of the oil.

3.1 Introduction

Currently, tiger nut oil (TNO) is extracted and sold as cold pressed oils. For research purposes, TNO is extracted either using a laboratory press or solvent extraction with n-hexane (Ali Rehab and El Anany, 2012; Yeboah *et al.*, 2012). Despite the higher recovery of oil achieved with solvent extraction (over 95%) as reported by Rosenthal and Niranjan (1996), there remain apprehensions regarding sustainable availability of petroleum based solvents, as well as the contribution of these solvents to the emission of volatile organic compounds. To overcome this problem, other methods of oil extraction have been investigated and re-visited along with pre-treatment effects on the yield of the extracted oil. Examples include employing enzymes and applying high hydrostatic pressure treatment. Enzymes such as cellulose and pectin are used to degrade cellular wall components and this facilitates oil release from the cells. Only a few studies have implemented the use of enzymes prior to mechanical oil extraction despite the potential benefits it may offer. With mechanical presses, there is no occurrence of de-emulsification that arises with aqueous extraction and this eliminates an additional processing step.

The high quality oil obtained is one of the reasons why mechanical presses are continually being used especially as there is an increasing niche market for novel oils. In some rural areas, it also remains the sole method of oil extraction. But it still is an inefficient process. Depending on the equipment used, authors have conducted studies to increase oil recoveries and optimise the process by varying operational variables like temperature, applied pressure and time (Adeeko and Ajibola, 1990; Ajibola *et al.*, 1990). Sample preparation must also be taken into account, as pre-treatments such as the application of extrusion or enzymes are employed (Nelson *et al.*, 1987; Smith *et al.*, 1993). Generally these treatments lead to increases in oil yields because they tend to either soften and/or destroy cellular structure thus aiding the extraction.

There are few research reports on techniques for extraction of TNO or optimising the yield of oil. However a study of the effects of extraction parameters on oil yield when using supercritical CO_2 was recently reported (Lasekan and Abdulkarim, 2012). In terms of pre-treatments, high pressure is not commonly used prior to oil extraction. It is commercially used in pasteurization and food preservation as the high pressures applied inactivate microbes, spores, and spoilage inducing enzymes (Pei-Ling *et al*., 2010).

This study aims to investigate the impact of moisture content in the tubers, particle size, high hydrostatic pressure and enzymatic pre-treatment prior to oil extraction by mechanical pressing on the recovery of tiger nut oil. In particular, the pre-treatments were assessed on their efficacy in reducing barriers to oil release from the tubers by destroying cellular structure of tiger nut cells. Oil quality parameters are reported for the pressed oil (without enzymatic treatment).

3.2 Materials and Methods

3.2.1 Samples

Dried brown tiger nuts (50 kg) from Spain was purchased from Real Foods, Edinburgh UK for use throughout the project. A desired quantity was then drawn when needed for individual experiments (Figure 3.1).

Figure 3.1: Sample of dried tiger nut tubers

3.2.2 Chemicals

Fatty acid methyl ester standards, α-tocopherol standard, gallic acid, methanol, alcohol oxidase (from *Pichia pastoris*), purpald (4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole), phosphate buffer, safranin and sodium hydroxide were purchased from Sigma-Aldrich (Dorset, UK). All chemicals were of analytical grade.

3.2.3 Enzymes

Alpha-amylase (*Bacillus licheniformis*), Alcalase (*Bacillus licheniformis*) and Viscozyme® L (*Aspergillus*) were procured from Sigma-Aldrich (Dorset, UK).

3.2.4 Sample Preparation and Treatments

3.2.4.1 Effect of Moisture Content and Particle Size

The initial moisture content of tiger nuts was measured using an infrared moisture analyser (Sartorius, Surrey UK) and this was given on a wet basis (wb). In order to restore the tubers to a hydrated state, they were soaked in distilled water at room temperature for 6 h. A preliminary study was carried out to obtain a drying curve and used to determine the time taken to achieve an initial moisture content of 4% (wb) for all samples. This was achieved by drying in a conventional oven at 70 ^{o}C for 13 h. At this temperature, the starch present was not affected by the hot air. Samples with the desired moisture content were prepared by adding calculated amounts of distilled water using equation 3.1 (Mwithiga and Sifuna, 2006) and mixed thoroughly. The weighed tubers were placed in polyethylene bags and kept in a refrigerator for a minimum of 48 h to establish uniform moisture content. Prior to oil extraction, they were withdrawn and left for 2 h at room temperature to equilibrate.

$$Q = \frac{w_i(m_f - m_i)}{(100 - m_f)} \qquad \text{(Equation 3.1)}$$

where Q = mass of water to be added in grams, w_i is the initial mass of the sample in grams, m_i and m_f are the initial and desired final moisture contents in percentages (wb), respectively.

Tiger nut flour was produced by grinding prepared tiger nut tubers in a coffee grinder (De'Longhi, Hampshire UK) and sieved to a desired particle size. To study the effect of moisture content and particle size on oil yield, tiger nut flour was sieved through three

ASTM sieves to produce average particle sizes of ≤1.16 mm, ≤0.84 mm and ≤0.5 mm at the different moisture levels (3.9%, 6.9%, 8%, and 12%).

3.2.4.2 High pressure processing

Dried tiger nuts (with moisture content of ca. 11.5 %) were dried further for 1.5 h at 70 °C or until the moisture content was between 6.5-8%. They were then ground using a coffee grinder, and sieved to produce flour with a particle size of ≤0.84 mm. 30 g of the samples were vacuum sealed in polyethylene bags and placed in a pressure vessel (Stanstead Fluid Power, Ltd) to be subjected to pressures of 50, 300, 500 and 700 MPa (15 min holding time, 40 °C). Whole tubers in 0.5 M citric acid were also high pressure treated at 700 MPa (15 min holding time, 40 °C). The tubers were not observed to swell. A preliminary study found there was no interaction between pressure, temperature and time on the oil yield. A mixture of water and 1, 2-propanediol (70:30, *v*/*v*) served as the pressure transmitting fluid. The adiabatic temperature rise during the pressure treatment was 3.3 °C per 100 MPa increase.

3.2.4.3 Enzymatic treatment

A combination of α-Amylase, Alcalase and Viscozyme was used (1:1:1). The starch content led to the use of α-amylase and protease, while Viscozyme was added to aid in softening cell wall structure. Enzymes and their hydrolysis conditions were based on specifications given by suppliers. Whole tiger nut tubers were soaked in distilled water for 6 h, ground and sieved to a particle size of ≤0.425 mm. Varying total weights of enzyme (0.15g, 0.30g, 0.45g) were added to 50 ml of distilled water, 30 g of ground tiger nut sample, and pH was adjusted to 8 using 0.5 M NaOH. Incubation was carried out for 6 h at 40 °C in a water bath with a linear agitation speed of 120 linear strokes per min. After incubation, the mixture was dried in a vacuum oven till the moisture content was between 6.5 - 8%. Temperature in the oven

was 55 °C while the maximum pressure reached was 700 mm Hg. Following drying, oil was extracted by pressing.

3.2.5 Mechanical Pressing of Tiger nut Oil

A metal chamber, depicted in Figure 3.2 was designed for the purpose of housing the ground tiger nut samples during mechanical pressing. The chamber was a cylinder hinged on four clusters of bolts and 6 holes (0.3 cm diameter) at the bottom. The cylinder had these dimensions: an outer diameter of 8.8 cm, inner diameter of 6.6 cm, and height of 7.6 cm. The metal cylinder had a lid and a square metal plate which was used to collect extracted oil.

Tiger nut oil was obtained by double pressing 30 g of ground tiger nuts with a hydraulic laboratory press (Specac, Ltd UK). A maximum pressure of 38 MPa was exerted due to the limitation of the strength of the nylon sieve material used. The samples were placed in the sieve and then in a metal chamber. The total time for pressing was between 40-50 s. Hexane was used to collect the expressed oil and recovered in a rotary evaporator. The amount of oil extracted was measured gravimetrically and stored in an amber glass bottle for analysis.

Hexane extraction was carried out to determine the total extractable oil in tiger nuts and to measure total oil recovery. The total oil content was measured gravimetrically from 10 g of ground tiger nuts extracted with 150 ml hexane for 6 h in a Soxhlet unit. Hexane was recovered in a rotary evaporator. Residual solvent was removed in an oven at 105 °C for 15 min and the residue was cooled in a desiccator. Throughout this chapter, oil recovery is synonymous with oil yield.

Pressing after enzymatic treatment was carried for 30 min. Controls consisted of pressing for 30 min without enzymatic pre-treatment. The time was increased to increase the quantity of oil that can be collected for analysis.

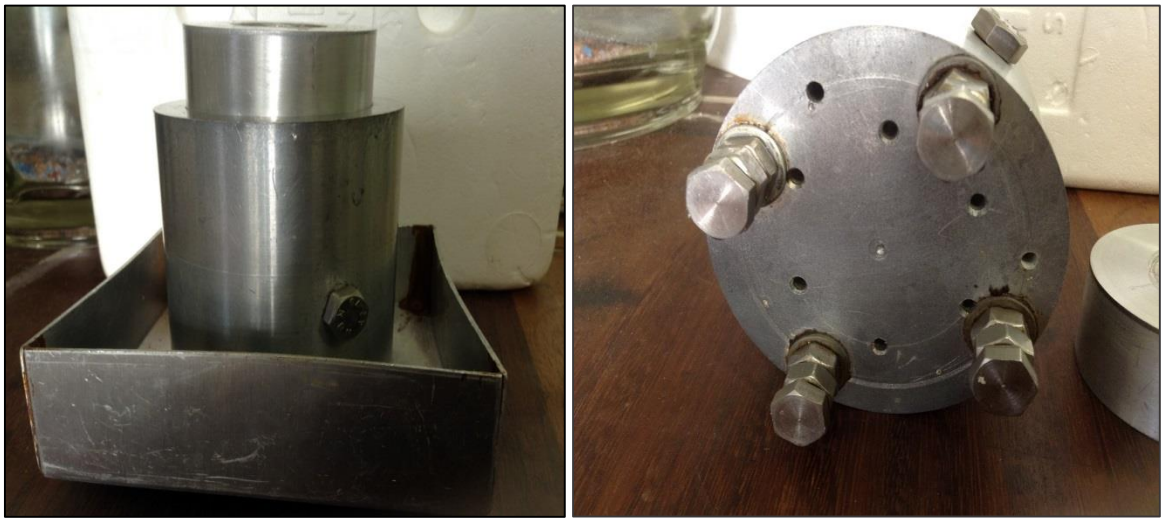


Figure 3.2: Top images shows metal chamber (right image shows four bolt clusters) and bottom picture shows the hydraulic press used for mechanical pressing of tiger nuts

3.2.6 Effect of High Pressure on Ground Tiger nut Cells

3.2.6.1 Methanol content in tissues

A spectrophotometric method was used to determine methanol content (Gonzalez *et al.*, 2010). Pectin methyl esterase activity was assayed by determination of the amount of methanol present in the tissues. Methanol is enzymatically oxidized to formaldehyde with alcohol oxidase and calorimetrically determined with Purpald (4- amino-3-hydrazinio-5-mercapto-1,2, 4 triazole). Ground tiger nut sample (1g) was vortexed with 50% trichloroacetic acid (TCA) and distilled water in the ratio 1:2:1. The mixture was centrifuged and the oxidation was begun by adding 0.25 ml of the vortexed mixture to 0.9 ml of 100 mM phosphate buffer (pH 7.5), 0.75 ml supernatant, 0.5 ml distilled water, and 1 ul alcohol oxidase (27 U/mg protein, 42 mg protein/ml). The samples were incubated in a water bath at 30 ^{o}C for 10 min after which 2 ml of 5 mg/ml Purpald in 0.5 M NaOH was added and the mixture was left for an additional 30 min. At the end of this period, 6 ml of distilled water was added and the absorbance at 550 nm was measured.

3.2.6.2 Confocal light scanning microscopy

The staining method reported by Sineiro, Domínguez, Núñez and Lema (1998) was adopted and modified. Ground tiger nut samples were mounted on glass slides using Evo-Stik rapid set adhesive. Cell walls were stained in Safranin solution for 1 min (10 g safranin in 155 ml 95% ethanol and 145 ml distilled water; this was diluted 1:1 with 50% ethanol). Sections were rinsed afterwards with distilled water and observed under a Leica SP2 Inverted Confocal Microscope (Carl Zeiss) operating in confocal mode. A Leica 10x/0.3 HC PL Fluotar dry lens (Carl Zeiss) was used. An Argon laser (488 nm, 496 nm and 514 nm excitation) provided the incident light and emission bandwidth was set from 525 to 606 nm. The obtained images

were 1024 × 1024 pixels in size and were scanned at various zoom factors to obtain the desired magnifications.

3.2.7 Oil Analysis

Non-treated pressed oil was used in all oil analysis.

3.2.7.1 Fatty acid content

The pressed oil was analysed for fatty acid composition by Gas Chromatography (Agilent HP 6890 fitted with FID). Fatty acid methyl esters were prepared by saponification as described in the International Union of Pure and Applied Chemistry method 2.301 (Dieffenbacher and Pocklington, 1992). The esters were analysed using fused silica capillary column Varian CP-Sil 88 (50 m × 0.25 mm × 0.20 μm). The injector temperature was 250 ^{o}C; detection temperature was 260 ^{o}C and oven temperature was initially 100 ^{o}C, held for 3 min and then ramped to 240 ^{o}C at 4 ^{o}C per min. The carrier gas was hydrogen at a flow rate of 0.8 ml/min. The fatty acids were identified by comparing retention times with those of standards.

3.2.7.2 Acid and peroxide values

Acid value (AV) and peroxide values (PV) were determined according to Cd 3d-63 and Cd 8b-90 AOCS official methods respectively (Firestone, 1998).

3.2.7.3 Tocopherol content

For tocopherol extraction and analysis, the procedure described by Costa *et al.* (2010) was followed. Analysis was performed with a high performance liquid chromatography with photodiode array detection (HPLC-DAD) system (Agilent 1200, Manchester, UK) using a Nucleosil C-18 reverse phase column (25 cm × 4.6 mm i.d.) with a particle size of 5 μm (Macherey-Nagel, Duren, Germany). Dilute concentrations of α–tocopherol standard were

prepared by dissolving in methanol. Tocopherol was identified by comparing the retention times with those of the standards and comparing the UV absorption spectra obtained. An external calibration was used for quantification.

3.2.7.4 Total phenolic compound analysis

The extraction of phenols was carried out using liquid-liquid extraction with methanol as solvent. The procedure reported by Baiano *et al.* (2009) was followed. 2 ml of methanol/water (70:30, v/v) and 2 ml of hexane were added to 5 g of tiger nut oil and vortexed for 10 min. The organic phase and the aqueous phase were separated by centrifugation (2400 × g, 4 ^{o}C, 10 min). The aqueous phase containing the phenolics was collected and centrifugation was repeated (11 300 × g, room temperature, 4 min). Finally, the aqueous phase was collected with a pipette for analysis. Total phenolic content was quantified using a spectrophotometric method (Stanković, 2011). The hydroalcoholic extract or blank methanol sample (0.5 ml) was mixed with 10% Folin-Ciocalteu reagent (2.5 ml) dissolved in water and 7.5% Na_2CO_3 (2.5 ml). The mixtures were incubated at 45 ^{o}C for 45 min and the absorbance was measured using a spectrophotometer at 765 nm. A standard curve was prepared using standard diluted solutions of gallic acid in methanol. Total phenolic content is expressed as milligrams of gallic acid equivalents per kg of oil.

3.2.8 Statistical Analysis

All analysis was done in triplicate and the mean values are presented. Statistical analysis was carried out by ANOVA using SPSS Version 20 Statistical software (SPSS Inc., Chicago, USA). Significance was defined at $p<0.05$.

3.3 Results and Discussion

3.3.1 Effects of Moisture Content and Particle Size

The total extractable oil in the tiger nut tuber was 23.1% (w/w) or 35.5% on a dry defatted basis (d.d.b) taking into account the initial moisture content of the samples. Thus it is a low oil bearing material, similar to soybean (18-20%) (Nelson *et al.*, 1987). The oil content falls within the range of 22.8-32.8% reported in literature (Sánchez-Zapata *et al.*, 2012) .

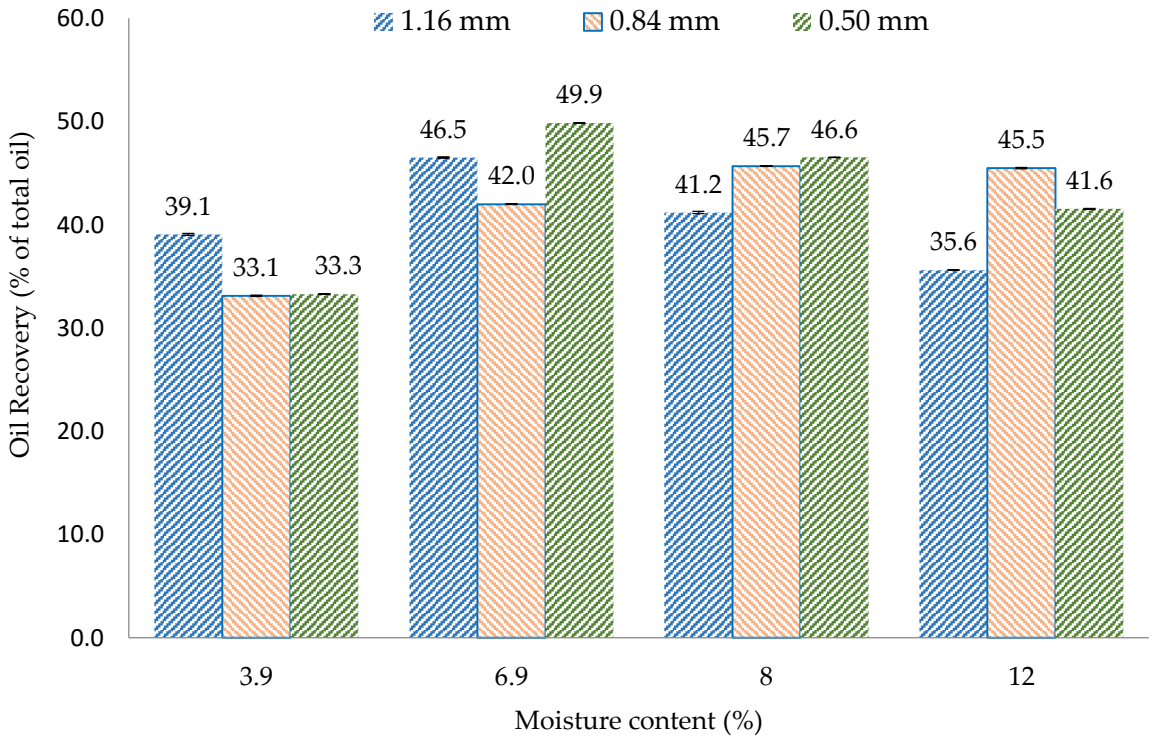


Figure 3.3: Mean oil recovery from pressed (maximum pressure of 38 MPa) tiger nut flour at different moisture contents and particle sizes. N= 3.

Smaller particle size tended to increase the oil recovery (Figure 3.3). By grinding the tubers further to create smaller particles, cell rupture increases. The pressing operation has been described as being analogous to a capillary filtration process, and the Hagen-Poiseuille equation below expresses this (Sorin-Stefan *et al.*, 2013).

$$V\ (m3) = \frac{\pi.p.d.t}{128.\eta.l} \qquad \text{(Equation 3.2)}$$

where V (m^3) - volume of separated liquid (passing through capillaries); p (N/m^2) – apparent pressure; d (m) – diameter of capillary channel; η (Pa s) – dynamic viscosity of liquid; l (m) – length of capillary channel; t (s) – time of applied pressure.

From equation 3.2, the volume of oil that gets released is proportional to the pore diameter and inversely proportional to the length of the capillary channel. The pore diameter and capillary channel length can be increased and decreased respectively with greater cellular destruction. The shorter channel length shortens the distance required by oil bodies to travel in order to get released. With larger pore diameters, more oil bodies are then extracted. This may explain the higher yields obtained with smaller particles.

These results were in agreement with those obtained for ground melon seeds (Ajibola *et al.*, 1990) and in contrast to results obtained for peanuts. Finely ground melon seed particles (1.10 mm) gave higher oil yields than coarsely ground particles (1.85 mm). In contrast, peanut oil yield was increased when the particle size was increased (Adeeko and Ajibola, 1990). Particle size is known to play a role in oil extraction processes such as solvent and aqueous extraction (Rosenthal *et al.*, 1998). In these techniques the smaller the particle size, the higher the oil recovery because of cell fracture and an increase in surface area allowing for more contact between the solvents and the oleaginous material.

Moisture content was found to significantly affect oil yields. The yields increased when moisture increased from 3.9 to 6.9% where maximum values were attained (using ≤0.5 mm flour). A further increase in moisture did not affect the yields significantly but at 12% moisture content, the oil yields experienced a significant decline. Different oilseeds exhibit different behaviour with varying moisture levels. It was reported for walnuts and peanuts, that an increase in moisture content from 2.4% to 7% increased oil extraction yield from 61%

to 84% while in some materials like sesame, no optimum moisture content exists. Although these extraction processes were carried out in a continuous process, similar observations were noted for hydraulic presses (Savoire *et al.*, 2013). It appears that tiger nuts behave in a similar manner to walnuts and peanuts as observed in Figure 3.3. In the case of tiger nuts, subsequent pressing experiments were carried within the favourable moisture level range of 6.9-8% and particle size of 0.5 mm. Other factors that influence oil yield include temperature and pressure. In this study, existing constraints prevented a manipulation of these factors. Pressure had to be limited due to the strength of the sieving material used and temperature due to the lack of temperature control of the hydraulic press.

3.3.2 Effect of High Pressure Processing and Methanol Content in Tissues

Samples pre-treated with high pressure showed no significant increase in oil recovery regardless of the pressure employed (Figure 3.4).

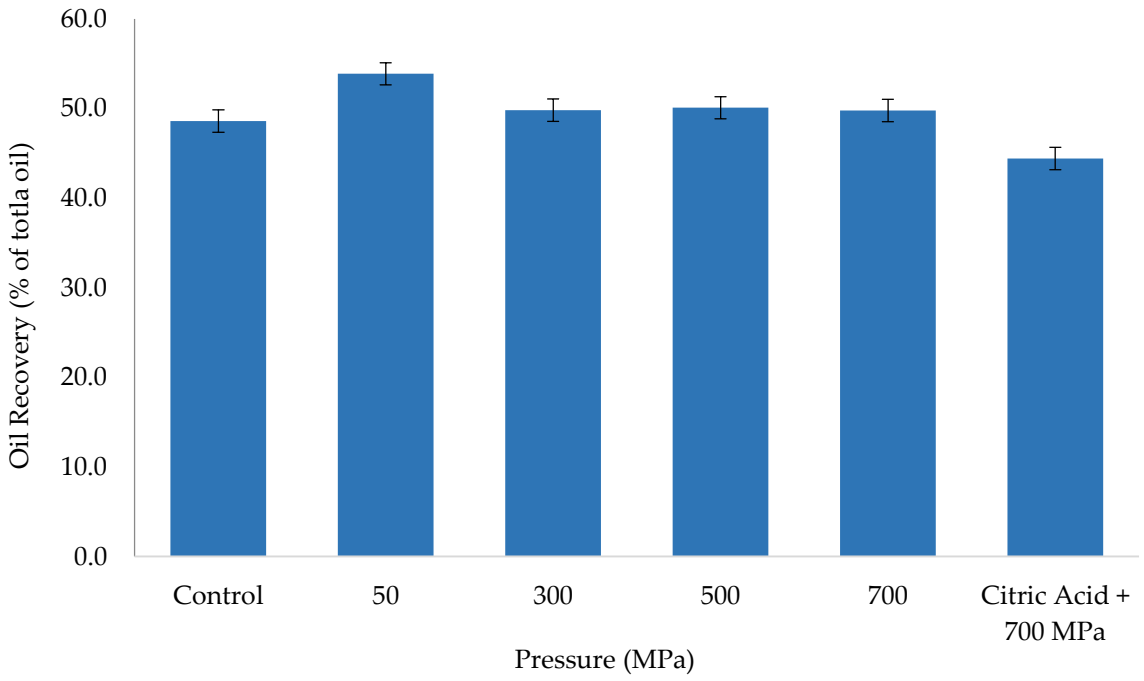
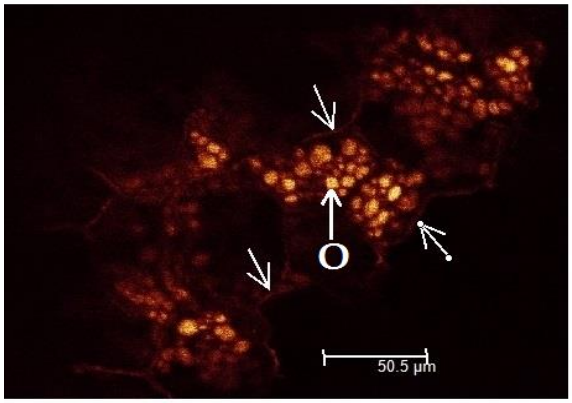


Figure 3.4: Effect of high pressure on mean oil recovery (% of total oil) from pressed (maximum pressure of 38 MPa) tiger nut flour (≤0.84 mm) (HPP at 40 °C, and 15 min)

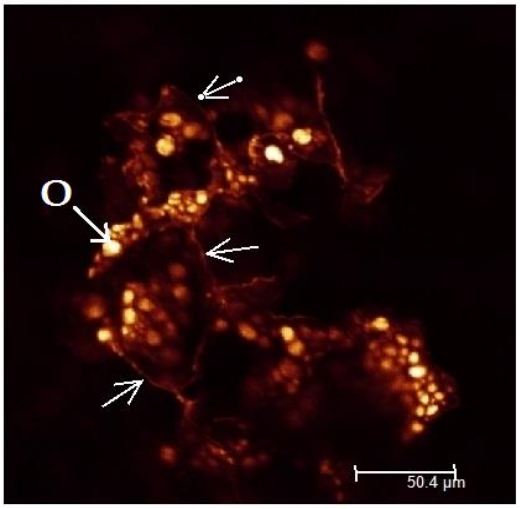
The high pressures (50-700 MPa) did not cause any further destruction of the parenchyma cells which had already suffered some disintegration due to the grinding process. Confocal images of control and high pressure treated samples (300-700 MPa) revealed similar cellular damage in all samples (Figure 3.5), thus supporting the hypothesis that HPP did not induce any additional functional damage to cell walls. Safranin was used to stain the cell walls and is also known to stain lipids which explain the multiple drops in the images. Focusing on different regions of the cells showed areas with intact cell walls and some cell damage. Two microscope slides per treated sample were viewed under the confocal laser microscope.

According to Jung *et al.* (2009), application of high pressures (200 MPa and 500 MPa) did not result in any significant increase in oil yield following both aqueous and enzyme assisted aqueous extraction of soybean oil. It was suggested that high pressure treatment did not promote any cell rupture in cotyledon cells of the soybeans. This observation can also be used to explain the lack of an effect on tiger nut tubers. Tiger nut tubers have a tough texture, even tougher than potatoes and this characteristic was attributed to the cross linking of diferulic acid with arabinoxylans in the parenchyma cells of the tubers (Parker *et al.*, 2000). High pressure alone is not sufficient to induce cell separation as it is only able to break weak non covalent bonds (Jung *et al.*, 2009).

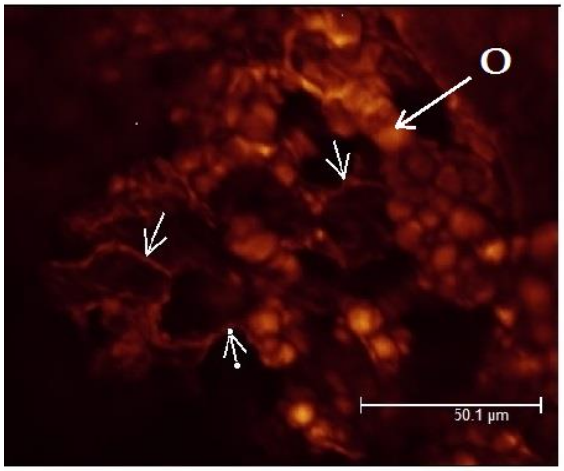
An alternative role HPP could play in the destruction of tiger nut cells is by maintaining cell adhesion in order to promote promotes cell fracture when forces are applied. HPP is known to activate pectin methyl esterase (PME) activity often present in plant cells. PME demethylates pectin molecules, releasing pectin with free carboxyl groups and methanol. Pectin precipitates in the presence of calcium forming strong bond linkages that preserves plant tissues when HPP is applied. This occurs within the middle lamella between cells where pectin is found. What this means is that cell adhesion is strengthened.



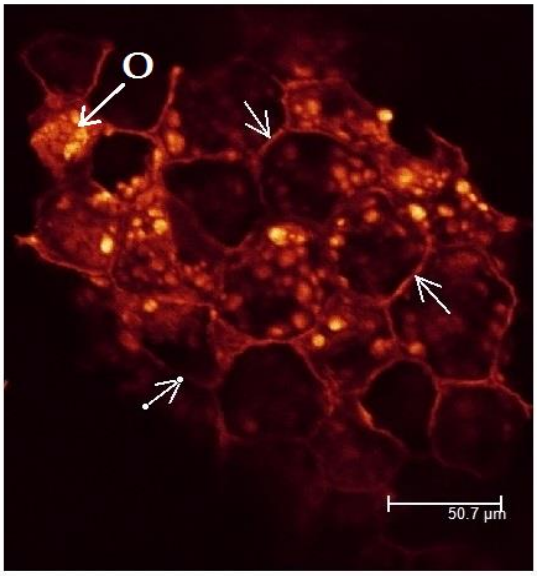
A: Control



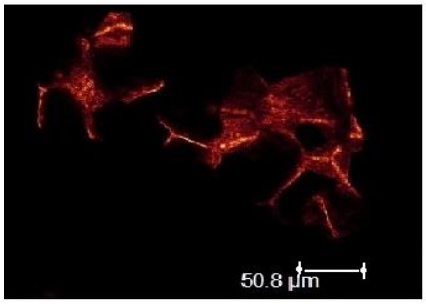
B: 300 MPa



C: 500 MPa



D: 700 MPa



E: Enzyme treated

Figure 3.5: Confocal images showing cell walls of pressed (maximum pressure of 38 MPa) tiger nut flour stained with safranin. (A) Control sample; (B)-(D) High pressure samples (15 min, 40 °C). Samples A-D show intact cells walls and presence of oil bodies; (E) Enzyme treated sample showing damaged and empty cells. Hydrolysis conditions: 1% E/S of Alcalase, α-amylase and Viscozyme mixture (1:1:1), 6 h, 40 °C, 1:4 S/L ratio. White arrows indicate cells walls. O, oil bodies.

Studies have shown HPP as an alternative to thermal treatment to maintain membrane integrity of tissues (De Roeck et al., 2008; Gonzalez et al., 2010) as a result of the effect of PME activity on cell adhesion. Following this, cell fracture can be promoted by strengthening cell adhesion. To investigate the role of PME in the strengthening of cell walls of tiger nuts, the tubers were high pressure processed in the presence of an acid with the aim of combatting the effects of PME by inhibiting the formation of pectin-calcium linkage. If PME played a role in cell rupture, the addition of an acid have the opposite effect and decrease oil yields from pressing. Results shown in Figure 3.4 show that citric acid had no significant effect on the oil yields from pressing. PME activity was confirmed to be present in tiger nut tissues by measuring its methanol content using a colorimetric method (Figure 3.6).

The action of grinding already plays a role in some cellular destruction, which may have led to PME release from within the cells, which may explain the observed similar measured absorbance in control and most pressure treated samples. The reduced absorbance at 700 MPa suggests a decrease in PME activity due to the high pressure. Enzymes tend to be resilient at high pressures and PME is no exception. The similarity of the effect of HPP with and without citric acid on oil yields illustrates that PME does not contribute to cellular destruction of the cells.

Parker *et al.* (2000) attributed the toughness of cell walls of tiger nut tubers to phenolic acids, particularly diferulic acid. The toughness was recognised to be greater than that of both raw potatoes and Chinese water chestnut. These acids were suspected to form stable bonds between polysaccharides in the cell wall. This tough property of the tubers appears to withstand attempts to destroy the cellular structure by HPP. Also contributing to the challenge of increasing the release of oil from the cells is the presence of starch. In tiger nuts, potential restriction of oil flow would be due to the presence of starch and the cell wall. The incompressibility of starch thus presents an additional barrier to oil release.

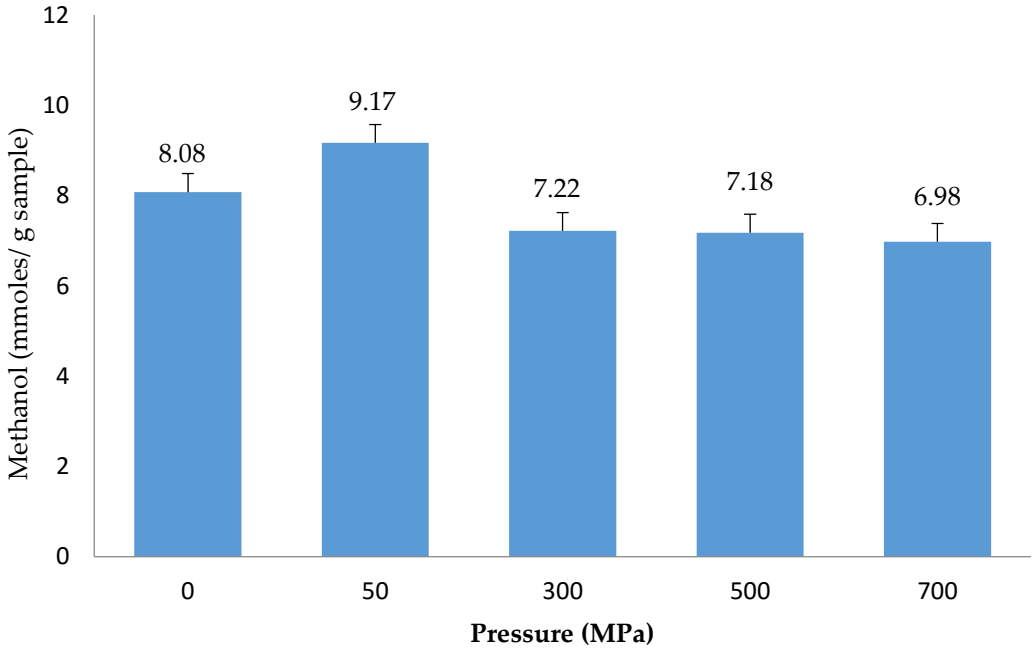


Figure 3.6: Methanol content in high pressure processed (15 min, 40 °C) pressed tiger nut flour. Methanol levels indicate pectin methyl esterase activity in tissues.

3.3.3 Effect of Enzymatic Pre-treatment

Another treatment employed in order to achieve cell destruction was the use of enzymes as they have been previously reported to be beneficial for this purpose. The degrading action of enzymes did significantly increase the oil yield from pressing. Tiger nut is known to have a relatively high starch content of about 25%. Cellulose also makes up a large fraction of its crude fiber. The occurrence of these cell components in tiger nuts would explain why α-amylase, protease and cellulolytic enzyme mixture enhanced oil recovery. A confocal image with Safranin stained cell walls confirmed greater cellular damage as a result of the enzymatic treatment (Figure 3.5). An enzyme concentration of 1% (*w*/*w*) was found to achieve the highest oil recovery of 90% as observed in Figure 3.7. Products from the degraded materials may prevent enzymes from reaching their substrates and any additional enzyme was not beneficial.

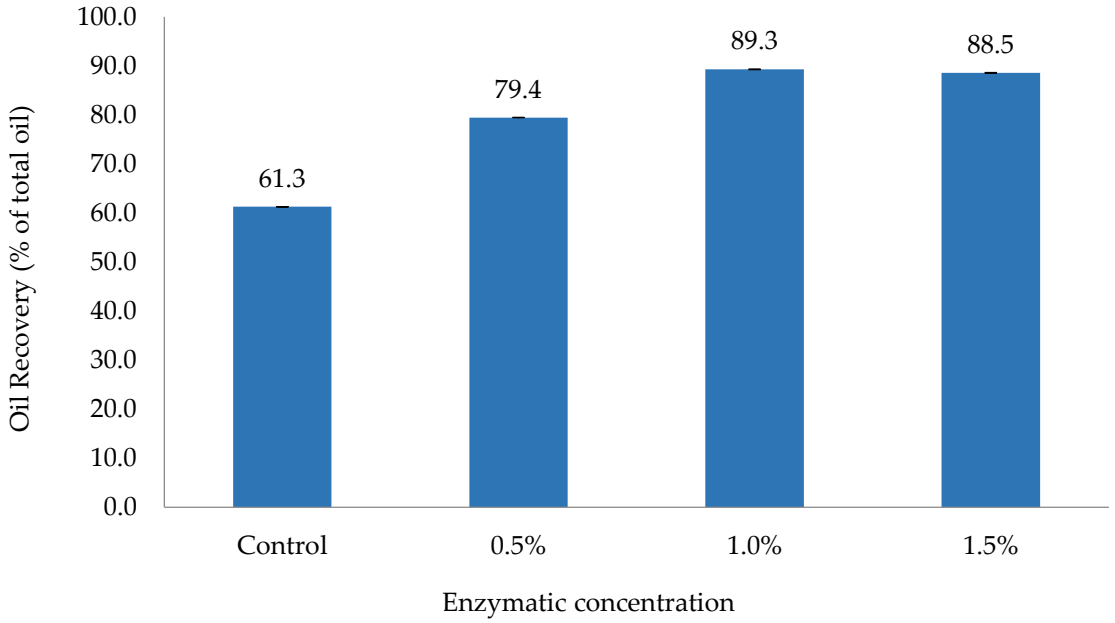


Figure 3.7: Effect of enzyme treatment on mean oil recovery (as a percentage of total oil) from pressed tiger nut flour. N=3. Hydrolysis conditions: 1% E/S of Alcalase, α-amylase and Viscozyme mixture (1:1:1), 6 h, 40 °C, 1:4 S/L ratio.

The recovered oil percentage was much higher than some reported recoveries obtained from other materials via pressing such as values for rosehip (74%) or soybeans (64%), (Smith *et al.*, 1993; Concha *et al.*, 2004). The amount of water used in these studies was not given and a fair comparison cannot be made on the impact of moisture on the hydrolysis. In other studies where higher oil recoveries up to 98% were obtained, thermal treatments as well as longer pressing times may have further improved the oil extraction. In addition, increasing pressing time from 40-50 s to 30 min contributed to an 11% increase in oil yield without any enzymatic pre-treatment.

3.3.4 Fatty Acid Composition

The most abundant saturated fatty acids in pressed tiger nut oil are palmitic (13.5%) and stearic acid (6.3%) while the major unsaturated fatty acid is oleic acid (67.4%). The fatty acid composition is given in Table 3.1.

Table 3.1: Fatty Acid Composition of Pressed Tiger nut Oil

Fatty Acid	Tiger nut oil %
Myristic	0.10 ± 0.00
Palmitic	13.5 ± 0.00
Palmitoleic	0.3 ± 0.00
Stearic	6.3 ± 0.03
Oleic	67.4 ± 0.07
Linoleic	10.7 ± 0.05
Alpha-linolenic	0.1 ± 0.00
Arachidic	0.7 ± 0.01
Eicosenoic	0.1± 0.00
Lignoceric	0.2 ± 0.01
Unknown	0.4 ± 0.02

Traces of myristic, linolenic, and arachidic acids were also detected. The concentration of oleic acid is in agreement with previous studies and similar to that of olive oil (Linssen *et al.*, 1988). Eteshola & Oraedu, (1996) found a rather high proportion of myristic acid (28.1%) in tiger nut oil with a much lower oleic acid content (44.8%). This discrepancy in values may be due to a difference in the origin of the tiger nut tubers, genetic history, the age of the tissue analysed and temperature and oxygen tension, since these variables can alter the lipid content of oilseeds (Eteshola and Oraedu, 1996). Aside from this difference, the composition of fatty acids is similar to those reported in a number of studies and similar to the fatty acid profiles of olive, hazelnut, macadamia and avocado oil (Sánchez-Zapata *et al.*, 2012). As fatty acid composition is a determinant of the quality of edible oils, the high concentration of monounsaturated fatty acids (MUFA) makes it desirable due to its good shelf life and potential health benefits. The carbon double bonds in fatty acids are prone to oxidation, producing aldehydes, ketones and hydrocarbons that cause odours and flavours linked with rancidity. Hence, oxidative stability increases with decreased levels of unsaturated fatty

acids, most especially PUFA (Moore and Knauft, 1989). This has been observed for olive oil and the lower PUFA content in tiger nut oil gives it the same advantage. MUFAs are much more stable and less prone to peroxidation due to their chemical structure compared to PUFAs. The above mentioned health benefits of olive oil are at least partly due to the MUFA content. Tiger nut oil can be substituted for olive oil in areas where the tuber is grown locally.

3.3.5 Quality Indices

The acid and peroxide values of TNO were found to be 1.2 mg KOH/g and 2.1 mEq/ kg respectively (Table 3.2). Acid value quantifies the concentration of free fatty acids and is an important indicator of oil quality. The low acid value obtained indicates the low level of free fatty acid in pressed tiger nut oil and thus reflects its high quality. Ali Rehab & El Anany, (2012) reported an even lower acid value of 0.31 in pressed tiger nut oil. Free fatty acids in oil occur as a result of hydrolysis which requires moisture to develop but the non-enzymatic reaction only occurs at high temperatures. Lipase in the tubers may have increased the hydrolysis reaction leading to the release of free fatty acid during grinding and extraction.

Table 3.2: Quality indices, tocopherol and total phenols in pressed tiger nut oil

AV (mg KOH/ g)	PV (mEq/ kg)	Total Phenols (mg GAE/ kg oil)	α-tocopherol(μg/g)
1.2 ± 0.00	2.1 ± 0.02	17.9 ± 0.04	145.7 ± 2.34

The peroxide value of tiger nut oil is lower than the value determined by Yeboah *et al.* (2012) of 5.54 mEq/kg which was deemed reasonable as it was in accordance with Codex recommended values for virgin olive oil of <20 mEq/kg. Peroxide value measures the concentration of hydroperoxides, which are the intermediate products during oxidation in oil and so is used to detect the early stages of oxidative rancidity. It gives an indication of the

development of oxidative rancidity in oils. The low value of 2.1 mEq/kg found in this study shows that oxidation had not progressed to a significant extent in this sample of tiger nut oil.

3.3.6 Total Phenolic Content

Tiger nut oil polyphenol content was 17.9 mg GAE per kg oil. This is lower than the value found by Ali Rehab and El Anany (2012). Pellegrini *et al.* (2001) reported on the polyphenol content in refined, virgin and extra virgin oils as 0.4, 1.4-2.4 and 7.3-26.5 mg GAE/ 100 g oil respectively. Soybean, sunflower and corn oils have been found to contain 6-8, 0.3-0.4 and less than 0.1 mg/ 100 g oil respectively (Valavanidis *et al.*, 2004). Compared with these values, tiger nut oil (1.8 mg/100 g oil) has similar polyphenol content to virgin olive oil and much lower than soybean and extra virgin olive oil. The phenolic content of oils is important in assessing its antioxidant activity. These bioactive compounds play a protective role in the degradation of tocopherols during cooking processes and storage (Marfil *et al.*, 2011). Polyphenol content and oxidative stability have been found to have a linear correlation with each other in virgin olive oil during storage at 60 ^{o}C and polyphenol content was proposed as an indicator of olive oil quality (Gutfinger, 1981). On the basis of the high MUFA content and the moderate polyphenol content, one can expect the oxidative stability of tiger nut oil to be comparable to that of olive oil.

3.3.7 Tocopherol Content

The quantification of α-tocopherol is given in Table 3.2. It shows that tiger nut oil contained 145.7 μg/g. β-tocopherol was not quantified but was identified to be present in the oil. The total tocopherol content of tiger nut oil is thus expected to be higher than 145.7 μg/g. Yeboah *et al.* (2012) reported an α-tocopherol content of 86.7 μg/g in solvent extracted tiger nut oil. Tocopherol content is affected by mode of oil extraction. Organic solvents are able to penetrate the cells of the oil-containing plant material dissolving more non-polar compounds. For a crude oil, the tocopherol value obtained is higher than some olive oil

values of 100-250 mg/kg, but there is a high variability in the amount of tocopherols reported (Boskou, 2008). The high tocopherol content also contributes to the stability of the oil as tocopherols acts as antioxidants. α -Tocopherol is more stable than β–tocopherol. A good correlation between tocopherol and PUFA content has also been described, suggesting that tocopherols are important in protecting the PUFAs against oxidation (Quiles *et al.*, 2002).

3.4 Conclusion

Small particle sizes were found to give higher oil recoveries. High pressure did not improve the extractability of oil and this may be due to the presence of diferulic bonds present in the cell walls. Enzyme pre-treatment on the other hand allowed for a 90% oil recovery. The triacylglycerol profile of tiger nut oil predominantly consists of oleic acid and 78.7% of the oil is unsaturated fatty acid. TNO can thus be used as a source of these beneficial fatty acids. The acid and peroxide values indicate its high stability and these were confirmed by the high polyphenol and tocopherol content. Polyphenols and tocopherols both have antioxidant capabilities, protecting oil from oxidative rancidity and prolonging its shelf life. Higher temperature and pressure were proposed to further increase the oil extracted from tiger nuts.

Chapter 4

AQUEOUS ENZYMATIC EXTRACTION OF TIGER NUT (*CYPERUS ESCULENTUS* L.) OIL

This chapter is based on a paper titled "Aqueous enzymatic extraction of tiger nut (*Cyperus esculentus*) oil and the application of high pressure processing as a pre-treatment" that is currently in submission.

In this section, the second hypothesis - aqueous enzymatic extraction of tiger nut oil can be improved by using a combination of enzymes and HPP treatments was tested. Aqueous enzymatic extraction was another tool employed to extract tiger nut oil. Factors affecting this process were studied and the use of high pressure processing as a pre-treatment was also investigated.

Abstract

The extraction of edible oil from tiger nut (*Cyperus esculentus* L.) by an aqueous enzymatic procedure was investigated. Tiger nut contains approximately 25.1% and 27.3% starch and oil respectively on a dry basis. Out of four enzymes examined, Alcalase and α-amylase were most effective in enhancing oil yields based on the difference between initial oil content and residual meal oil content. An enzyme mixture of Alcalase, α-amylase, and Celluclast was used to increase oil yields. Agitation during incubation led to gravity separation, decreasing oil yields, and a static incubation unexpectedly gave the highest oil yield. A hydrolysis time of 6 h and solid/liquid ratio of 1:6 also gave higher oil yields. When high pressure processing was used as a pre-treatment prior to the extraction process, the lowest pressure of 300 MPa employed in this study increased oil yields by 24.1% in relation to a similar process without pressure pre-treatment. Higher pressures exceeding 300 MPa are likely to encourage lipid-amylose complex formation but this requires a more sensitive analytical method for confirmation.

4.1 Introduction

An environmentally friendly alternative to solvent extraction is the aqueous extraction process in which water is used as the oil extraction medium. Aqueous extraction gives relatively poor oil recoveries but offers a safer process with higher quality oil as compared to solvent extraction. Researchers have sought ways to improve upon this process by studying pre-treatments such as extrusion and enzymatic treatment (Lamsal and Johnson, 2007). Hydrolytic enzymes such as cellulose are able to enhance oil extraction yields as they degrade the structure of cell walls which may inhibit the release of oil. Some materials that have been studied using aqueous enzymatic extraction (AEE) include soybeans, horse radish seeds (*Moringa oleifera*) and corn germ (Mat Yusoff et al., 2015).

In AEE, the enzyme chosen is of utmost importance. The cell structure of the oilseed needs to be investigated to ensure the right enzyme or combination of enzymes is used. Other factors affecting oil yields include particle size, enzyme concentration, agitation speed, pH and temperature (Mai *et al.*, 2013). Depending on composition of the oilseed, these factors may affect the oil extraction process in different ways, and so it is important before carrying out an optimisation study, to explore firstly how these factors work independently. In addition to the already mentioned factors, other treatments exist which can be used alongside AEE, high pressure processing being one. Although its conventional use in industry is for food preservation, its capacity to destabilise cell membranes and strengthen cell walls thereby increasing enhancing cell rupture could allow scientists to extend its applications and investigate its efficacy in enhancing oil extraction processes.

Tiger nut tuber contains both starch and oil that tend to be present at similar concentrations, making it an unusual storage system.

This study aims to apply the principles of aqueous enzymatic extraction to the extraction of tiger nut oil and evaluate how operational factors influence the oil yield. High pressure processing was also employed as a pre-treatment tool and its effect on oil yield was investigated.

4.2 Materials and Methods

4.2.1 Samples

Samples described in 3.2.1 were used for extraction of oil by aqueous enzymatic process.

4.2.2 Starch and Protein Determination

The starch content was determined using a gravimetric method after extraction with sodium metabisulfite (Manek *et al.*, 2012). Tiger nut tubers were grinded using a coffee grinder and defatted using hexane in a Soxhlet apparatus. The defatted powder was soaked overnight in sodium metabisulphite (0.075%, *w/v*) solution. The soaked powder was milled finely using a hand blender and sifted through a muslin cloth. The suspension was allowed to settle and supernatant decanted. The obtained starch was washed with distilled water and dried in a conventional oven at 50 ^{o}C for 24 h. It was then weighed and the result expressed on a dry weight basis.

Protein content was determined using a modified version of the Kjedahl method AOAC method 955.04 (AOAC, 1995). Ground tiger nut samples were weighed out into a digestion tube with 8 g of catalyst added. Concentrated sulfuric acid (25 ml) was added and heated for approximately 45 min till the solution was clear. It was removed and left to cool before distillation. Distillation was carried out using 50 ml water and 125 ml NaOH in a distillation unit. A conical flask containing 50 ml 2% boric acid and a few drops of screened methyl red was placed in the distillation unit to receive the condensate. The collected mixture in the conical flask was titrated against 0.05M sulfuric acid.

Percentage nitrogen was calculated using the following formula

$$\%N = N\ H_2SO_4 \times \left[\frac{ml\ Titrant\ volume(sample) - ml\ titrant\ volume\ (blank)}{Sample\ weight(g)}\right] \times 14\ g\ \frac{N}{mol} \times 100$$

(Equation 4.1)

where 14 is the molecular weight of nitrogen. The percentage of nitrogen was converted to total protein using 6.25 as a conversion factor for tubers.

4.2.3 Enzymes

All enzymes used are given in Table 4.1 with their corresponding recommended conditions supplied by manufacturers.

Table 4.1: Enzymes, activity and hydrolysis condition

Enzyme	Main activity	Recommended pH range	Recommended temperature range (°C)	pH and temperature used in study.
α-Amylase (*Bacillus licheniformis*)	Endo-amylase	7 – 9	40 - 60	8, 40°C
Celluclast ® 1.5 L (*Trichoderma reesei*)	Cellulase	4.5 – 6.0	50 - 60	5, 50°C
Viscozyme® L (*Aspergillus*)	Mixture of carbohydrases including xylanase, arabanase, cellulase, β-glucanase.	3.3 – 5.5	40 - 50	4, 40°C
Alcalase (*Bacillus licheniformis*)	Endo-protease	7.5 – 9.0	45 - 60	8, 50°C
Amyloglucosidase (*Aspergillus niger*)	Exo-amylase	4.5 – 5	50 - 60	4, 55 °C

4.2.4 Aqueous Enzymatic Procedure for Oil Extraction

A schematic representing the sequence followed for aqueous enzymatic extraction is given in Figure 4.1. Samples used for aqueous enzymatic extraction of oil were ground tubers, sieved to a desired particle size. The samples were first weighed into 200 ml Duran bottles followed by the addition of enzymes and distilled water. The bottle was shaken to till thoroughly mixed before pH was adjusted using either 0.5 M NaOH or 0.5 M HCl. Incubation was carried out in a shaking water bath (Grant OLS, Cambridge, UK), and the sample was allowed to cool, mixed thoroughly and then centrifuged at 2300 × g for 20 min. The resulting mixture was seen to be in 4 layers; a solid residue at the bottom, an aqueous skim layer, a creamy emulsion layer and a clear oil layer at the top.

At least 2-3 centrifuge tubes were used per bottle and so top layers from the tubes were decanted into another clean tube and centrifuged for 20 min at 2300 × g to separate the emulsion/free oil layer. The solid residue was collected and dried at 90 °C for 12 h followed by oil extraction using Soxhlet extractor. Oil extraction yields were expressed as a percentage of the initial oil content of the sample used.

$$Oil\ extraction\ yield\ (\%) = \frac{Initial\ oil\ in\ sample(g) - residual\ oil\ in\ meal\ (g)}{Initial\ oil\ in\ sample\ (g)} \times 100$$

(Equation 4.2)

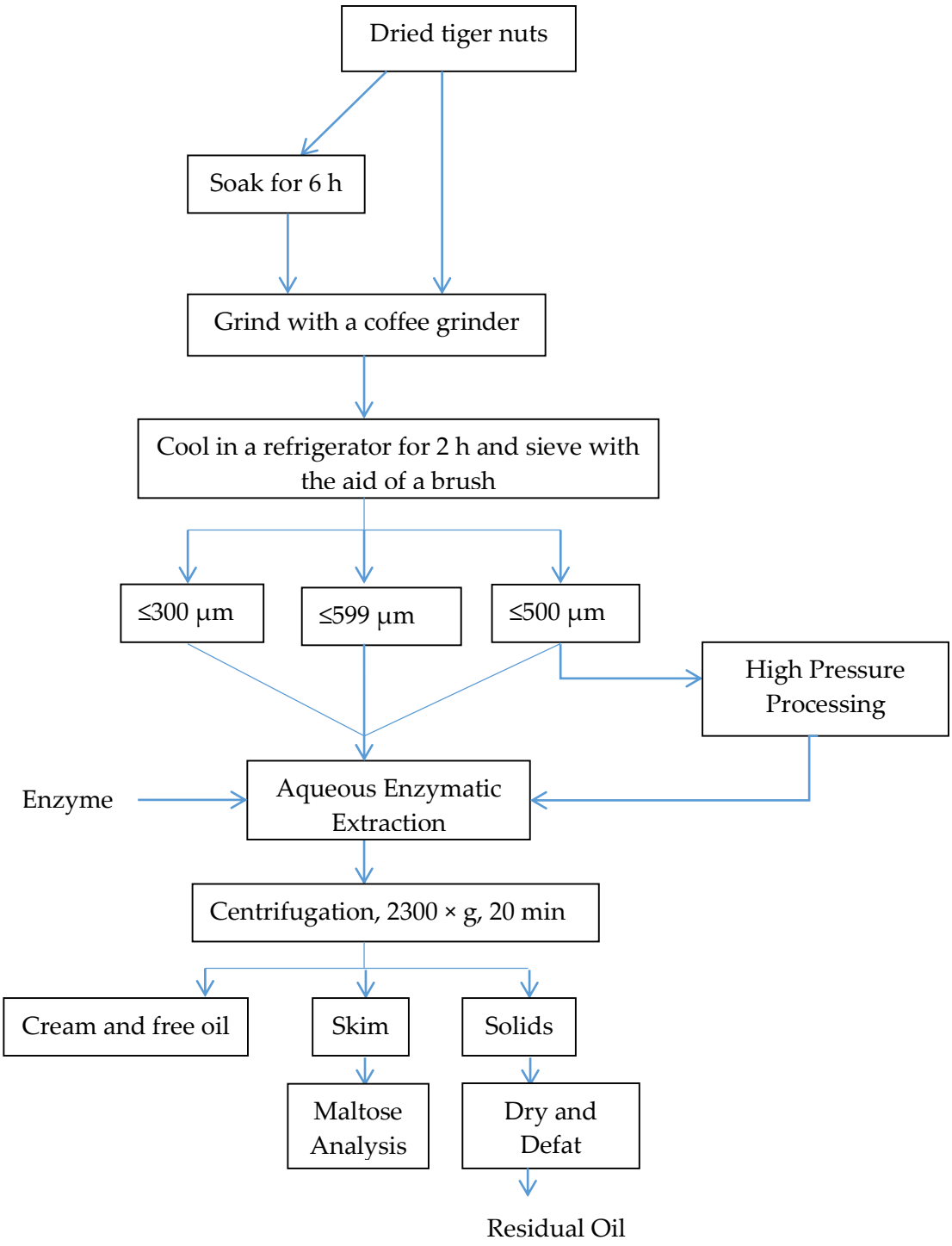


Figure 4.1: Sequence for aqueous enzymatic extraction of tiger nut oil

4.2.5 Influence of Soaking on Oil Yield

Tiger nut tubers were soaked in distilled water for 6 h at room temperature. After draining off the water, the surfaces of samples were dried with paper towels before grinding. Grinding produced a moist powder (not a paste) and this was allowed to cool for 2 h to aid sieving. A brush was used to unclog sieve during sieving. The powder was sieved to a particle size of ≤599 μm before incubation. Linear agitation speed was maintained at 120 linear strokes/min, and enzyme concentration was 0.5% (*w*/*w*) of the dry solid weight. Incubation was carried out for 6 h. Control experiments were conducted on soaked samples without the addition of enzymes. All subsequent analysis utilised soaked tubers.

4.2.6 Effect of Particle Size

Whole tiger nut tubers were soaked for 6 h, drained, dried and ground. After this, they were sieved to three different particle sizes; ≤300 μm, ≤500 μm and ≤599 μm. Aqueous enzymatic extraction of oil proceeded as described above. Only Alcalase and α-amylase were used in this part of the study because they gave the highest oil yields in previous studies.

4.2.7 Mixture of Enzymes Treatment

A combination of α-amylase, Alcalase, and Celluclast was investigated for maximum oil extraction yields. In order to determine pH and temperature values and what ratio of enzymes to use, a 3^3 full factorial design was used. For exploratory purposes, only one replicate was run and so the experimental design consisted of 27 runs. The main effects of three factors: enzyme ratio (α-amylase: Alcalase: Celluclast) (X_1), pH (X_2) and temperature (X_3) were assessed. The real levels of the independent variables are presented in Table 4.2. The extraction was carried out with tiger nut flour sieved to a particle size of ≤300 μm to

achieve as much cellular destruction as possible. The design was created and data analysed using Minitab 17 Statistical software (Minitab Ltd. Coventry, UK). The experimental design and data are shown in Table 1-C in Appendix 1.

After selecting values for pH, temperature and enzyme ratio, different factors displayed in Table 4.3 were investigated for their effects on oil yield.

For selected samples, the aqueous skim layer and free oil were frozen for maltose analysis and quantification respectively.

Table 4.2: Independent variables and their levels for the selection of hydrolysis conditions

Independent Variables		Variable levels		
		-1	0	1
Ratio	X_1	1:1:1	1:1:2	1:2:2
pH	X_2	4	5	8
Temperature (°C)	X_3	40	45	50

Table 4.3: Process variables and values investigated for AEE of Tiger nut oil

Process parameter	Parameter Values
Time (h)	2, 4, 6
Linear agitation speed (strokes per min)	0, 120, 160, 200, 240
Enzyme concentration (%, *w*/*w*)	0.5, 1.0, 1.5, 2.0, 2.5
Solid-Liquid Ratio (S/L)	1:4, 1:6, 1:8

4.2.8 Sugar Analysis

Starch hydrolysis by α-amylase generates maltose and so maltose concentration in the aqueous skim layer after hydrolysis was determined using HPLC-RI (Agilent 1050, Manchester UK). Chromatographic separation was achieved using a TSKgel Amide-80 column (2 mm ID × 25 cm L) with a particle size of 5 μm (TOSOH, Griesheim, Germany) at

80 °C. The mobile phase used was acetonitrile/water, 7:3 (*v*/*v*) at a flow rate of 0.25 ml/min and injection volume of 5 µl. Data was analysed using ChemStation Software (Agilent) and results expressed in mg per ml of solution. Sugar identification was made by comparison with a maltose standard. Aqueous skim samples were filtered using a 0.45 µm filter before HPLC analysis.

4.2.9 High Pressure Processing

Ground and sieved tubers were subjected to high hydrostatic pressures between 300 – 700 MPa before aqueous enzymatic oil extraction. Temperature and time were maintained at 25 °C and 20 min respectively. The instrument information and procedure followed is given in 3.2.4.2.

4.2.9.1 Microscopy

Scanning electron microscopy (SEM) was adopted to visualise the structure of cells following AEE, with and without HPP treatment. Samples were freeze dried, attached to an electrically conductive double-sided adhesive carbon disc on a specimen stub, before gold plating using a sputter coater (S150B, BOC Edwards, Crawley UK). Samples were examined under the SEM (FEI, Quanta 600 F, USA). Images were taken at 12.5 kV.

To observe intact cells, whole tiger nut tubers were cut into slices using a sharp blade and fixed in 3% glutaraldehyde, dehydrated in a series of ethanol before transferring to an acetone solution (100%). It was then critically point dried (Balzers CPD-030, Balzers, Liechtenstein, Germany). The dried samples were mounted on specimen stubs, gold coated and examined under the SEM (Parker *et al.*, 2000). Images were taken at 20 kV.

4.2.9.2 Differential scanning calorimetry (DSC)

The degree of gelatinization of starches in HPP treated meals was analysed using Differential Scanning Calorimetry (DSC) (DSC Q2000, TA Instruments). The meals which are rich in starch were first freeze dried, and about 5 mg was mixed with water (4 times sample weight) and weighed into aluminium pans. An empty pan was used as a reference. The starchy meal residues were heated from 20 to 130 ^{o}C at a scanning rate of 5^{o}C/min (Eerlingen *et al.*, 1994). Peak temperature (T_P) and enthalpy of the transition were determined with TA Universal Analysis software.

4.2.9.3 X-ray powder diffraction

The diffraction patterns of HPP treated samples were analysed using a Bruker D8 Powder X-ray Diffractometer (Bruker Biosciences, Billerica, US) at room temperature. Freeze dried meal of HPP treated samples were packed in glass round cells, then irradiated with X-ray beams at 40 kV and 40 mA and scanned between diffraction angles of 5^{o} and 35^{o} 2θ.

4.2.10 Statistics

All experiments were performed in triplicate except the factorial study and mean values are presented with their standard deviations. Statistical analysis was done using Microsoft Excel 2010 and SPSS Version 20 Statistical software (SPSS Inc, Chicago, USA). Tukey's post-hoc tests were also carried out. Significance was defined at $p<0.05$.

4.3 Results and Discussion

4.3.1 Influence of Soaking on Oil Yield

Tiger nuts when soaked had a hydration capacity of 0.24 g/g material. Although the tubers appeared to swell only slightly (Figure 4.2), this led to an increase in the amount of oil extracted as observed in Figure 4.3 for all enzymes except Alcalase. Oil yields were increased by 55.1, 32.8 and 58.9% for α-amylase, Celluclast and Viscozyme respectively. Generally, swelling which increases the size of the tubers increases the number of cells broken during grinding. A higher number of broken cells mean more passage ways exist for oil to be released.

Figure 4.2: Dried and Swollen tiger nuts; soaked for 6 h at room temperature (left image is dried nuts and right image is tiger nuts after 6 h soaking)

During soaking, water is taken up to form hydrogen bonds with cellulose fibres within the cell wall. The formation of these bonds increases the size of inter-fibre pores, and in turn, increases the accessibility of cellulase to cellulose. Palonen (2004) observed from several

studies that drying lignocellulosic substrates, and the consequential decrease in pore size, decreased the effectiveness of enzymatic hydrolysis.

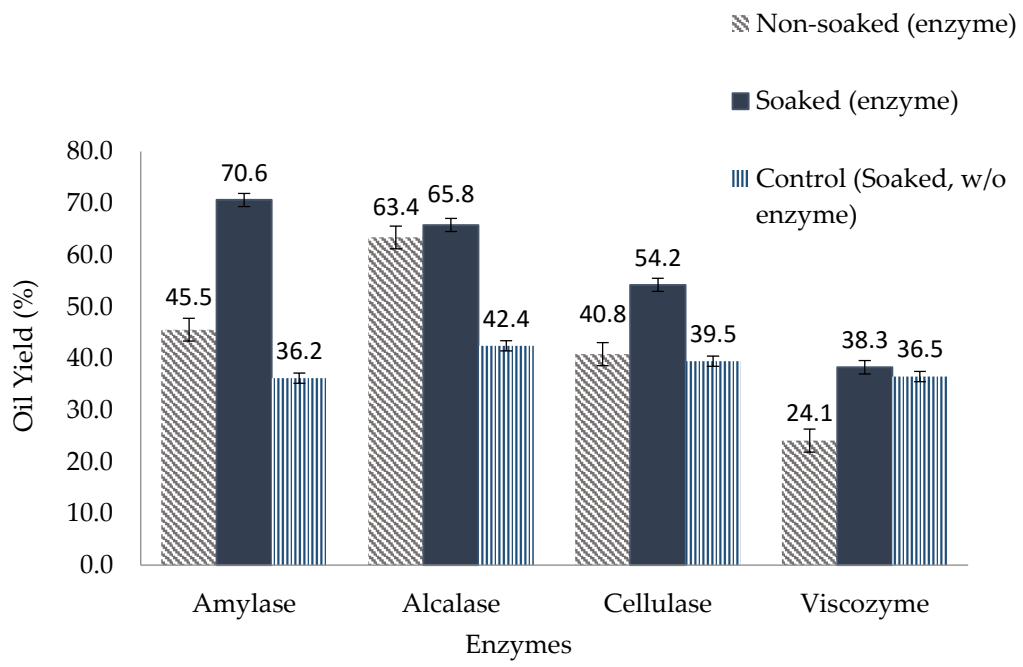


Figure 4.3: Influence of soaking on mean oil yields following aqueous enzymatic oil extraction (particle size of ≤599 µm, enzyme concentration of 0.5% (*w*/*w*), time of 6 h)

Similar to the effect on cellulose, starch molecules get hydrated, in particular the amorphous regions, and they expand when immersed in water. Starch surface area is increased as a consequence and made more available for hydrolysis by α-amylase. The action of soaking alone enhances oil extraction if one observes the similarity between control and Viscozyme treated samples. It can be assumed that the activity of Viscozyme did not contribute positively to the extraction of oil, and thus the only factor which improved oil yields was soaking. The content composition and volume fraction of hemicellulose tends to be lower than that of cellulose fibres in cell walls of grasses, thus hydrolysis of this component may not significantly affect the mobility of oil out of cells.

In the case of Alcalase, soaking did not significantly affect oil yields. Proteins are much smaller in size compared to starch, cellulose and hemicellulose, e.g. a starch bound protein has a diameter of 5.5 nm (BeMiller and Whistler, 2009) versus starch with 10 μm. Tiger nuts have a cell size of approximately 40-50 μm and grinding the tubers to a cut off size of 599 μm means that a large number of cells are still intact. The increase in the number of broken cells from swelling may have been insufficient to have an impact on the nanoscale sized molecule. Subsequent experiments were carried out with soaked tubers.

The aim of the enzymatic treatments was to reduce barriers to oil release in the cell. A number of cell structures pose different barriers such as starch and cellulose hence the enzymes used. Of the four enzymes shown in Figure 4.3, α-amylase and Alcalase were more effective in oil extraction followed by cellulase. Compared to control experiments, increases in oil yields were 95.3%, 55.2% and 37.4% for α-amylase, Alcalase and Celluclast respectively. Viscozyme hardly improved oil yields as it had no significant difference compared to control experiments. Hemicelluloses tend to be resistant to hydrolysis because of its association with lignin which may explain why Viscozyme did not have an impact on oil yields. Oil bodies and starch granules generally tend to be covered with protein rich membrane and the same may be assumed for tiger nuts. This may explain why proteolytic action increased the extracted oil despite the low protein content of tiger nut tubers of 3.5%. The SEM image (Figure 4.4) of cell structure of intact tiger nut reveals the presence of starch granules, which occupy a sizable volume of the cells. The empty spaces are a result of lipid removal by the solvents (ethanol and acetone) used during dehydration of the samples. Since starch granules lie adjacent to lipid bodies and a starch content of 25.1% based on the mass of dry seed, they present a barrier to the movement of oil out of the cell. Thus starch degradation would contribute to minimising this barrier. The measurement of the concentration of the various products of protein, starch, cellulose and hemicellulose hydrolysis would have been useful to provide more evidence to the effects of their respective

enzymes. Additional potential barriers to oil release are the intercellular spaces present in the cell walls. From Figure 4.4, it can be noted that oil can be easily trapped within these spaces, highlighting the importance of destroying the cell walls.

Table 4.4: Oil, starch and protein content of tiger nut tuber

Oil (%) dry basis	27.3 ± 0.28
Starch (%) dry basis	25.1 ± 0.33
Protein (%)	3.5 ± 0.08

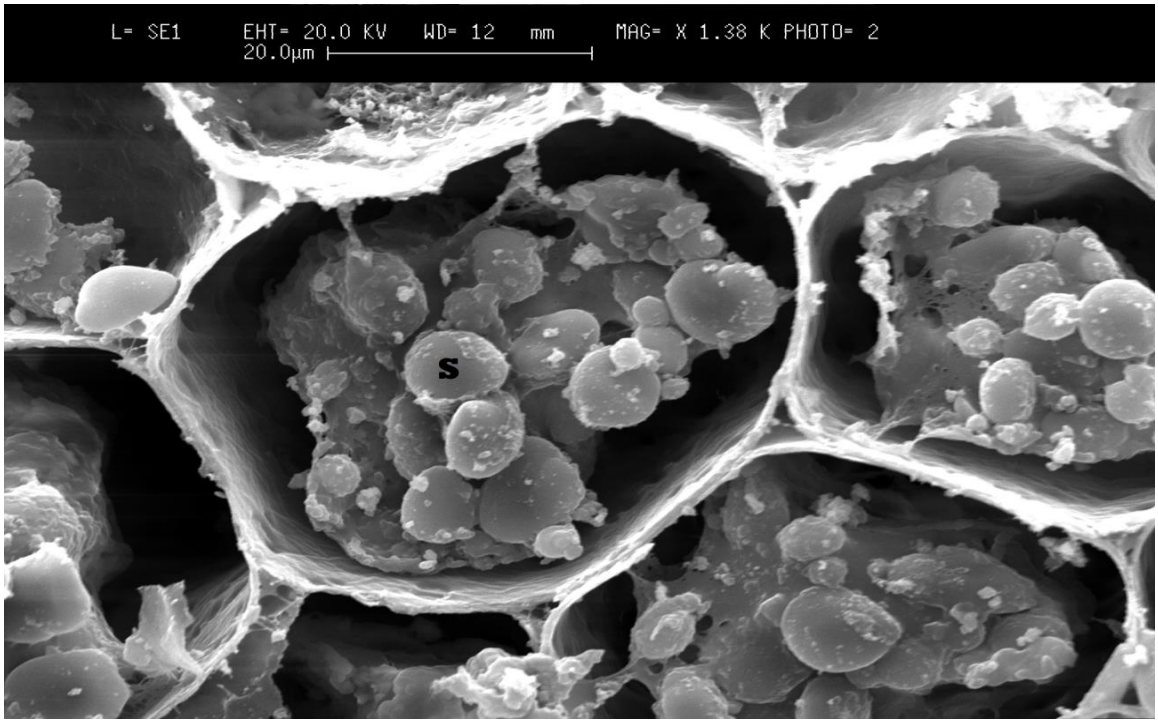


Figure 4.4: Scanning electron image of tiger nut cells obtained after slicing whole tiger nut tuber, (s) starch

4.3.2 Effect of Particle Size

Alcalase and α-amylase were used to study the effect of particle size because they gave the highest oil yields. After further grinding and sieving soaked tiger nut tubers to a size of ≤300 µm led to an increase of only 2% for Alcalase. There was no significant difference in oil yields between extraction from particles ≤599 µm and ≤500 µm. For α-amylase, the yields

were the same regardless of particle size and no improvements were made on the yields. A number of factors play a role in the efficacy of starch hydrolysis including the chemistry of the starch, and the matrix surrounding the starch granules etc. Native starch often takes much longer than 6 h before complete hydrolysis occurs as it is resistant to degradation at mild temperatures and pH. Thus particle size may not make much difference to α-amylase activity.

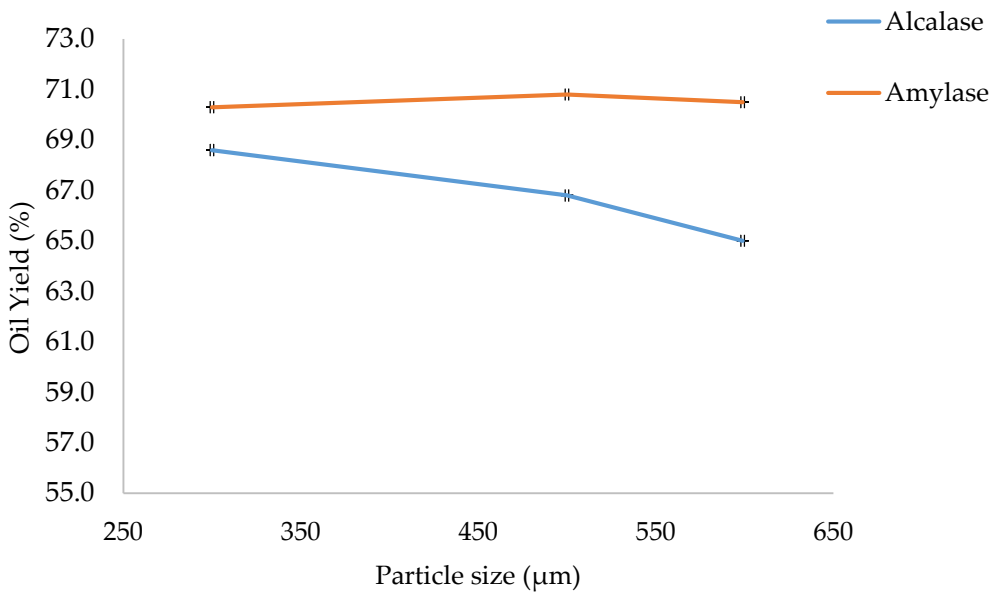


Figure 4.5: Effect of particle size on oil yield after aqueous enzymatic oil extraction with α-Amylase and Alcalase (enzyme concentration of 0.5% (*w*/*w*), time of 6 h, soaked samples)

Rosenthal *et al.* (2001) recognised that particle size played a critical role in the aqueous enzymatic oil extraction of soybean oil. Smaller particle sizes correspond to an increase in surface area, thereby shortening the travel pathway for enzymes to reach the substrate. Reducing the particle size to ≤300 μm means further cell rupture and this may have increased accessibility of Alcalase as pathways to starch bound proteins are minimised.

4.3.3 Mixed Enzymes

Combining α-amylase with other enzymes has been previously reported to augment oil yields, and even more when cell wall degrading enzymes are used (Mai *et al.*, 2013). In earlier studies, only α-amylase, protease, and cellulose led to a significant increase in oil yields, so Viscozyme was not added in this part of the study. The primary objective of the full factorial study conducted was to determine if, and which of the three factors (temperature, pH and mixed enzyme ratio) had a significant effect on oil yield. To be able to carry out an ANOVA, the three factor interaction was assumed to be insignificant, so the term was neglected allowing for the use of a reduced model. Out of the three factors, pH had the most significant effect, as well as the pH-temperature interaction (Table 1-A and 1-C and in Appendix 1). This was not surprising since pH and temperature are two key criteria that affect enzyme activity. The next question to be answered was what combination of pH and temperature would give the highest oil yield? After adjusting the model for non-significant terms, a new regression model was used to obtain a combination of pH and temperature estimated to produce maximum oil yields. A temperature of 40 ^{o}C and pH 8 were given as the solution (Table 1-B in Appendix 1). The ANOVA for the reduced model showed the significant effect of the interaction between pH and temperature on oil yield (Table 4.5). The procedure was repeated in triplicate with the predicted values and the results obtained were consistent with the prediction (Table 4.6).

The chosen values (pH 8 and 40 ^{o}C) are well within recommended values for α-amylase which may explain why the highest oil yield was obtained. At these conditions, α-amylase is at its optimum, positively affecting starch hydrolysis.

Table 4.5 : ANOVA for reduced model of effect of temperature, pH and enzyme ratio on oil yield using an α-amylase, Alcalase and Viscozyme enzyme mixture

```
Analysis of Variance

Source                DF    Adj SS    Adj MS  F-Value  P-Value
Model                  8  0.256662  0.032083    12.32    0.000
  Linear               4  0.185770  0.046442    17.84    0.000
    pH                 2  0.180364  0.090182    34.64    0.000
    Temp               2  0.005406  0.002703     1.04    0.374
  2-Way Interactions   4  0.070892  0.017723     6.81    0.002
    pH*Temp            4  0.070892  0.017723     6.81    0.002
Error                 18  0.046864  0.002604
Total                 26  0.303526
Model Summary

        S    R-sq  R-sq(adj)  R-sq(pred)
0.0510252  84.56%     77.70%      65.26%
```

(Enzyme concentration of 0.5% (*w*/*w*), particle size of ≤300 μm, incubation time of 6 h, S/L ratio of 1:4)

Table 4.6: Predicted and actual values for maximum oil yield with recommended pH and temperature

Solution		Oil Yield (%)	
pH	Temp (°C)	Predicted Value	Actual Value
8	40	74.2	76.3 ± 0.221

The pH also favours Alcalase activity although the lower temperature when compared to its recommended value would slightly affect it. Protein is known to affect starch hydrolysis or digestion by α-amylase and hydrolysing protein aids that of starch by increasing surface accessibility of enzymes. On the digestion of cereal grains, Kotarski *et al.* (1992) mentions that proteins and structural carbohydrates limit starch digestion by microbial flora. The augmentation of oil yields could be mainly due to the combined effects of these two enzymes. As the ratio of enzymes did not appear to have a significant effect on the oil yields, it was kept at 1:1:1 for successive experiments.

4.3.4 Effect of Incubation Time on Oil Yields with Mixed Enzymes

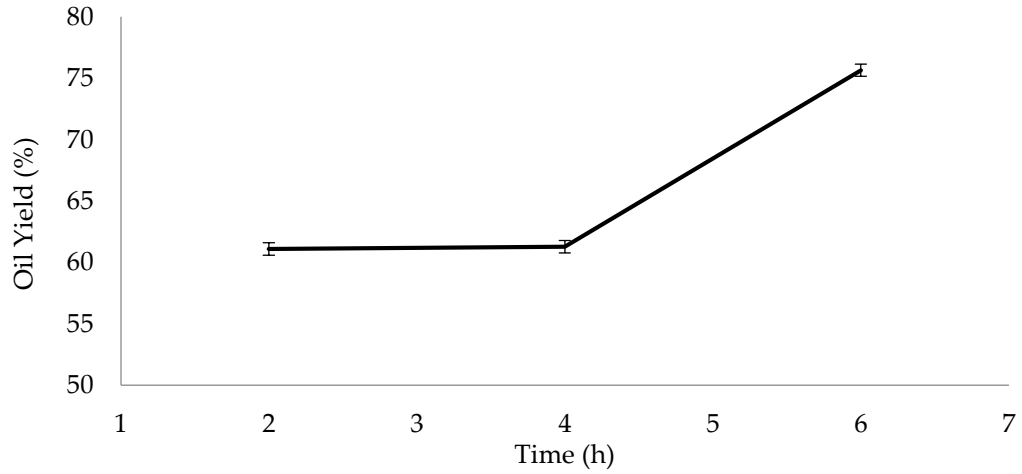


Figure 4.6: Effect of time on oil yield using an enzyme mixture of α-Amylase, Alcalase and Celluclast (enzyme concentration of 0.5% (*w*/*w*), particle size of ≤425 μm, S/L ratio of 1:4)

As expected, the longer hydrolysis was allowed to take place, the greater was the amount of oil extracted. Despite significant differences between oil yields at 4 and 6 h, oil recovery suffered as hydrolysis time was prolonged. The emulsion layer was observed to thicken after 6 h of incubation. Allowing more time for incubation, even though it led to higher yields, also provided more time for the formation of thicker emulsions. The extracted oils and water form emulsions that are stabilised by starch and modified starch which acts as emulsifiers. The emulsion was not destabilised by centrifugation at higher speeds than 2300 × g or α-amylase. The hydrocolloids formed from both starch and cellulose in the emulsions may not be discernible to α-amylase when used, preventing it from acting on any α-1-4 bonds present. At 2 h, the released oil was hardly recoverable as can be seen in Figure 4.7, which may be explained by insufficient time for oil coalescence to occur. To demulsify this stable emulsion, a comprehensive study on the role of each component of the emulsion would be required. Subsequent experiments were carried out at 4 h because of the shorter hydrolysis time and reduced emulsion.

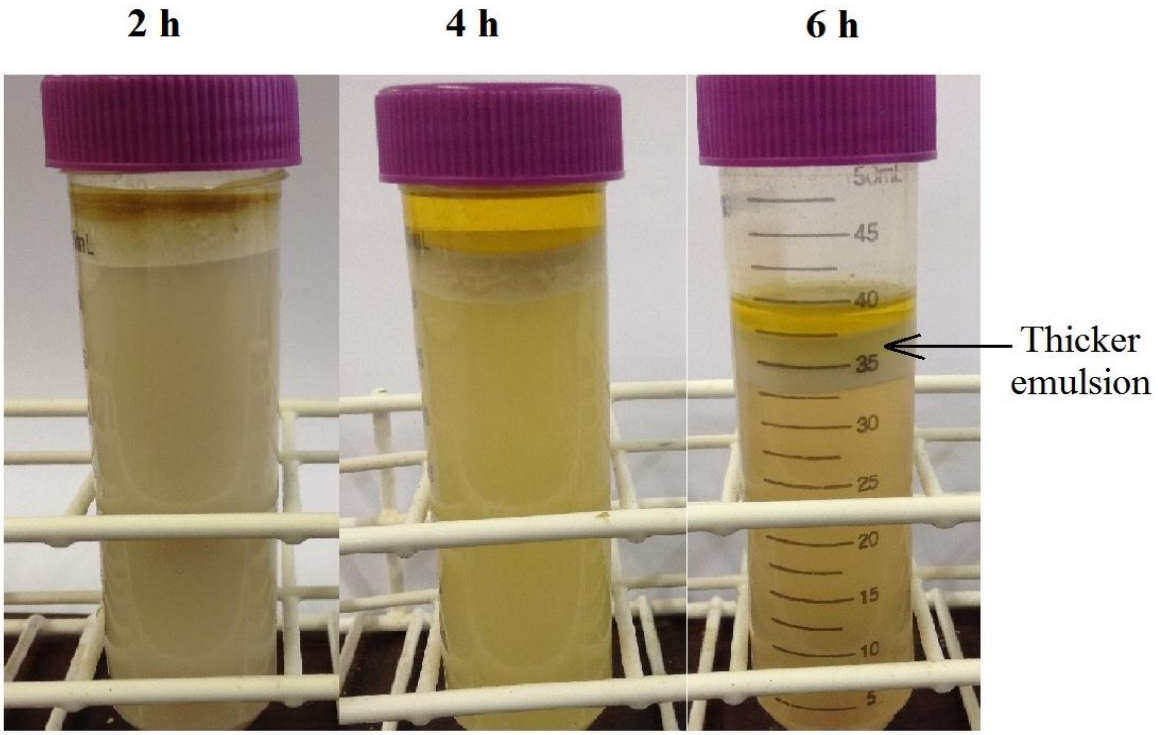


Figure 4.7: Effect of incubation time on emulsion (α-amylase, Alcalase and Celluclast, enzyme concentration of 0.5% (*w*/*w*), particle size of ≤425 μm, S/L ratio of 1:4)

4.3.5 Effect of Enzyme Concentration on Oil Yields with Mixed Enzymes

Higher yields were obtained when mixed enzyme concentrations were increased from 0.5% to 1% after which it fell. The yield also remained constant above a 1.5% concentration. This is consistent with results obtained with enzyme assisted pressing experiments carried out on tiger nuts in 3.3.3 where the maximum oil yields were obtained with enzyme concentration of 1%, although different incubation times and enzymes were used. Maximum oil yields of 67% were obtained with an enzyme concentration of 1%. Above this concentration, an accumulation of enzyme degradation products may increase the viscosity of the extraction medium such that it affects enzyme diffusion, thus their activities and rate of reactions. This then may reduce oil extraction yields.

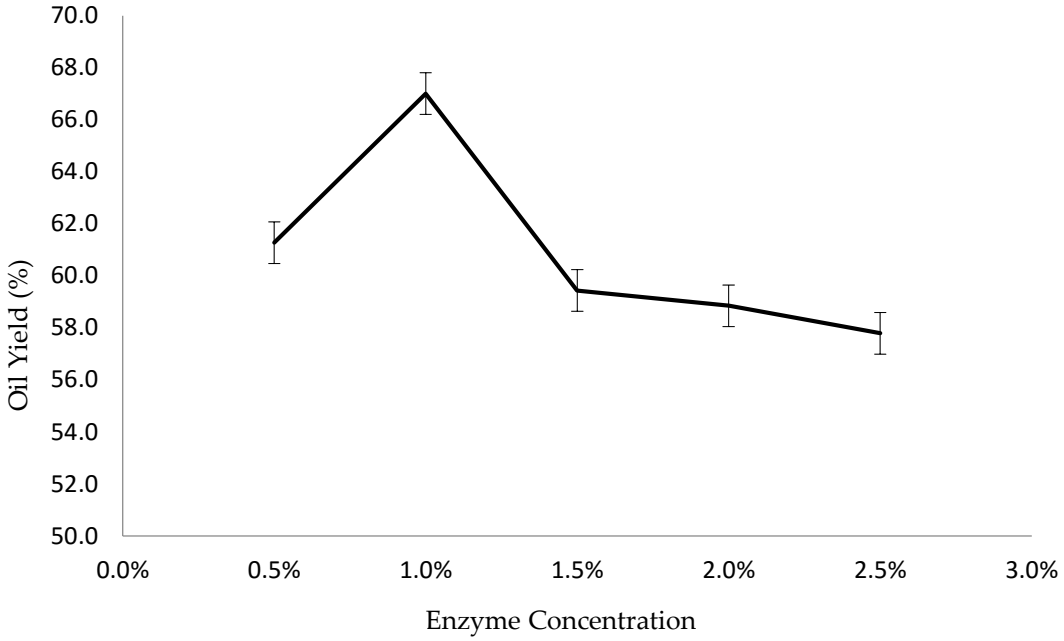


Figure 4.8: Effect of enzyme concentration on oil yields using a mixture of α-amylase, Alcalase and Celluclast (enzyme concentration of 0.5% (*w*/*w*), particle size of ≤425 μm, incubation time of 4 h, S/L ratio of 1:4)

4.3.6 Effect of Solid-Liquid Ratio on Oil Yields using Mixed Enzymes

Increasing solid-liquid (S/L) ratio from 1:4 to 1:6 increased the oil yield by 16.6% and a further increase to 1:8 made no significant difference. For an enzymatic hydrolysis process to occur efficiently, a specific amount of water is necessary in order to facilitate the cleavage of substrate bonds. In this study, increased quantities of water to 1:6 improved enzyme activities and hence oil yields. The viscosity of the mixture decreases when water quantities increases, which would increase diffusivity of the enzymes to the substrates. Although enzyme concentration becomes reduced, this effect is not significant enough to affect rates of reaction and oil extraction yields. Above a ratio of 1:6, additional water was not required for hydrolysis to occur. At higher water levels, enzyme concentrations are reduced beyond the limit needed for efficient enzyme diffusion, limiting hydrolysis rate of reaction and eventually oil yields.

Table 4.7: Effect of S/L on Oil Yields with Aqueous Enzymatic Extraction

Solid-Liquid Ratio	Oil Yield (%)
1:4	61.3 ± 0.04^{a}
1:6	71.5 ± 0.63^{b}
1:8	69.7 ± 0.35^{b}

Different superscript letters indicate significant difference. Enzyme mixture of α-amylase, Alcalase and Celluclast was used. Enzyme concentration of 1.0% (*w*/*w*), particle size of ≤425 μm, incubation time of 4 h, S/L ratio of 1:4

4.3.7 Effect of Linear Agitation Speed on Oil Yields with Mixed Enzymes

As expected, agitating the samples during incubation slightly increased oil yields, but differences were small (Table 4.8). A significant increase was only observed when the agitation speed was increased from 200 to 240 linear strokes/min. On examination of the hydrolysis process, it was noticed that linear agitation motion mimicked a gravity separation process, as denser solids were seen settling to the bottom during agitation as shown in Figure 4.9. This observation led to an extraction being carried out without agitation to find out if settling would occur and how this may affect the yield. It was discovered that was the highest oil yield of 76.5% was obtained without agitation. The agitating action prevented sufficient mixing, and consequently inhomogeneous enzyme distribution. At the bottom where settled starch and other solid fractions presented themselves as larger aggregates, solid to liquid ratio is low and with minimum water available for enzymatic hydrolysis, the rates of reaction is lowered. In addition, the surface areas of the substrates become reduced and less area is available to bind to the enzymes affecting their efficacy. A combination of the effects of the particle sedimentation would thus affect oil extraction yields.

Table 4.8: Effect of Linear Agitation Speed on Oil Yield with Aqueous Enzymatic Extraction and Maltose Concentration in Samples

Agitation (linear strokes/min)	Oil Yield (%)	Maltose (mg/ml)	Settled solids(g)
0	76.5 ± 1.13[a]	5.60 ± 0.22[a]	-
120	61.3 ± 0.04[b]	5.18 ± 0.04[b]	17.6[a]
160	62.9 ± 0.77[b]	4.99 ± 0.03[b]	21.8[b]
200	63.8 ± 0.63[c]	5.23 ± 0.02[b]	9.7[c]
240	69.9 ± 0.49[d]	5.10 ± 0.03[b]	4.9[d]
280	64.2 ± 0.07[b,c]	nd	nd

nd: not determined, different superscript letters in each column indicate significant differences. Enzyme mixture of α-amylase, Alcalase and Celluclast was used. Enzyme concentration of 1.0% (*w*/*w*), particle size of ≤425 μm, incubation time of 4 h, S/L ratio of 1:4

Table 4.8 shows the maltose concentrations of the aqueous skim layer of samples following agitation at different speeds. Maltose concentration was used as an indication of the extent of starch hydrolysis. It is clear that without agitation, both oil yield and maltose concentration were the largest values, suggesting that starch hydrolysis affects oil yields and this is maximised when no agitation occurs. Hydrolysing starch reduces one of the various barriers to oil release from cells which may explain why highest oil yields and maltose concentrations occurred simultaneously. Increasing agitation decreased the degree of settling and at 200 linear strokes/min, the speed was sufficient to reduce the degree of settling, slightly increasing oil extraction yields. When agitation was 240 linear strokes/min, the mass of settled solids was reduced even further and the oil yield was the maximum (out of the agitated samples).

Figure 4.9: Settled solids after enzymatic incubation (*Bottles on left are static samples, bottles on the right are agitated samples at 200 strokes/min; Bottom image show settled solids lack of homogeneity) Enzyme mixture of α-amylase, Alcalase and Celluclast was used. Enzyme concentration of 1.0% (*w*/*w*), particle size of ≤425 μm, incubation time of 4 h, S/L ratio of 1:4

4.3.8 Effect of High Pressure Processing on Oil Yields with AEE

Given the low yield obtained when aqueous enzymatic oil extraction was carried out at 120 linear strokes/min, 0.5% enzyme concentration and S/L ratio of 1:4 for 4 h, using HPP as an additional pre-treatment led to a considerable increase in oil yields. A particle size of ≤435 μm was used in this study because of the difficulty of sieving to a particle size of ≤300 μm. The lowest pressure used (300 MPa) gave the best oil yields of 76.1% compared to an extraction without HPP with a yield of 61.3%. Higher pressures were observed to decrease

the oil yields. The maltose concentrations of the aqueous skim layers showed a corresponding declining trend with the highest concentration obtained at 300 MPa and the lowest at 700 MPa (Table 4.9).

Table 4.9: Effect of HPP on Oil Yield with Aqueous Enzymatic Extraction

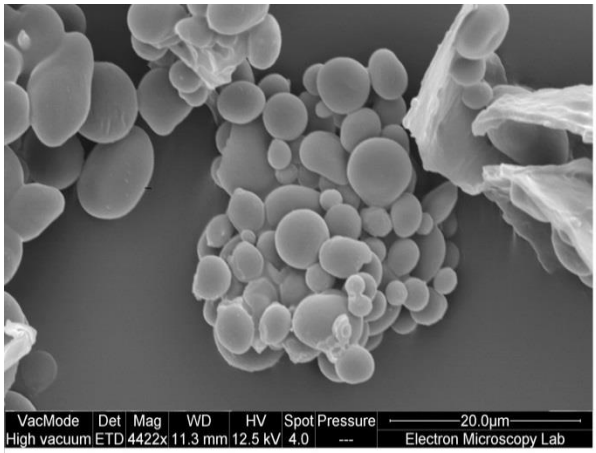
Pressure (MPa)	Oil Yield (%)	Maltose (mg/ml)
0	61.3 ± 0.04[a]	5.2 ± 0.04[a]
300	76.1 ± 1.16[b]	7.3 ± 0.08[b]
500	70.6 ± 1.01[c]	6.1 ± 0.75[c]
700	61.0 ± 0.69[a]	5.4 ± 0.02[a]

Different superscript letters in each column indicate significant difference. Enzyme mixture of α-amylase, Alcalase and Celluclast was used. Enzyme concentration of 0.5% (*w*/*w*), particle size of ≤425 μm, incubation time of 4 h, S/L ratio of 1:4

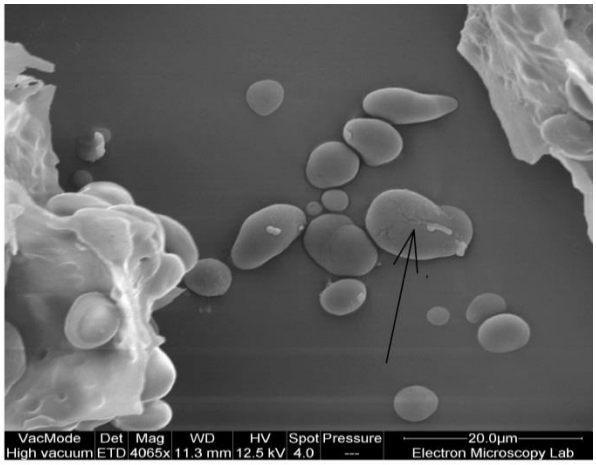
Visualising the solid residue of the HPP treated samples under an SEM revealed partial ruptures on the surface of some starch granules (Figure 4.10). Care was taken during analysis to avoid damage from the electron beams which were observed to damage the granules at higher voltages by reducing the voltage from 20 to 12.5 kV, and ensuring the magnification did not exceed 10 μm for too long. Intact starch granules from non-treated samples shown in Figure 4.10 have smooth surfaces, typical of native starches and some cavities, probably as a result of enzyme hydrolysis. The non-treated sample was one which underwent AEE of oil with the same conditions, but without HPP pre-treatment. With the partial damage caused by HPP, α-amylase had an improved accessibility to the granules which is illustrated by the higher maltose concentration at 300 MPa. As pressure was increased from 300 to 500 and 700 MPa, it is possible that in addition to the damage induced to starch granules, higher pressures damaged membranes of both amyloplasts and oleosomes. In a whole tuber, these two organelles surround each other and keep starch and lipid separate. The action of grinding increases the exposure of these organelles to HPP. Destroying their membranes is likely to bring these cell nutrients in contact, driving the formation of amylose lipid

complexes when mixed with water during incubation, and increasing starch resistance to enzymes. It has been proposed that the outer layers of starch granules contain a higher proportion of amylose (Kotarski *et al.*, 1992) and with the surface cavities created by HPP, it is possible that more amylose lipid complex formation occurs. Another possible explanation might be an increase in resistant starch caused by HPP. Studies have shown that HPP can be used as a tool to increase resistant starch in different types of wheat (Dupuis *et al.*, 2014). However, HPP is often applied to starch slurries and this leads to partial gelatinisation. In this study, dry samples were used to avoid possible gelatinisation and this also may prevent the formation of resistant starch. The reduction in maltose concentration in 500 and 700 MPa samples indicated that starch hydrolysis rates were reduced. A recent publication reports on the quick formation of amylose lipid complexes (within 10 s) which takes place at the periphery of native starches well below gelatinisation temperature (Manca *et al.*, 2015a). This process is driven by the hydrophobicity of amylose helical cavities to which the non-polar regions of lipids are drawn.

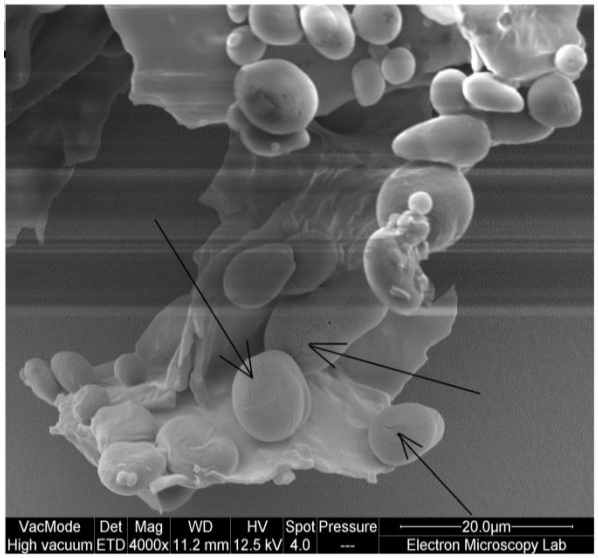
The SEM images also revealed that not all starches were affected by HPP as a large number were seen to be intact and a few had holes from enzyme hydrolysis. Being enclosed within a cell matrix may have provided protection to starch not affected by HPP. HPP does not damage cell walls in tiger nuts, and the starch granules affected by HPP may be the starch granules released during grinding.



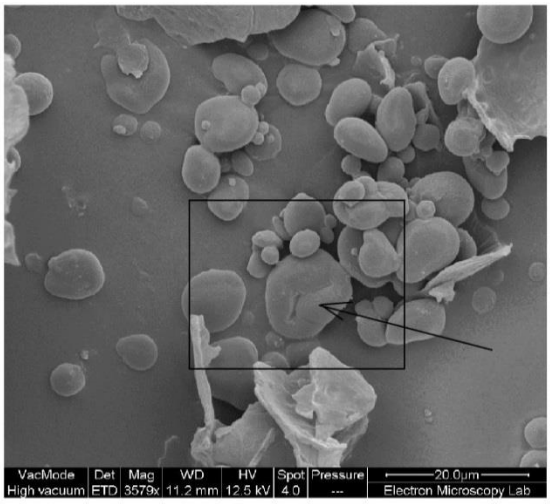
a) Control



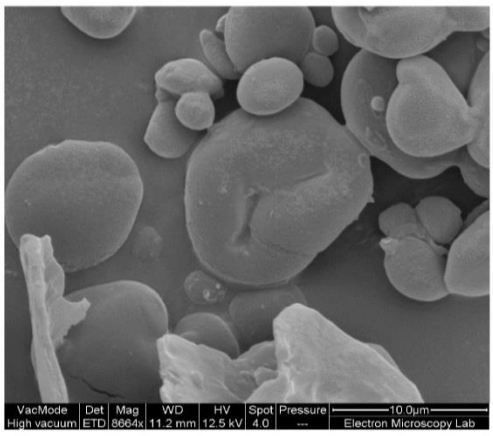
b) 300 MPa



c) 500 MPa



d) 700 MPa



e) 700 MPa enlarged

Figure 4.10: Scanning electron microscope images of dried HPP (20 min, 25 °C) treated samples after aqueous enzymatic oil extraction using an enzyme mixture of α-amylase, Alcalase and Celluclast. Arrows indicate areas of starch rupture. (Enzyme concentration of 0.5% (*w*/*w*), particle size of ≤425 µm, incubation time of 4 h, S/L ratio of 1:4)

Starches can be characterised by X-ray diffraction patterns given by their crystalline lattices and they fall into three types: A, B and C. Another structure exists namely V type which corresponds to amylose lipid complexes. Each of these (type A-C) respond differently to HPP and type A is the most sensitive (Pei-Ling *et al.*, 2010). Tiger nut, although being a tuber, contains type A starch (Builders *et al.*, 2013), commonly found in cereals. X-ray diffraction analysis and differential scanning calorimetry are two common techniques employed to study the formation of amylose lipid complexes, but these may not be sensitive enough to detect very small quantities of complexes (Manca *et al.*, 2015b). Kotarski *et al.* (1992) also came to this conclusion, stating that X-ray diffraction of granular starches does not usually show the presence of amylose lipid complexes especially with amylose content lower than 30%. Absence of the typical V structure of inclusions in diffraction patterns does not prove lack of these structures in the starches themselves.

An attempt was made to detect the presence of any inclusions using both DSC and X-ray diffraction and neither displayed any sign of these. DSC was carried out with samples mixed with water while dried samples were used for X-ray analysis. From the DSC analysis, there was a reduction in enthalpy associated with starch disassociation with pressure treated samples, compared to the control (Figure 4.11), suggesting a change in structure of the starch. DSC measures the enthalpy change associated a specific temperature change within a substance, so a reduction in enthalpy for HPP treated samples indicate that the samples had been altered such that energy required to raise the temperature was reduced. This alteration would be a change in the structure of starch following HPP treatment and in this case, one can observe from Figure 4.11 that this change is significant. With DSC analysis of starches, gelatinisation temperature can be obtained which is specified by the peak temperature. Peak temperatures for all HPP samples and control were comparable suggesting that no partial

gelatinisation took place during pressurisation. Other studies on HPP treated starch showed a reduction in peak temperatures (Molina-García *et al.*, 2007; Liu *et al.*, 2008) as a result of partial gelatinisation.

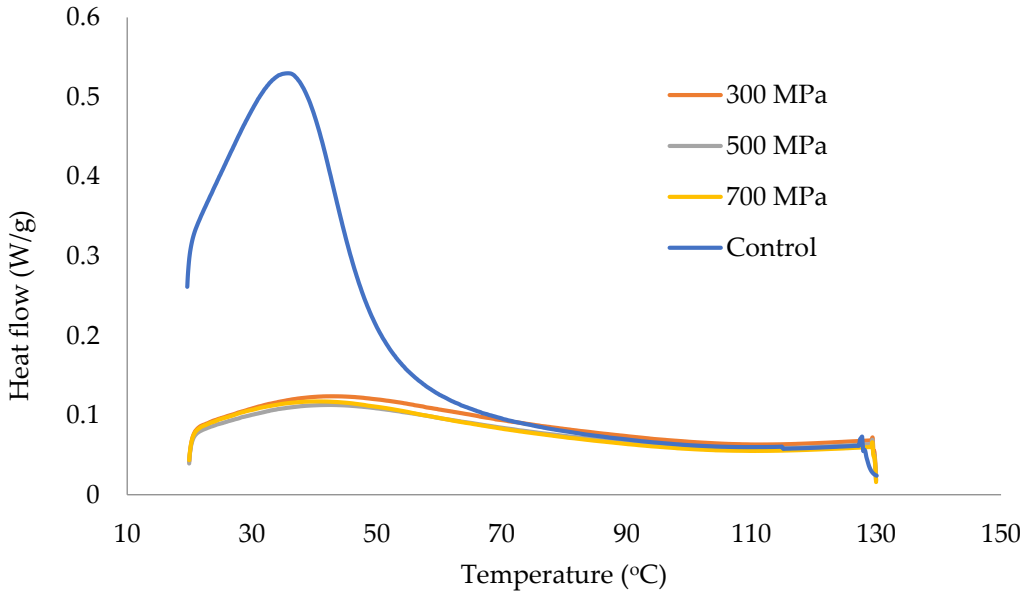


Figure 4.11: DSC thermograms of HPP treated (20 mins at 25 °C) samples after aqueous enzymatic oil extraction (Enzyme mixture of α-amylase, Alcalase and Celluclast was used, enzyme concentration of 0.5% (*w*/*w*), particle size of ≤425 µm, incubation time of 4 h, S/L ratio of 1:4); samples for DSC measurements were mixed with water 4 times its weight

Despite the fact that the samples used were not purified starches, the X-ray diffraction patterns in Figure 4.12 confirmed the type A structure of tiger nut starch, with signature peaks at approximately 15, 17 and 23° 2 Theta (angles of diffraction). The angles of diffraction can be used to calculate the spacing between atoms in the starch crystalline structure and thus are in indirect indication of the spacing. All samples were indistinguishable, demonstrating that crystallinity was maintained across the treatments. An unknown peak at 2 Theta =26.8° in 300 MPa treated samples was observed after conducting the analysis in triplicates. As mentioned earlier, minute quantities of amylose lipid complex will probably not be detected by this method. A more sensitive method would be valuable to conduct an

in-depth study on the association between high hydrostatic pressure treatments, lipid amylose complexes and starch hydrolysis.

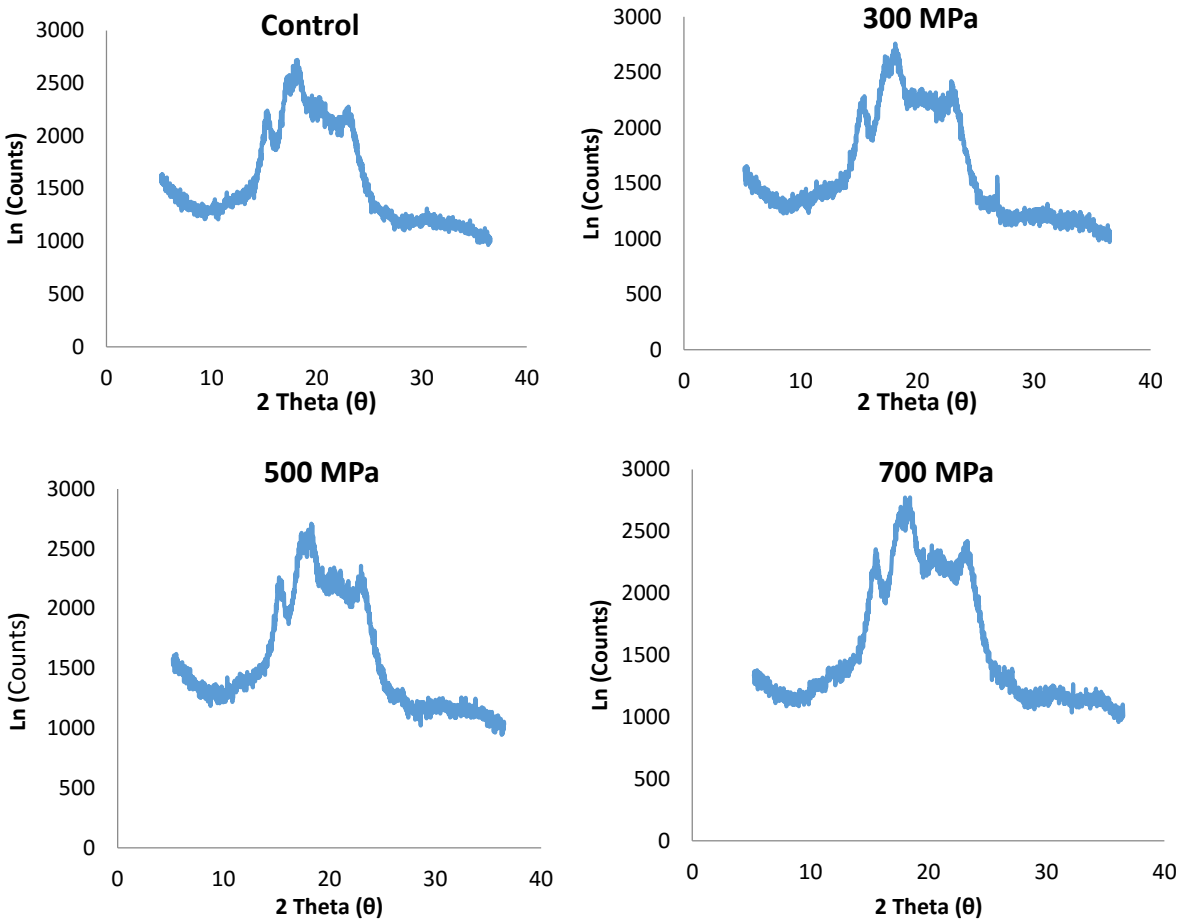


Figure 4.12: X-ray diffraction patterns of dried HPP (20 mins, at 25 °C) treated samples after aqueous enzymatic oil extraction (Enzyme mixture of α-amylase, Alcalase and Celluclast, enzyme concentration of 0.5% (*w*/*w*), particle size of ≤425 μm, incubation time of 4 h, S/L ratio of 1:4)

4.4 Conclusions

Aqueous extraction of tiger nut oil can easily be enhanced using a mixture of enzymes, mainly α-amylase and Alcalase, with α-amylase having the major impact. When a mixture of α-amylase, Alcalase, and Celluclast were used, time, solid/liquid ratio, agitation speed and enzyme concentration were factors found to affect the extraction process. Of the four examined factors, the greatest contributions to increasing oil yields were extraction time and agitation. The optimum time found was 4 hours and static conditions during incubation remarkably gave the highest oil yields, as agitation mimicked gravity separation. High pressure processing as a pre-treatment had a positive impact on oil yields and the highest yield of 76.1% was obtained using 300 MPa. Slight cavities on the surface of starch granules in tiger nuts may be responsible for the increased yields. However, pressures greater than 300 MPa did not have the same positive effect.

Chapter 5

CHARACTERISTICS OF EXTRACTED OILS AND OLIGOSACCHARIDES IN RESIDUAL MEALS OF TIGER NUT

This chapter is based on a paper titled "Characteristics of edible oil from Tiger nut (*Cyperus esculentus*) extracted using enzyme aided pressing and aqueous enzymatic extraction and oligosaccharides present in residual meals" that is currently in submission.

The hypothesis that the use of enzymes and high pressure processing as pre-treatments do not affect the quality of oils extracted using mechanical pressing and aqueous extraction was examined in this chapter. Selected oils obtained using both pressing and aqueous enzymatic oil extraction methods were analysed and characterised. Soluble sugars in the residual meals were also identified and quantified.

Abstract

Using enzyme-aided pressing (EAP) as a method for oil extraction increased the concentration of some phenolic acids and tocopherols present in extracted oils. High pressure processing as a pre-treatment before aqueous enzymatic extraction (AEE) also enhanced tocopherols and total polyphenolic content in oils. Oil quality parameters by both methods indicated the oils were stable and they all met the standards for virgin olive oil set by the International Olive Oil Council. Residual meals from both of the extraction processes contained low protein contents ranging from 2.4-4.6%. Additionally, EAP and AEE meals contained low DP (degree of polymerisation) sugars that appeared as 1-kestose (DP3) and nystose (DP4). EAP had the highest total DP3 and DP4 content of 82.5 mg/g. These sugars would need further assessment to verify their identity and determine their suitability as a potential food.

5.1 Introduction

Quite often, claims for a number of beneficial qualities to different aspects of life ranging from human health to hair condition and skin care are made for plant oils. These benefits are commonly attributed to the composition and chemical constituents of the oils. An example is the high lauric acid content of coconut oil which allows it to easily penetrate into the hair shafts, and together with coconut oil's affinity for protein, offers protection from hair protein loss (Rele and Mohile, 2003). Applications of this oil is not limited to hair care, but also used for cooking, baking and frying as well as skincare preparations. Similarly olive oil is as versatile, because of its fatty acid composition, and content of bioactive compounds such as polyphenols and Vitamin E.

With the numerous possible sources of plant oils, it is important to identify new sources to diversify the range available to consumers and encourage local production in areas where the plants are grown. Furthermore, development of new sources allows sustainable growth, manufacture and use of the oils. Tiger nut oil, although not entirely new, remains unknown in major parts of the world and underutilised even in regions where it is cultivated. It has already been described as being similar to olive oil both in the literature and in previous chapters.

The quality of oils is dependent on the processing conditions employed during their extraction process. It is important that key components of the oil are protected when a processing factor is altered. Another important aspect of oil extraction processing is the use of the residual meals. Commonly they are popular as animal feed because of the high protein content that most oilseeds possess. Here, the oils obtained after enzyme pressing and

aqueous enzymatic oil extraction were evaluated for their chemical composition and quality. Residual meals were also evaluated for their soluble sugars composition.

5.2 Materials and Methods

5.2.1 Samples

Oils were extracted using enzyme assisted pressing (3.2.4.3) and aqueous enzymatic extraction (4.2.7) methods. Control consisted of samples extracted using the same extraction method but without the use of enzymes. Some analyses were also conducted on aqueous enzymatic extracted (HPP-AEE) oil with high pressure processing (300 MPa) as a pre-treatment. The oils were stored in amber bottles and maintained at 4 ^{o}C till analysis.

Table 5.1: Conditions for Extraction of Oils

	Enzyme Assisted Pressing	Aqueous Enzymatic Extraction
Enzymes	α-amylase, Alcalase, Viscozyme,	α-amylase, Alcalase, Celluclast
Enzyme concentration (%)	1.0	0.5
Agitation speed (linear strokes/min)	120	120
Solid/liquid ratio	1:1.7	1:4

5.2.2 Chemicals

Supelco 37 Component fatty acid methyl ester (FAME) Mix, α-tocopherol standard, gallic acid, phenolic acid standards (vanillic acid, *trans*-ferulic acid, vanillin, *trans*-cinnamic acid), glucose, 1-kestose (DP3) and nystose (DP4) standards were purchased from Sigma-Aldrich (Dorset, UK). All chemicals were of analytical grade.

5.2.3 Oil Analysis

5.2.3.1 HPLC phenolic profile

Phenolic extracts to be used for HPLC analysis were obtained using the liquid-liquid extraction procedure outlined by Pirisi *et al.* (2000). Oils were weighed into tubes with 1 ml

hexane and 2 ml methanol (6:4, v/v) and vortexed for 2 min. The mixture was centrifuged at 2300 × g and the methanol layer was separated. This was repeated twice and the extract was washed with 2 ml hexane. Methanolic extracts were evaporated to dryness at 35 ^{o}C and re-dissolved in 1 ml methanol prior to injection.

Analysis was carried with a HPLC-DAD system (Agilent 1200, Manchester, UK) using a Nova-Pak C-18 reverse phase column (4 μm, 25 cm × 4.6 mm i.d.) (Waters Limited, Hertfordshire, UK). Mobile phases were 0.001 M H_2SO_4 (A) and acetonitrile (B) at a flow rate of 1 ml/min. The detector was set at 225 nm and the sample loop was 20 μl volume. The gradient program was as follows: t= 0 min, A = 85%, B = 15%; t = 35 min, A= 34%, B= 66%; t=35.1-40 min, A = 85%, B = 15%. Identification of phenolic compounds was done by comparison with peak times and spectra of standards. An external calibration was used for quantification.

5.2.3.2 Total polyphenol content

The extraction procedure in 3.2.7.4 for total polyphenol content (TPC) was followed. For analysis, the method described by Baiano *et al.* (2009) was used. The phenolic extract (100 μl) was mixed with Folin-Ciocalteu reagent (100 μl) and after 4 min, 800 μl of 5% Na_2CO_3 was added. The mixture was incubated at 40 ^{o}C for 20 min, after which the absorbance was determined at 750 nm. A calibration curve using gallic acid in methanol/water (7:3, v/v) was constructed and TPC was expressed as gallic acid equivalents.

5.2.3.3 Tocopherol content

The method described in 3.2.7.3 was used. β-Tocopherol is expressed as an α-tocopherol equivalent.

5.2.3.4 Fatty acid profile

The procedure described in 3.2.7.1 was followed.

5.2.3.5 Free fatty acid and peroxide values

Acid values were measured using the method described in 3.2.7.2 and converted into percentage free fatty acid (FFA) using the formula $\% FFA = AV \times 1.99$ (Equation 5.1)

For peroxide values (PV), the International Dairy Federation (IDF) method for determination of peroxide value was adopted (Shantha and Decker, 1994). Oils (0.3g) were weighed in test tubes and 9.8 ml of chloroform/methanol (7:3, v/v) was added. The mixture was vortexed for 2-4 s, followed by the addition of ammonium thiocyanate (50 µl) solution. The sample was vortexed again for 2-4 s and 50 µl of iron (II) solution was added, vortexed and left to incubate for 5 min at room temperature. After incubation, the absorbance was taken at 500 nm against a blank. The blank was a mixture containing all reagents without the oil sample. Analysis was conducted under limited lighting. A calibration curve of Fe^{3+} concentration against absorbance was constructed using standard solutions of iron (III) chloride (5-40 µg Fe^{3+}).

Peroxide value was calculated using the formula $PV = \frac{(A_s - A_b) \times m}{55.84 \times m_0 \times 2}$ (Equation 5.2)

Where A_s = absorbance of sample; A_b = absorbance of blank; m = slope of calibration curve; m_0 = mass of sample in grams; 55.84 = atomic weight of iron.

5.2.4 Residual Meal Analysis

5.2.4.1 Chemical analysis

The Kjeldahl method outlined in 4.2.2 was used to measure protein content while the Soxhlet extraction was used for sample preparation for oil measurements. Ash content was determined using AOAC method 942.05 (AOAC, 1995), moisture using a Sartorius moisture analyser and total carbohydrate was calculated by difference; 100% -% (crude protein + ash + crude fat + moisture).

5.2.4.2 Extraction of sugars

Residual meals from oil extraction were defatted and soluble sugars were extracted by a modified method based on Teixeira *et al.* (2012). Fifty milligrams of sample was placed in a centrifuge tube and 2.5 ml of 80% ethanol was added. The mixture was vortexed for 5 s and heated in a water bath at 95 ^{o}C for 20 min. Following this, it was centrifuged at 2300 × g for 20 min and the extraction was repeated three times. The ethanolic extracts were combined and evaporated under vacuum at 42 ^{o}C till dryness. Extracted sugars were re-dissolved in 2.5 ml deionised water, filtered (0.45 μm) and kept at -20 ^{o}C till analysis.

5.2.4.3 Quantification of sugars

HPLC analysis was performed with on an Agilent 1100 system (Cheshire, UK) coupled to a refractive index detector. The mobile phase was HPLC grade water with a flow rate of 0.25 ml/min. The stationary phase was an Aminex HPX-42A column, 7.8×300 mm (Bio-Rad) attached to an anion-exchange guard column. The column was found to hydrolyse fructo-oligosaccharides at high temperatures and to prevent this, the temperature was maintained at 25 ^{o}C. Peaks were identified by comparison with the retention times of external standards. To quantify the sugars, calibration curves of varying concentrations were constructed.

To further confirm the presence of fructo-oligosaccharides, the samples were analysed using a Dionex ion chromatography system (Sunnyvale, CA) consisting of ED50A pulsed amperometric detector (PAD) operating in the integrated amperometry mode, AS50 autosampler and GS50 gradient pump. The system was also equipped with a CarboPac PA1 (4 × 250 mm) analytical column and a CarboPac PA1 (4 × 50 mm) guard column (Dionex Corp., Sunnyvale, CA). The mobile phase was HPLC grade water (A), 1000 mM NaAc (B) and 500 mM NaOH (C). The following profile was used; 0-30 min, 80% A, 20% B; 30-35 min, 63% A, 17% B, 20% C; 35-40 min, 60% A, 20% B, 20% C; 40-41 min, 22.5% B, 20% C; 41-46 min, 80% B, 20% C; 47-65 min, 80% A, 20% C. The injection volume was 20 μl and the flow rate was maintained at 1.0 ml/min at room temperature. Peaks were identified using available sugar standards. Data analysis was performed using a Chromeleon V6.8 (Dionex).

5.3 Results and Discussion

5.3.1 Phenolic Compound Profile

Enzyme aided pressed (EAP) and aqueous enzymatic extracted (AEE) oils were analysed for their phenolic contents using HPLC-DAD. Four simple phenolic compounds were identified: *trans*-ferulic acid, vanillic acid, vanillin and *trans*-cinnamic acids (Figure 5.1). There were smaller peaks that could not be identified due to the limitation of the DAD detectors as they required known standards.

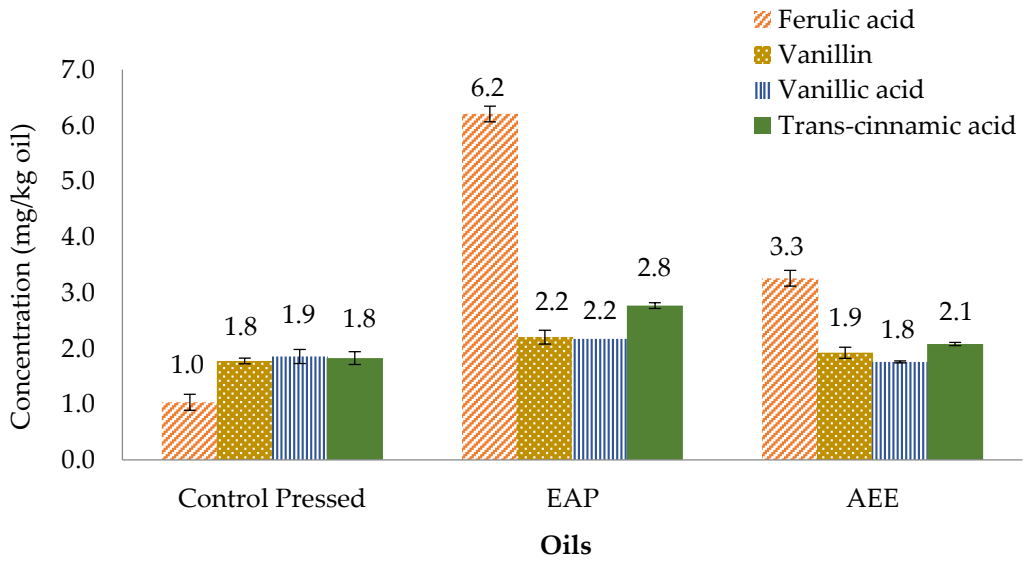


Figure 5.1: Phenolic profile of oils extracted using different methods; EAP: enzyme aided pressed (α-amylase, Alcalase and Viscozyme, enzyme concentration of 1%, incubation time of 6 h, S/L ratio 1:1.7, pressing time of 30 min, maximum pressure of 38 MPa); AEE: aqueous enzymatic extracted (α-amylase, Alcalase, and Celluclast, enzyme concentration of 1% (w/w), incubation time of 4 h, S/L ratio 1:4)

Phenolic compounds which have been identified previously in tiger nuts include *p*-coumaric acid, *p* -hydroxybenzaldehyde and *p* -hydroxybenzoic acid. The release of these compounds may require the decomposition of lignin which is a phenolic polymer.

Using enzymes as a pre-treatment increased the total concentration of some phenolic compounds, most especially for *trans*-ferulic acid which had the most concentration of the

phenolic compounds at 6.2 mg/kg in EAP. The hemicellulase mixture, Viscozyme is most likely responsible for the increase in *trans*-ferulic acid as it exists as an esterified component of tiger nut cell walls (Parker *et al.*, 2000). Ferulic acid is often found esterified to arabinoxylans in grasses. Cell walls can be considered as composites enclosing polysaccharide polymers such as cellulose in a cross-linked lignin cage, the lignin being covalently linked to polysaccharides such as arabinoxylans which in turn are esterified to ferulic acid and its dimers. This results in a strong network forming the structure of cell walls. Diferulic acid crosslinks with hemicellulose in particular makes cell wall mechanically rigid (Krishnamurthy, 1999). Enzymatic hydrolysis by xylanase and arabanase present in Viscozyme would thus result in the release of bound ferulic acid.

In AEE extracted oil, the quantities of phenolic compounds were less than EAP oils with *trans*-ferulic acid about half of that present in EAP oils. For AEE, Celluclast was used instead of Viscozyme, which may explain the lower ferulic acid content. Vanillin has been identified previously in roasted tiger nut oil (Lasekan, 2012) as one of the key compounds responsible for the sweet vanilla aroma of the oil. This property of tiger nut oil can be taken advantage of, both for cosmetic and edible food applications. Vanillin contents were similar in all oil samples examined.

5.3.2 Total Polyphenolic Content

Amongst the oils assessed, HPP-AEE oil contained the largest concentration of polyphenolic compounds (shown in Figure 5.2). The impact of high pressure appears to have greatly enhanced the release of phenolic compounds into the aqueous enzymatic extracted oil. On the other hand, the use of enzyme as a pre-treatment decreased the total polyphenolic content (TPC) in pressed oil. The vacuum drying step may have contributed to the loss of polyphenolic compounds either via action of oxidative enzymes such as polyphenol oxidase or thermal degradation. Suvarnakuta *et al.* (2011) observed that xanthones, a group of

polyphenols in mangosteen decreased significantly when vacuum dried at 60 °C while hot air drying and vacuum drying at higher temperatures retained more of the polyphenols. The lower temperature of 50 °C used during vacuum drying of tiger nuts paste after enzyme hydrolysis, before pressing may be insufficient to inactivate these enzymes, making it more likely for oxidative degradation to occur thus resulting in lower TPC in EAP oil. TPC has been linked to the antioxidant capacity of oils as polyphenols act as free radical scavengers. HPP-AEE oil may thus have a higher antioxidant capacity than the other oils extracted.

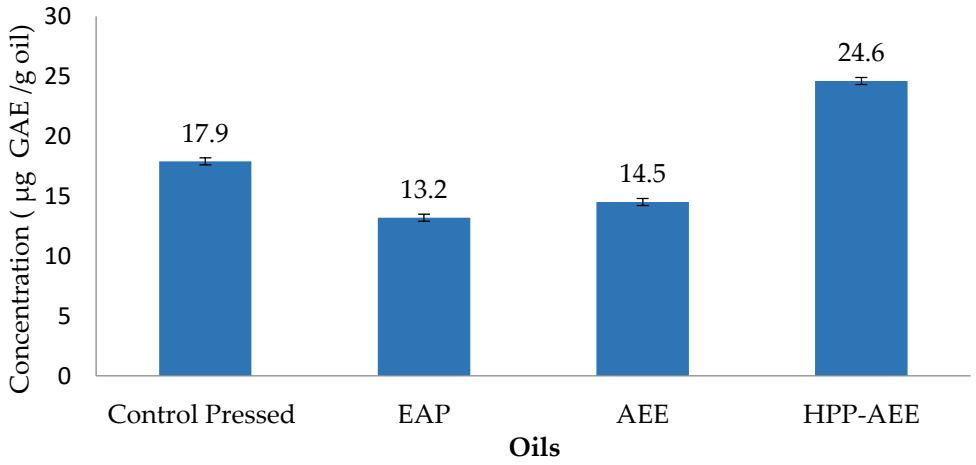


Figure 5.2: Total polyphenol content of oils extracted using different methods; EAP: enzyme aided pressed oil (α-amylase, Alcalase, and Viscozyme, enzyme concentration of 1%, incubation time of 6 h, S/L ratio 1:1.7, pressing time of 30 min, maximum pressure of 38 MPa); AEE: aqueous enzymatic extracted oil (α-amylase, Alcalase, and Celluclast, enzyme concentration of 1% (w/w), incubation time of 4 h, S/L ratio 1:4); HPP-AEE: High pressure processing-Aqueous enzymatic extracted oil (HPP for 20 min at 25 °C)

5.3.3 Tocopherol Content

The α-tocopherol content in EAP oil was higher than that of control pressed oil. The use of enzymes in pressing likely increased the amount of tocopherol released due to hydrolysis of cellular structures. The highest quantity of α-tocopherol was present in HPP-AEE oil similar to TPC (Figure 5.3). HPP as a pre-treatment increased oil yields by increasing starch hydrolysis which was found to affect oil yields positively. This allowed easier access of oil bodies out of tiger nut cells and consequentially, may have contributed to the increase in

tocopherol content. β-Tocopherol content was similar in all samples. In general, β-Tocopherol was much less than α-tocopherol for all samples. Tocopherols have been described as the most important natural antioxidant found in vegetable oils (Frankel, 1998). This is not surprising as it participates in different pathways as an antioxidant including acting synergistically with ascorbic acid with the end result of preventing lipid peroxidation. Of the two identified tocopherols, α-tocopherol is the only one that contributes to daily human Vitamin E requirements (Insel *et al.*, 2014), as it is preferentially absorbed and incorporated into membranes by the body.

The quantities of α-tocopherol found in tiger nut oil (except AEE oil) exceed those measured in canola (117-125 mg/kg), soybean (64-75 mg/kg) and corn oil (122-129 mg/kg), the oils most commonly found in supermarkets (Grilo *et al.*, 2014), and hence used by consumers, restaurants and in the frying industry. Consequently, tiger nut oil can be used in the same manner and even expanded to be employed in skin care as a source of Vitamin E.

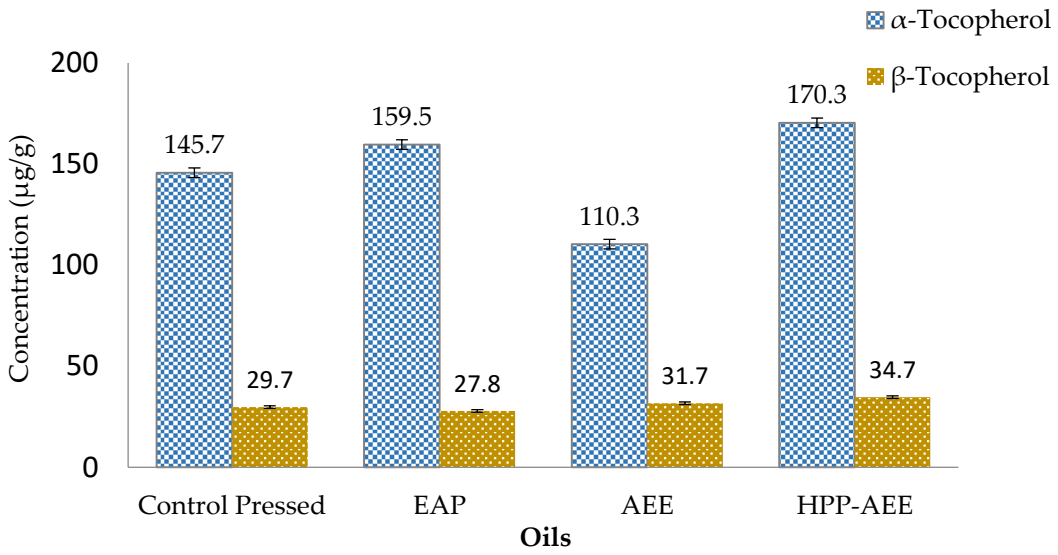


Figure 5.3: Tocopherol content of extracted oils using different methods; EAP: enzyme aided pressed oil (α-amylase, Alcalase and Viscozyme, enzyme concentration of 1%, incubation time of 6 h, S/L ratio 1:1.7, pressing time of 30 min, maximum pressure of 38 MPa); AEE: aqueous enzymatic extracted oil (α-amylase, Alcalase and Celluclast, enzyme concentration of 1% (w/w), incubation time of 4 h, S/L ratio 1:4); HPP-AEE: High pressure processing-Aqueous enzymatic extracted oil (HPP for 20 min at 25 °C)

5.3.4 Fatty Acid Profile

The fatty acid (FA) profiles for oils are shown in Table 5.2. There was minimal variability between all samples extracted and pre-treatments did not seem to affect the concentration of the acids. Oleic acid was the most abundant FA and the main monounsaturated FA present. As was mentioned before, when describing the FA content of pressed oil only, minor quantities of myristic, alpha-linolenic acid and arachidic acids were found in all oils making them comparable to previously reported values for tiger nut oil (Linssen *et al.*, 1988). All samples also have the same benefits already described for pressed tiger nut oil with regards to their high concentrations of monounsaturated FAs.

Table 5.2: Fatty Acid Profile of Oils extracted with EAP, AEE and HPP-AEE methods

Fatty Acid	Control Pressed (%)	EAP (%)	AEE (%)	HPP-AEE (%)
Myristic	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00
Palmitic	13.5 ± 0.00	14.5 ± 0.08	13.9 ± 0.06	13.7 ± 0.00
Palmitoleic	0.3 ± 0.00	0.3 ± 0.00	0.1 ± 0.09	0.3 ± 0.00
Stearic	6.3 ± 0.03	6.6 ± 0.08	6.4 ± 0.04	6.2 ± 0.03
Oleic	67.4 ± 0.07	66.0 ± 0.04	66.1 ± 0.07	66.0 ± 0.41
Linoleic	10.7 ± 0.05	11.0 ± 0.07	11.6 ± 0.05	12.0 ± 0.05
Alpha-linolenic	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.00	0.1 ± 0.00
Arachidic	0.7 ± 0.01	0.6 ± 0.01	0.7 ± 0.01	0.7 ± 0.01
Eicosenoic	0.1± 0.00	0.1± 0.03	0.1± 0.00	0.1± 0.00
Lignoceric	0.2 ± 0.01	0.2 ± 0.01	0.3 ± 0.01	0.3 ± 0.01
Unknown	0.4 ± 0.02	0.4 ± 0.03	0.4 ± 0.02	0.4 ± 0.02

Values are expressed as means and standard deviations. EAP: enzyme aided pressed oil (α-amylase, Alcalase and Viscozyme, enzyme concentration of 1%, incubation time of 6 h, S/L ratio 1:1.7, pressing time of 30 min, maximum pressure of 38 MPa); AEE: aqueous enzymatic extracted oil (α-amylase, Alcalase and Celluclast, enzyme concentration of 1% (w/w), incubation time of 4 h, S/L ratio 1:4); HPP-AEE: High pressure processing-Aqueous enzymatic extracted oil (HPP for 20 min at 25 ^{o}C)

5.3.5 Free Fatty Acid and Peroxide Values

The percentage of free fatty acids is one good indicator of the sensory quality of the oil since high values indicate rancidity by hydrolysis. All samples had a percentage value lower than 2.0% thus meeting the criteria set for virgin olive oils set by the International Olive Oil Council (IOC). However, they did exceed the % FFA standard for extra virgin olive oil of <0.8% with the exception of AEE oil (International Olive Oil Council, 2015). Storage conditions and time between harvests of tiger nuts, drying time and their purchase are unknown and hydrolytic reactions leading to free fatty acid production may have already begun in the nuts, since there were no significant difference between oils subjected to different treatments.

Table 5.3: Free Fatty Acid and Peroxide Values of extracted Oils with EAP, AEE and HPP-AEE methods

	Control Pressed	EAP	AEE	HPP-AEE
% FFA	1.80 ± 0.19	1.70 ± 0.01	1.63 ± 0.09	1.82 ± 0.28
PV ($mEqO_2$/kg oil)	1.2 ± 0.02	1.68 ± 0.01	0.13 ± 0.13	1.38 ± 0.01

EAP: enzyme aided pressed oil (α-amylase, Alcalase and Viscozyme, enzyme concentration of 1%, incubation time of 6 h, S/L ratio 1:1.7, pressing time of 30 min, maximum pressure of 38 MPa); AEE: aqueous enzymatic extracted oil (α-amylase, Alcalase and Celluclast, enzyme concentration of 1% (w/w), incubation time of 4 h, S/L ratio 1:4); HPP-AEE: High pressure processing-Aqueous enzymatic extracted oil (HPP for 20 min at 25 ^{o}C)

The peroxide values (PV) on the other hand all fall well below PV standards set by IOC for olive oil of <20 $mEqO_2$ per kg oil. The low values also suggest low progression of any oxidative rancidity in the oils. Enzyme pre-treatment prior to pressing increased PV slightly, while HPP had a larger effect on AEE oil. Peroxide values measured in this study were much lower than those reported for tiger nut oil (5.54 $mEqO_2$/kg oil) by Yeboah *et al.* (2012).

5.3.6 Residual Meals

5.3.6.1 Chemical analysis

Table 5.4 shows the composition of tiger nut meals after oil extraction. AEE had the highest oil and lowest protein content. The high oil content in the meal is a consequence of the lower oil extracted with AEE compared to EAP while the lower protein content might be due to a larger degree of protein hydrolysis. With the higher solid-liquid ratio used in AEE compared to EAP, more water is available for protein hydrolysis to take place effectively. Similar reasoning may also explain the lower carbohydrate content including the fact that a different carbohydrase was used.

Table 5.4: Composition of tiger nut meals (dry basis) from oil extraction

Sample	Control Pressed	EAP	AEE
Crude fat	8.0 ± 0.80	5.1 ± 0.02	20.3 ± 1.29
Crude protein	4.1 ± 0.21	4.6 ± 0.19	2.4 ± 0.04
Ash	2.6 ± 0.01	3.2 ± 0.04	1.9 ± 0.41
Total Carbohydrate	74.7 ± 0.23	76.4 ± 0.33	65.8 ± 0.15

EAP: enzyme aided pressed oil (α-amylase, Alcalase and Viscozyme, enzyme concentration of 1%, incubation time of 6 h, S/L ratio 1:1.7, pressing time of 30 min, maximum pressure of 38 MPa); AEE: aqueous enzymatic extracted oil (α-amylase, Alcalase and Celluclast, enzyme concentration of 1% (w/w), incubation time of 4 h, S/L ratio 1:4)

EAP with the highest protein and lowest oil content reflects the highest oil yield that was achieved with this method of oil extraction. Notwithstanding the method used, the protein contents in all samples were lower than those in major oil seed meals such as soybean, canola, and sunflower with typical protein content of 47.5, 35.6, and 42.2% respectively (Bajjalieh, 2004). One should bear in mind that tiger nut is a tuber with a low protein content of 3.2% so its meals would also have low protein levels. As a consequence, tiger nut meals would be deemed unsuitable for animal or fish feeding.

5.3.6.2 Sugars in residual meals

To assess a prospective use for the by-products of the oil extraction process, the soluble sugars in the residual meals were determined. Meals remaining from EAP and AEE were the only samples found to contain peaks that appeared to be sugars with low degree of polymerisation (DP). Both DP3 and DP4 oligosaccharides were present in EAP meal (Figure 5.4) and only DP3 in AEE meal (Figure 5.5). Both enzyme treated meals contained sucrose, fructose and glucose as shown in Table 5.5. CP meal only had sucrose present. Using HPAEC-PAD, the sugars coincided with the peak times of the standards nystose and 1-kestose (Figure 5.6 and 2-A in Appendix 2). These are fructo-oligosaccharides (FOS) comprising of linear chains of α-D-glucopyranosyl-[β-D-fructofuranosyl] n-1-β-fructofuranoside (GFn) (Yıldız, 2010) so contain sucrose units. Higher quantities of sugars with similar DP as 1-kestose were quantified in the EAP meal using the BioRad column as shown by the areas of the peaks from the HPAEC-PAD profiles. Traces of higher DP molecules were observed in the HPAEC-PAD profile of EAP meals that were not completely resolved using the BioRad column and would thus require standards for verification.

The results in Table 5.5 suggest that the actions of enzymes raised the yield of soluble higher DP sugars in the meals. The difference between EAP and AEE samples reflects the different carbohydrase used. EAP employed Viscozyme, a mixture of carbohydrases including xylanase, arabanase and hemicellulase while AEE was done with Celluclast, predominantly a cellulase. Having an array of different enzymes in Viscozyme allowed for hydrolysis of different components of tiger nut cell walls, and helped to increase the release of short chain sugars.

Pollard (1982) detected FOS in plants belonging to the Cypereae tribe, the same that tiger nuts belong to, a tribe being a taxonomy rank between family and genus. Although Fuchs (2012) concurred with this, by listing *Cyperus esculentus* as a plant containing FOS, it should

be noted that the short chain sugars found in the meals may appropriately be products of polysaccharides degradation. During the enzymatic treatment, starch for example was hydrolysed and products such as the oligosaccharide maltotriose with a DP of 3 could be released. Arabinoxylan oligosaccharides may be another possible explanation for these sugars. In general, plant oligosaccharides are considered to be beneficial to human health including the immune system. They may stimulate the growth of bifidobacteria and lactobacilli as well as the production of short chain fatty acids by the gut microbiota (Moreno and Sanz, 2014).

Table 5.5: Sugars in residual meals of tiger nuts after oil extraction

	Mean ± SD (mg/g, dry basis)				
Sample	Fructose	Glucose	Sucrose	DP3	DP4
Control	<dl	<dl	246.5 ± 1.02	<dl	<dl
EAP	20.4± 0.48	63.1± 0.85	80.3± 0.45	73.2 ± 1.05	9.3± 0.60
AEE	30.8± 1.12	8.4± 1.08	50.0± 0.76	11.1± 0.65	0.0

dl: detection limit

EAP: enzyme aided pressed oil (α-amylase, Alcalase and Viscozyme, enzyme concentration of 1%, incubation time of 6 h, S/L ratio 1:1.7, pressing time of 30 min, maximum pressure of 38 MPa); AEE: aqueous enzymatic extracted oil (α-amylase, Alcalase and Celluclast, enzyme concentration of 1% (w/w), incubation time of 4 h, S/L ratio 1:4)

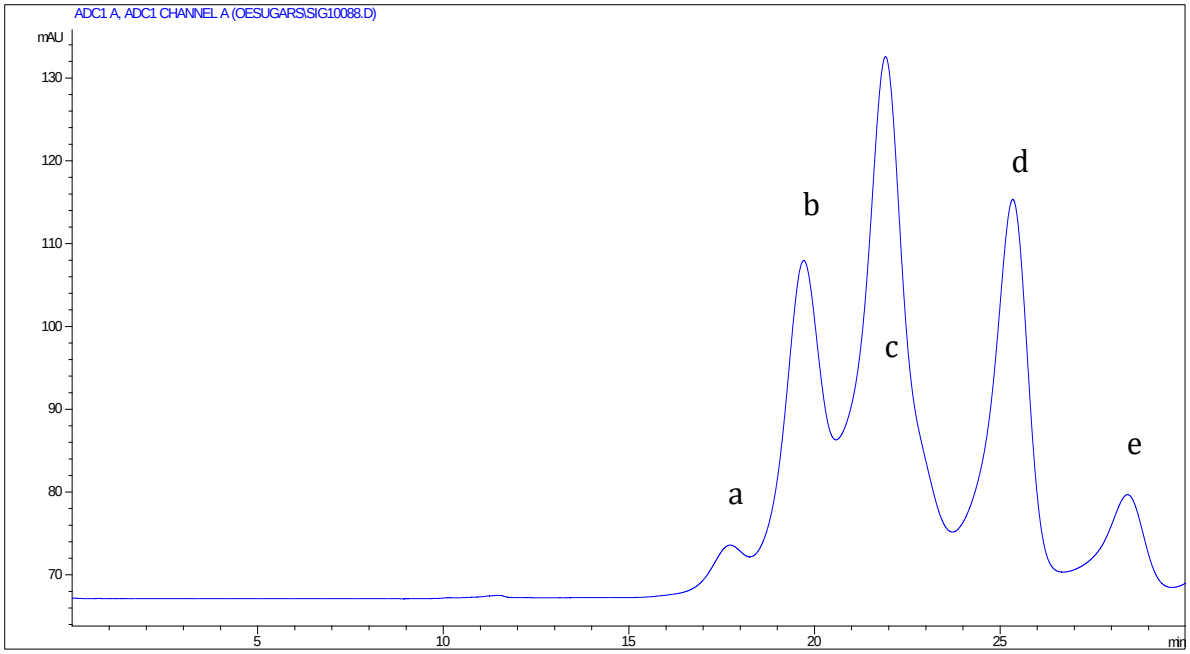


Figure 5.4: HPLC-RI Soluble sugars in EAP meal: a, DP4; b, DP3; c, sucrose; d, glucose; e, fructose. EAP: enzyme aided pressed oil (α-amylase, Alcalase and Viscozyme, enzyme concentration of 1%, incubation time of 6 h, S/L ratio 1:1.7, pressing time of 30 min, and maximum pressure of 38 MPa)

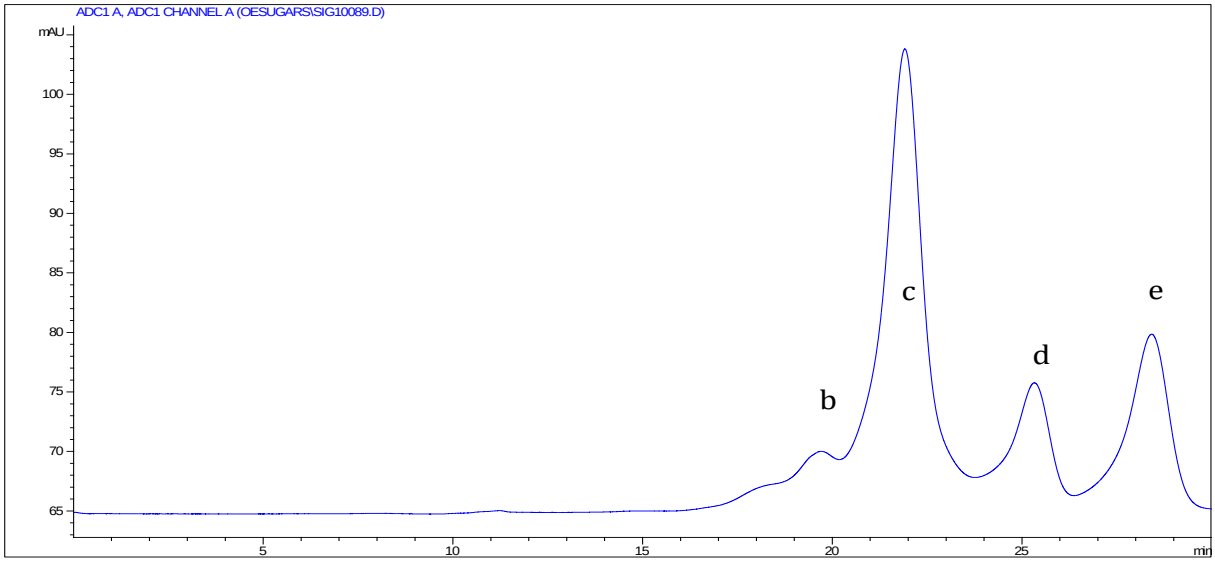


Figure 5.5: Soluble sugars in AEE meal: b, DP3; c, sucrose; d, glucose; e, fructose. AEE: aqueous enzymatic extracted oil (α-amylase, Alcalase and Celluclast, enzyme concentration of 1% (*w*/*w*), incubation time of 4 h, S/L ratio 1:4)

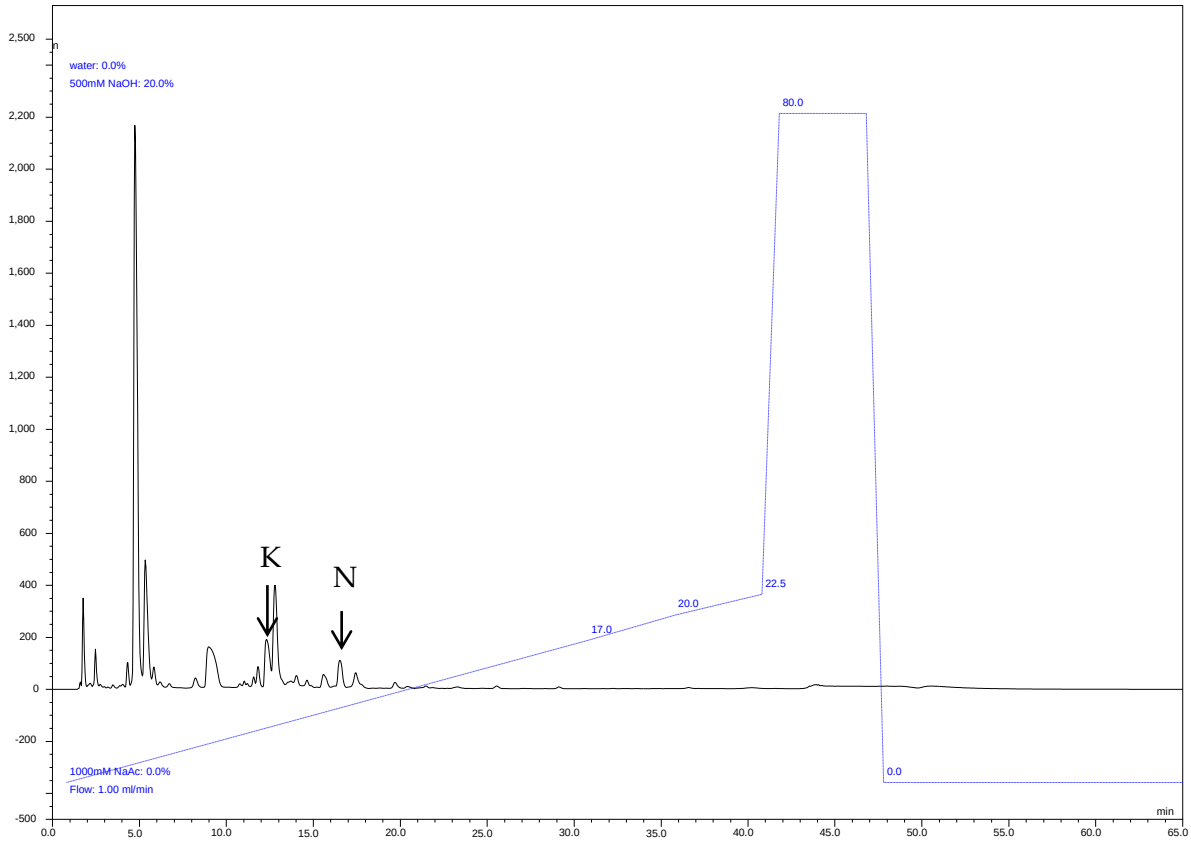


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Figure 5.6: HPAEC-PAD profile of EAP meal: K, 1-kestose, N, nystose. EAP: enzyme aided pressed oil (α-amylase, Alcalase and Viscozyme, enzyme concentration of 1%, incubation time of 6 h, S/L ratio 1:1.7, pressing time of 30 min, and maximum pressure of 38 MPa)

Nonetheless, the oligosaccharides in tiger nut meal would require further qualitative assessment such as enzyme assays to confirm their identity. An evaluation of their functional characteristics and effects on human health and well-being would also be required in order to safely recommend them for use. Tiger nut residual meals may potentially serve as a source of possible valuable prebiotics.

5.4 Conclusion

Oils extracted using EAP had an increased content of key bioactive compounds such as tocopherol and some phenolic acids. HPP-AEE oil also had higher tocopherol and TPC content than AEE oil. The quality parameters (% FFA and PV) of the oils indicated that they were all less than the IOC recommended values for virgin olive oil, and thus are good quality and quite stable oils. Their fatty acid profiles remained unchanged by extraction method.

Residual meals obtained from the process contained low protein contents (2.4-4.6%). Sugar analysis of EAP and AEE samples showed that they contained 82.5 and 11.1 mg/g (DP3 and DP4) respectively. Quantitative analysis would be required to assess the identity and quality of these oligosaccharides and their effect on human health.

Chapter 6

CONCLUDING REMARKS AND FUTURE RESEARCH

The anomaly that tiger nut is an oil containing tuber makes it an interesting crop to study. This, coupled with the fact that it is commonly grown in a large number of countries in Africa which stand to benefit from research into its use, makes it even more fascinating. Most other tubers are typically known to contain high quantities of starch and little or no fat. Therefore, *Cyperus esculentus* L. plant, with its hard swollen endings (tiger nut tubers) containing similar percentages of starch and fat, poses an unusual case study for oil extraction.

Even though tiger nut is not a conventional oilseed, established methods of oil extraction have been shown to be feasible with the tuber. In line with the first hypothesis investigated; the mechanical extraction of tiger nut oil can be enhanced with the use of enzymes and HPP treatments, particle size and moisture content were factors found to influencing mechanical extraction, which was consistent with literature reports. As a pre-treatment option, the use of high pressure processing (HPP) was not viable as it did not have any destructive effect on the cell walls of tiger nuts in order to reduce barriers to oil release during pressing. Hence, the oil yields were not affected. On the other hand, incubating the tubers with a combination of Alcalase, α-Amylase and Viscozyme gave the largest oil yield and 90% recovery. The relatively high content of starch led to the use of α-amylase and a protease, while Viscozyme, a hemicellulase mixture was added to aid in softening cell wall structure for the pressing process. It appeared then that mechanical extraction oil yields could be improved with enzyme treatments but not HPP. Employing enzymes as a processing aid can be adopted as a way to improve traditional mechanical extraction of oil, especially as it preserves bioactive

compounds in the oils. The drawback to this is the feasibility of adopting vacuum drying on a larger scale.

Another mode of oil extraction that has been researched extensively is the aqueous enzymatic extraction (AEE) process and this was investigated in the second hypothesis tested: aqueous enzymatic extraction of tiger nut oil can be improved by using a combination of enzymes and HPP treatments. When different enzymes were applied to tiger nuts, α-Amylase had the most effect in increasing oil yields followed by Alcalase. Of the three carbohydrases used, Viscozyme did not significantly improve oil yields. Not surprisingly, soaking the tubers before incubation with enzymes aided oil extraction due to hydration of both starch and cellulolytic materials of the tubers. This hydration seemed to be beneficial for both α-Amylase and Celluclast activities as both starch and cellulase become hydrated and undergo an increase in surface areas. Due to the unique nature of tiger nut, different operational factors were then investigated to evaluate their effect on oil yields and a mixture of Alcalase, α-Amylase and Celluclast was used. Typically, a longer hydrolysis time and increase in solid-liquid ratio led to larger oil yields. Longer incubation time however affected the oil recovery as more time allowed the formation of stable emulsions. Increasing the enzyme concentration up to 1% (*w*/*w*) also increased oil yields. The effect of agitation speed during hydrolysis gave an unexpected result where agitation decreased on oil yield, and the best oil yield was achieved when the solid-liquid mixture was held stationary during incubation. This defies known theories on mass transfer as agitation is expected to enhance mass transfer by ensuring thorough mixing and promoting diffusion which in turn increases hydrolysis rates and then oil yields. However in this case, agitation led to a gravitation separation where solids were observed to settle at the bottom of the incubation bottles. This settling prevented sufficient mixing and eventually enzymatic hydrolysis and oil yields. As already stated, tiger nut is not a conventional oilseed but a tuber, hence this unexpected effect reflects the nature of the crop.

Application of high pressure processing to the tuber prior to aqueous enzymatic oil extraction was studied as a processing aid. The oil yield was increased with pressure up to 300 MPa, probably due to some cavities being induced on starch granules which could have enhanced α-amylase activity. The increased enzyme activity led to an increase in starch degradation which meant that one of the barriers (others include cell wall) to oil's mobility was being reduced and an increase in oil extraction yields. However, greater pressures had a detrimental effect on oil yields. High pressure processing stands as a possible tool to improve enzymatic hydrolysis of starch, and is thus not limited to the oil extraction processes. Its economic viability would though need to be assessed.

Finally, the last hypothesis that enzymes and HPP as pre-treatments do not affect the quality of oils extracted using mechanical pressing and aqueous enzymatic extraction was proved to be true. The oils obtained from both enzyme assisted pressing and aqueous enzymatic extraction, exhibited higher tocopherol and total polyphenolic content than control samples, particularly in the case of HPP-AEE oils. All extracted oils were deemed stable based on their low percentage of free fatty acid and low peroxide values. The meals obtained from EAP and AEE would not be suitable for animal feed as is commonly the case for oilseed meals, because of their low protein content. On the other hand they could potentially act as sources of low DP oligosaccharides but the identities of these sugars would need confirmation. The meals could also be employed as a source of energy for a number of different applications such as biofuel production. Another part of the tiger nut is the husk or outer skin layer removed during sieving which amounts on average 23.6% of the initial ground sample. Quite likely, this layer would be discarded during most processing, but analysis showed that it also contained simple sugars, mainly sucrose (144.5 mg/g). The presence of sugars and the high fibre content can be exploited in the production of bioethanol.

To conclude, tiger nut oil can be extracted using both mechanical pressing and aqueous enzymatic processing methods, with the aid of enzymes and high pressure processing as pre-treatments respectively. Most oil was obtained when enzyme assisted pressing was utilised. Although, it should be noted that aqueous enzymatic extraction poses more of a challenge due to complex emulsion formation and limitations which arise from the presence of starch which limits the release of oil.

The study raises questions that require further investigation in order to provide answers:

- What are the effects of longer hydrolysis time with both mechanical pressing and aqueous enzymatic extraction on oil yields?
- Would employing other cell wall degrading enzymes such as pectinase enhance oil yields using both extraction methods?
- Could an optimisation study with different factors for aqueous enzymatic extraction further increase oil yields?
- What is the feasibility of increasing the scale of the EAP processing and could a pilot plant study be designed?
- Can different genotypes of tiger nuts be screened for more oil and less starch and how would this affect extraction yields?
- What are the potential oligosaccharides present in the meals and their effects on human health?

Research focused on answering these questions is thus highly recommended as it would greatly contribute to the sparse but growing body of knowledge on tiger nut and its related products.

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Appendix 1

Table 1-A: ANOVA for effect of temperature, pH and enzyme ratio on AEE oil yield

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	18	0.290635	0.016146	10.02	0.001
Linear	6	0.196143	0.032690	20.29	0.000
Ratio	2	0.010373	0.005186	3.22	0.094
pH	2	0.180364	0.090182	55.96	0.000
Temp	2	0.005406	0.002703	1.68	0.246
2-Way Interactions	12	0.094492	0.007874	4.89	0.016
Ratio*pH	4	0.018471	0.004618	2.87	0.096
Ratio*Temp	4	0.005130	0.001282	0.80	0.560
pH*Temp	4	0.070892	0.017723	11.00	0.002
Error	8	0.012891	0.001611		
Total	26	0.303526			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0401423	95.75%	86.20%	51.62%

(Enzyme mixture of α-amylase, Alcalase and Celluclast was used. Enzyme concentration of 0.5% (*w*/*w*), particle size of ≤300 μm, incubation time of 6 h, S/L ratio of 1:4)

Appendix

Table 1-B: Minitab Output for predicting pH and temperature for maximum oil yield.

Parameters

Response	Goal	Lower	Target	Upper	Weight	Importance
Oil Yield	Maximum	0.334305	0.773762		1	1

Solution

Solution	pH	Temp	Oil Yield Fit	Composite Desirability
1	8	40	0.741469	0.926515

Multiple Response Prediction

Variable	Setting
pH	8
Temp	40

Response	Fit	SE Fit	95% CI	95% PI
Oil Yield	0.7415	0.0295	(0.6796, 0.8034)	(0.6177, 0.8653)

Table 1-C: Experimental Design with Results (3^3 Full Factorial Design with no replication)

StdOrder	RunOrder	PtType	A(Ratio)	B(pH)	C(temp)	solid weight	Bottle weight Before	Bottle weight After	OIL	Oil extracted (g)	Oil Yield(%)
3	1	1	(1:1:1)	4	50	16.87	94.8705	98.1865	3.316	4.1664	55.68%
22	2	1	(1:2:2)	5	40	14.1	98.0736	100.5257	2.4521	5.0303	67.23%
26	3	1	(1:2:2)	8	45	13.21	98.0807	99.9208	1.8401	5.6423	75.41%
25	4	1	(1:2:2)	8	40	13.19	101.1927	103.37	2.1773	5.3051	70.90%
9	5	1	(1:1:1)	8	50	13.81	105.869	109.0224	3.1534	4.329	57.86%
19	6	1	(1:2:2)	4	40	16.14	101.258	104.5722	3.3142	4.1682	55.71%
4	7	1	(1:1:1)	5	40	14.45	110.9177	113.4229	2.5052	4.9772	66.52%
13	8	1	(1:1:2)	5	40	14.11	98.0775	100.6915	2.614	4.8684	65.06%
27	9	1	(1:2:2)	8	50	13.74	98.8579	101.6534	2.7955	4.6869	62.64%
20	10	1	(1:2:2)	4	45	17.67	101.1996	105.2414	4.0418	3.4406	45.98%
5	11	1	(1:1:1)	5	45	15.89	101.2692	104.0417	2.7725	4.7099	62.95%
24	12	1	(1:2:2)	5	50	15.36	105.5214	108.2044	2.683	4.7994	64.14%
17	13	1	(1:1:2)	8	45	13.72	110.8646	112.7938	1.9292	5.5532	74.22%
18	14	1	(1:1:2)	8	50	13.95	98.859	101.1855	2.3265	5.1559	68.91%
23	15	1	(1:2:2)	5	45	15.86	94.8534	97.4158	2.5624	4.92	65.75%
6	16	1	(1:1:1)	5	50	14.4	106.9574	109.5094	2.552	4.9304	65.89%
14	17	1	(1:1:2)	5	45	15.83	107.3969	110.068	2.6711	4.8113	64.30%
11	18	1	(1:1:2)	4	45	17.35	86.7263	90.7108	3.9845	3.4979	46.75%
12	19	1	(1:1:2)	4	50	16.63	97.4527	100.8305	3.3778	4.1046	54.86%
15	20	1	(1:1:2)	5	50	16.87	105.5116	108.6382	3.1266	4.3558	58.21%
2	21	1	(1:1:1)	4	45	18.39	97.4591	102.4401	4.981	2.5014	33.43%
10	22	1	(1:1:2)	4	40	15.9	110.9146	114.7957	3.8811	3.6013	48.13%
8	23	1	(1:1:1)	8	45	14.44	101.2024	103.3929	2.1905	5.2919	70.72%
1	24	1	(1:1:1)	4	40	16.29	101.2734	105.3719	4.0985	3.3839	45.22%
7	25	1	(1:1:1)	8	40	13.62	98.1172	99.81	1.6928	5.7896	77.38%
21	26	1	(1:2:2)	4	50	13.02	106.9469	109.1171	2.1702	5.3122	71.00%
16	27	1	(1:1:2)	8	40	13.4	110.9518	112.885	1.9332	5.5492	74.16%

α- Amylase, Alcalase and Celluclast, enzyme concentration of 0.5% (*w*/*w*), particle size of ≤300 μm, incubation time of 6 h, S/L ratio of 1:4

Appendix 2

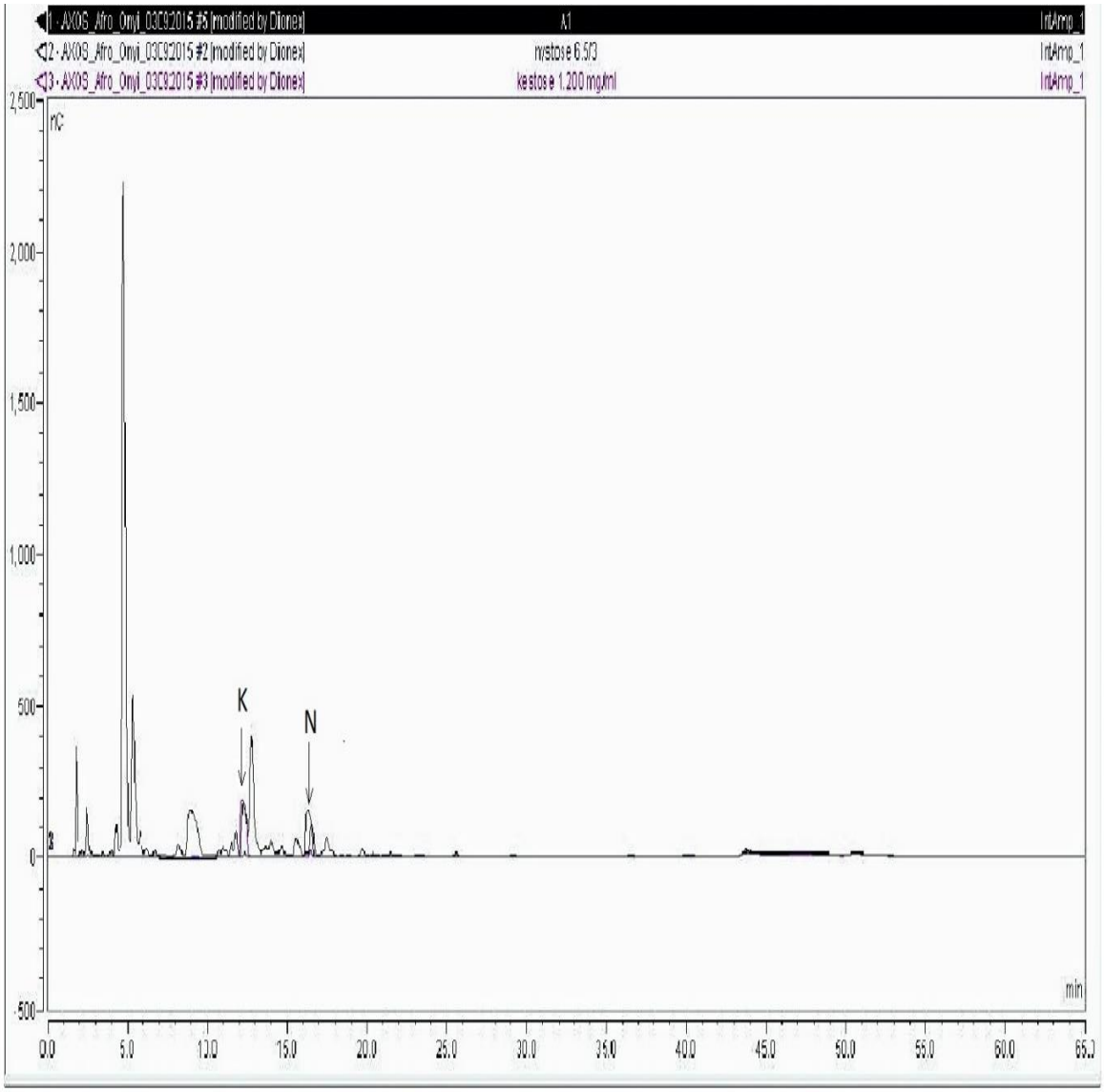

Figure 2-A: HPAEC-PAD profile showing overlap of FOS standards with EAP sample; K, 1-kestose, N, nystose (α-amylase, Alcalase and Viscozyme, enzyme concentration of 1%, incubation time of 6 h, S/L ratio 1:1.7, pressing time of 30 min, maximum pressure of 38 MPa)