Supercritical Fluid Extraction of *Sclerocarya birrea* Kernel Oil

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Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Chemical Engineering at the Potchefstroom Campus of the North-West University

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May 2015

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ABSTRACT

Sub-Saharan Africa is a treasure chest of natural materials remaining to be explored for commercial applications and as alternative foods to diversify and improve food sustainability. The Marula tree is available in abundance in South Africa and bears a fruit with a highly nutritious kernel containing high oil and protein content. The oil from the kernels has various applications from food to cosmetics. The accepted oil processing practice is required to be a green technology, producing no effluent or using toxic solvents. Therefore, the oil is extracted using an expeller. However, with average 55 wt. % oil in the kernel the extracted oil yield is far from optimal, typically ranging from as low as 7 wt. % to 47 wt. %. The latter is obtained only with proprietary modified expellers. Therefore, an alternative green technology which retains the native characteristics of the Marula oil is needed. Communication with local producers, South African and Namibian, confirmed the need for investigation of an alternative means of extraction of Marula oil from the seed kernels which can improve the yield and potentially the quality of the oilcake. The latter of which is typically adversely affected by the expelling process.

A review of various processing technologies available for oil extraction was completed and supercritical fluid extraction utilizing carbon dioxide as the extraction solvent was identified as a potential solution. An overview on supercritical fluid extraction using carbon dioxide (SFE-CO_2) of similar materials to the Marula kernels, such as hazelnuts, walnuts and pine kernels indicates that yields similar to that of solvent extraction and of the quality of the oils obtained by cold pressing can be obtained with the technique. The theory, practical applications, and how one can use the system to improve yield from various natural materials were reviewed. It was determined that the two main parameters one can manipulate on supercritical extraction systems to optimize the yield, were pressure and temperature.

Subsequently kernels of the *Sclerocarya birrea* tree, common name Marula, cultivated in South Africa, were obtained for extraction with supercritical carbon dioxide. The effects of pressure and temperature on extraction yield were investigated. The total maximum yield of Marula kernel oil obtained was found to be 54 wt. %, compared to a solvent extracted yield of 52 wt. %, such that a 100 % oil recovery was obtained with SFE-CO_2. The optimal conditions were found to be 450 bar and 60 °C as the yield per kg solvent initially was 41 g kg^{-1} CO_2.

Following the extractions, the oils were characterized for fatty acid composition using gas chromatography. Quality parameters of a cold pressed sample and a sample obtained at the optimal extraction conditions were determined and compared; and the results indicate that the two oils are of similar composition and quality.

Supercritical fluid extraction using carbon dioxide was successfully verified as a potential processing method for the extraction of Marula oil from the kernels. The SFE-CO_2 provided an improved yield compared to cold pressing and a quality of oil similar to cold pressed Marula oil. Additionally, after SFE-CO_2 processing, the defatted Marula kernels contain high protein content, 69 wt. %, in the form of a pure white powder. Due to the favourable nutritional content the residue may be used for human consumption to create new products such as meat analogues, porridges, and shakes, or can be sold as a high protein powder.

Key words: Marula, supercritical, *Sclerocarya birrea*, oil, quality, yield, carbon dioxide, protein, protein powder, SFE, SFE-CO_2.

ACKNOWLEDGEMENT

I hereby express my gratitude to the people who made this research project possible through their willingness to take time to provide guidance, support and inspiration:

My family, whose love, support and patience throughout my entire life have enabled me to achieve all my personal and academic successes leading up to this;

Dr. Zarko Krkeljash, the love of my life, for providing inspiration, love and support, without which I would not have been here;

Prof. Johan Markgraaff, whose on-going support, patience, guidance and concern, for both my personal and academic success, shaped and guided this thesis, and inspired further interest in Material Science;

Prof. LJ Grobler, for the financial support and provision of facilities which enabled this project to come to fruition;

Prof. Manie Vosloo, for the on-going support with the chemical analyses and guidance; and

Dr. Louwrens Tiedt and Dr. Anine Jordaan, Light and Electron Microscopy Lab, for their assistance with the microscopy images and providing insight into understanding the cellular structure of the Marula kernel.

ACRONYMS

AEE	Aqueous Enzymatic
AH	Afrinatural Holdings
ALASA	Agri Laboratory Association of Southern Africa
AOAC	AOAC International
AOCS	In International Society for the Science and Technology of Fats, Oils and Related Material
AOCS Methods	Official Methods and Recommended Practices of the American Oil Chemists' Society for fats, oils and soap technology.
BIC	Broken-Intact Cells
CODEX STAN	Codex Standard
df	Film Thickness (GC Column)
EC	European Community
ECCD	European Commission Cosmetic Ingredients Database (ECCD)
EN	EN Standards, European standards
EOS	Equation of State
EtOH	Ethanol
EU	European Union
FA	Fatty acid
FAO	Food and Agriculture Organization of the United Nations
FAME	Fatty acid methyl esters
FDA	Food and Drug Administration of the United States
FFA	Free Fatty Acid Value
FID	Flame Ionisation Detector
GC	Gas Chromatograph
GRAS	Generally Recognised as Safe
HPLC	High Performance Liquid Chromatography
IV	Iodine Value
ID	Internal Diameter (GC Column)

L	Column Length (GC Column)
MAE	Microwave Assisted
MAEE	Microwave and Aqueous Enzymatic
MCP	Marula Cosmetic Products
mEq	Miliequivalents
min	Minute
NATEX	Natural Process Technologies Gmbh
NMPT	Natural Marula Products
PLC	Programmable Logic Control System
PV	Peroxide Value
PUFA	Polyunsaturated Fatty Acids
SABS	South African Bureau of Standards
SC	Shrinking Core
$scCO_2$	Supercritical Carbon Dioxide
scFluid	Supercritical Fluid
SEM	Scanning Electron Microscope
SFE	Supercritical Fluid Extraction
$SFE\text{-}CO_2$	Supercritical Fluid Extraction using Carbon Dioxide as Solvent
WIPO	World Intellectual Property Organisation
WO	World Intellectual Property Organisation Patents

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND AND MOTIVATION

The Sub-Saharan region of Africa is home to a vast variety of flora and fauna with potential applications in medicines and cosmetics. The Marula tree, *Sclerocarya birrea (*also*, S.birrea)*, family *Anacardiaceae,* subspecies *caffra*, is native to the sub-Saharan region of Africa. Nearly all the parts of the Marula tree the bark, leaves, roots, fruit, and kernels have been utilized in some form in traditional cultures in Mozambique, Namibia, South Africa, Zimbabwe and Botswana (Vermaak *et al.* 2011; WIPO, 2010). The chemical composition and antimicrobial effects of the oil from the Marula kernels were summarized by Mariod *et al.* (2010). Published research indicates that of all the Marula tree components, the highest antioxidant activity is found in the Marula kernels (Mariod *et al.* 2008). Marula oil, in addition to being used as edible oil, is used as a component in cosmetic formulations for foundations, bronzers, moisturizers as well as body oils by several cosmetic companies, and is listed as a cosmetic ingredient in the European Commission Cosmetic Ingredients Database (ECCD).

In general, seed and kernel oils are typically extracted by either cold-pressing or solvent extraction, or a combination of the two processes. For organic processing of oils, cold pressing is the common acceptable processing technique as it does not produce effluents, compromise the intrinsic nature of the oil, or utilize toxic solvents. However, due to the low yield, there is a need for a more effective oil extraction method. Solvent extraction typically involves using a solvent such as hexane to extract the oil from a given seed or kernel matrix, with very good efficiency. However, solvent extraction is not a favorable technique due to the toxicity of the solvents typically used. The combination of the two processes, results in improved yield, such that the majority of the oil is pressed out of the raw material, and subsequent solvent extraction is used to recover the remaining oil.

1.2 PROBLEM STATEMENT

Research indicates that the total Marula kernel oil content, as attained by solvent extraction, is 50-65 % of the total kernel mass. However, the Marula oil is typically extracted with a press and the yield attained by pressing/expelling (both manual and electric) is as low as 7 wt. %, up to a maximum yield of 47 wt. % of total kernel mass. Therefore, a need exists for a more effective processing technique (Mariod & Abdelwahab 2012; Gandure & Kelogetswe 2011; Pradhan *et al.* 2010; Personal communication, Mr. V. Dhlamini 2013, Natural Marula Products Trust (NMPT) (Pty) Ltd.; Personal communication, Mr. A. Joubert 2014, Afrinatural Holdings (AH); Personal communication, Mr. A. Brink 2014, Marula Cosmetic Products (MCP); Personal communication, Mr. J. Visser 2014, Private Producer).

1.3 AIMS AND OBJECTIVES

This study aims to determine a suitable processing technique for optimizing extraction of oil found in the kernels of the Sub-Saharan tree, *Sclerocarya birrea* and to assess the quality of the oil extracted with the identified technology compared to cold pressed Marula oil.

To achieve the overall aim of the study to identify and evaluate an optimal processing technique to recover maximum oil content, while simultaneously retaining the integrity of the oil, the following objectives were identified:

1. Review of the *Sclerocarya birrea* (Marula) tree with respect to the applications of its components, with focus on the Marula oil.
2. Review of different processing techniques with respect to oil extraction from kernels and oilseeds.
3. Review of the selected techniques, assessment of different processes for optimization and evaluation of the chosen method.
4. Apply the selected method to Marula kernels to determine the optimal parameters at which the greatest percent of extractable oil can be recovered from the kernels.
5. Characterize the oil composition of the extracted Marula kernel oil.
6. Compare and assess the quality of the oil obtained against a traditionally processed sample with respect to the fatty acid profile and relevant physico-chemical characteristics.

CHAPTER 2: BACKGROUND AND LITERATURE REVIEW

2.1 MARULA

The Marula tree produces a round fruit, 3 cm in diameter, with a seed consisting of 3 kernels, on average 1.5 cm in length, and 0.5-1.0 cm in diameter (FAO 1988; Mariod & Abdelwahab 2012). Current processing practices in South Africa and Namibia involve combined efforts between local communities, entrepreneurs, and small companies, to collect the fruits and process them. The process is as follows:

- The fruits are collected in the wild.
- The fruits are washed and then utilized to make jams, jellies, liquor, wine, or juice.
- The seeds which remain are then cracked and the three kernels are manually removed from the shell.
- The kernels, which contain 50-65 % oil (kg oil kg^{-1} kernel) (Mariod & Abdelwahab 2012), are pressed to extract the oil. The results of the manual and electric pressing range from 7-47 % oil (kg oil kg^{-1} kernel) (Pradhan *et al.* 2010; Abu-Arabi *et al.* 2000; Swilling 2013; Personal Communication, Mr. Dlamini, NMPT; Personal Communication, Mr. Joubert, AH); Personal Communication, Mr. Brink, MCP; Personal Communication, Mr.Visser, Private Producer).

Figure 2.1 Eudafano Womens Cooperative oil press, Namibia (Swilling 2013).

Published literature indicates that screw pressing of oil from oilseeds or kernels requires multiple pressings followed by leaching with a solvent in order to extract all the available oil (Abu-Arabi *et al.* 2000). The maximum Marula oil yield obtainable with pressing is 47 wt. % (kg oil kg^{-1} seed) (Personal Communication, Mr. V. Dhlamini 2013, NMPT; Personal Communication, Mr. A. Brink 2014, MCP; Personal Communication, Mr. J. Visser 2014). According to Swilling (2013), at the Eudafano Women's Cooperative in Namibia, a large 40 kg bag of Marula kernels yields 12 L, or approximately 30 %, of oil. The actual values may be significantly lower than reported, as personal correspondence with local South African and Namibian producers indicates that the yield may be as low as wt. 7 % (Personal Communication, Mr. A. Joubert 2014, AH; Personal Communication, Mr. J. Visser 2014, Private Producer). The latest research indicates that the

total oil content of the Marula kernels varies with the harvesting date (Mariod *et al.* 2010). Yet, even with an optimized harvesting date, the main factor limiting yield is the processing technique.

2.1.2 THE *APPLICATIONS OF MARULA*

A general value chain for the fruits, bark, and leaves, of the Marula tree is summarized in Figure 2.2. Inspired by traditional uses, commercial applications and new products with a favourable commercial value have been and have the potential to be generated from this natural resource.

Wyk *et al.* (2013:264-265) reports that the bark, roots and leaves are traditionally used to treat a variety of ailments including diarrhoea, dysentery, unspecified stomach problems, fever, indigestion, diabetes, and malaria. While several studies investigating some of the medicinal applications of the bark, root, and leaf extracts have been completed with positive results, in some instances the traditional use is not backed up scientifically (Braca *et al.* 2003; Fotio *et al.* 2009; Tanih & Ndip 2012*;* van Wyk *et al.* 2013:264; Russo *et al.* 2013).

While the medicinal applications of the bark, root, and leaves, are not developed substantially for sustainable commercialisation, the Marula fruit yields raw material for several applications both in cosmetics and food (FAO 1998; Kleiman *et al.* 2008; WIPO 2010; Vermaak *et al.* 2011; van Wyk *et al.* 2013:264; Lall & Kishore 2014). The pulp of the fruit is used to manufacture liquor, wine, jams, beer, juice and other foodstuff. A popular alcoholic beverage, *Amarula*, is sold commercially and is available globally. In South Africa, Marula fruit juice is produced and sold in various Grocery Chains. The Marula fruit seed components -- shell, kernel and kernel oil -- are recognized for their cosmetic value by the European Union's database on Cosmetic Ingredients, European Commission Cosmetic Ingredients Database (ECCD). The Marula derived ingredients listed by the ECCD are summarized in Table 2.1.

Powder from the dried ground seeds is used as an abrasive in skin cleaning formulations. According to the ECCD, native and modified Marula oil is used in various cosmetic applications. The unmodified Marula oil has humectant and skin and hair conditioning functions, as well as the potential to reduce skin redness (Gruenwald 2006; Hein *et al.* 2009). Due to its emollient properties it is used in several high value moisturisers. Various products exist on the market with native Marula oil in their formulation and may be easily purchased from cosmetic giants such as Sephora, a French cosmetic company.

Marula oil is transesterified with polyglycerin for applications in cosmetic skin formulations; the modified Marula oil has emulsifying and conditioning properties (Vermaak *et al.* 2011; ECCD). According to Hein *et al.* (2009: US0285876 A1) a base catalysed reaction between Marula oil and glycerine transforms the Marula

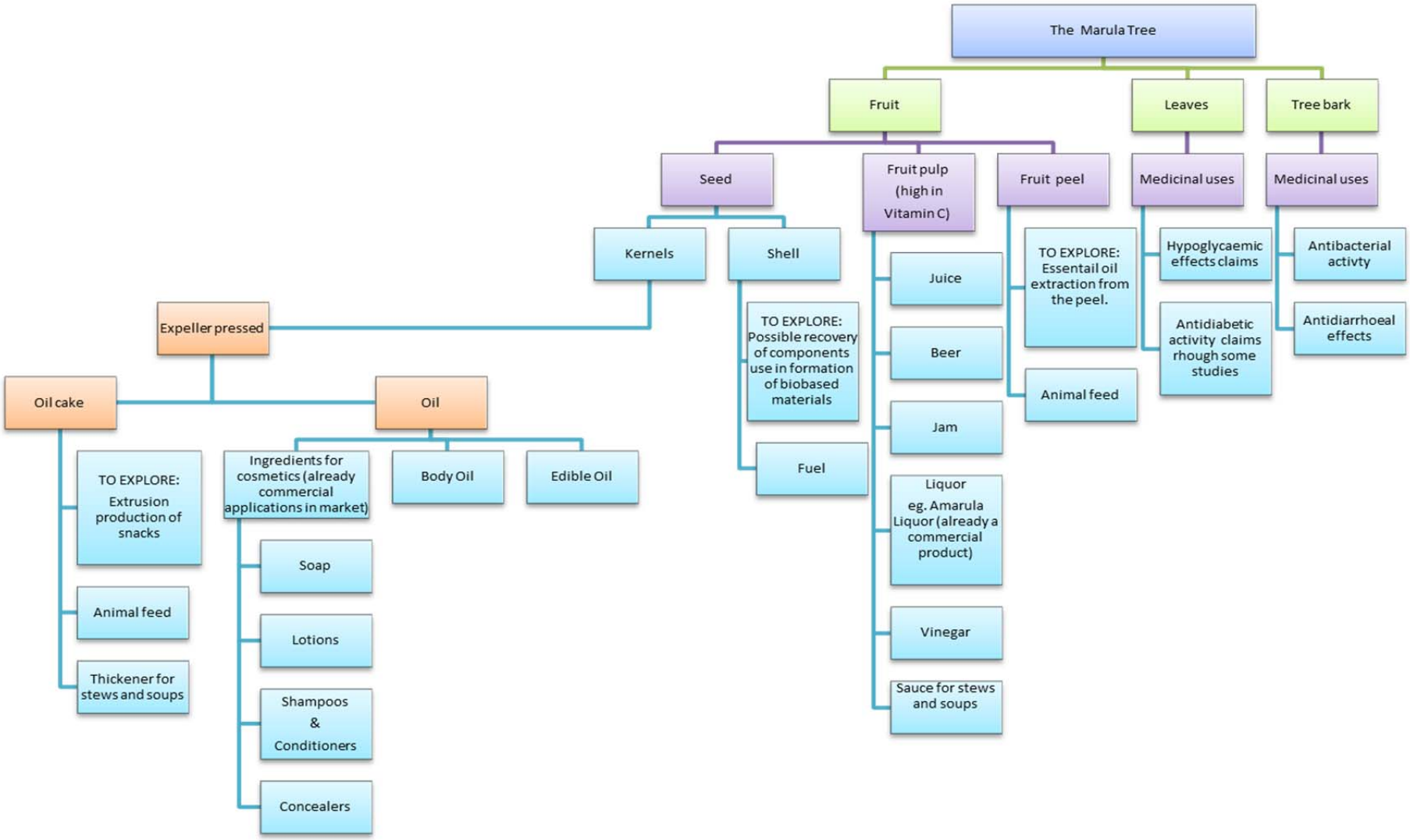

Figure 2.2: General Marula tree (fruit, bark, and leaves) value chain (Tereblanche, 1983; Zharare & Dhlamini 2000; Braca *et al.* 2003; Glew *et al*, 2004; Mojeremane & Tshwenyane 2004; Kleiman *et al.* 2008; Mariod *et al.* 2008; Hillman *et al.* 2008; Fotio *et al.* 2009; Ojewole *et al.* 2010; Vermaak *et al.* 2011; Tanih & Ndip 2012; Lall & Kishore 2014).

oil into a self-emulsifiable oil while retaining the unsaponifiable fraction. The aforementioned patented process transforms the oil into a mixture of mono- and di-esters and un-reacted materials, such that the modified oil is able to form a stable mixture with the desirable creamy appearance and viscosity for personal care and cosmetic products (Vermaak *et al.* 2011; Hein *et al.* 2009).

Table 2.1: Native and modifed *Sclerocarya birrea* (Marula) derived cosmetic ingredients and their functions (ECCD).

INCI Name	Description	Functions
Marula fruit extract	Marula fruit extract	Skin Conditioning
Marula leaf extract	Marula leaf extract	Skin conditioning
Marula seed powder	Powder obtained from the dried ground seeds of Marula, *Anacardiaceae*	Abrasive
Marula seed oil Polyglyceryl-6 esters	The product obtained by the transesterification of the Marula seed oil with Polyglycerin-6	Emulsifying Skin Conditioning
Marula seed oil Polyglyceryl-10-esters	The product obtained by the transesterification of the Marula seed oil with Polyglycerin-10	Emulsifying Skin Conditioning
Polyglyceryl-6 Marula seedate	Polyglyceryl-6 Marula seedate is the ester of polyglyceryl-6 and the fatty acids obtained from the *Schinsiophyton rautanenii* kernel oil	Emollient Emulsifying
Marula seed oil PEG-8 Esters	S. Birea seed oil PEG-8 esters is the product obtained by the transesterification of PEG-8 with Marula seed oil	Emulsifying Skin conditioning
Marula seed oil	The oil expressed from the seeds of Marula	Hair Conditioning Humectant

Stemming from research completed by a French cosmetic company, Aldivia, and efforts of Namibian researchers, Marula (fruit, roots, bark, leaves, seeds, cakes, testa) derived antioxidants have been patented by Charlier *et al.* (2006: WO097806). This patent claims that the antioxidants from Marula extracts have anti-free radical properties, are more stable than vitamin E when exposed to temperature and light, have a high Rancimant induction period, and may be used alone or in a mixture (Charlier *et al.* 2006). These properties are typically attributed to the unsaponifiable fraction of fats or oils. This portion of fats or oils typically consists of hydrocarbons, steroids (vitamin D, phytosterols, desmetylsterols, and methyl and dimethylsterols), tocopherols (vitamin E), tocotrienols, and carotenoids (vitamin A) (Belitz *et al.* 2009: 225-245). Most oils consist of 0.2-1.5 % unsaponifiable compounds, with a few exceptions which contain higher content of these constituents (Belitz *et al.* 2009: 225-226). According to Vermaak *et al.* (2011) and Robinson *et al.* (2012), Marula oil is one of these exceptions with 0.7-3.1 % unsaponifiable fraction. As a result of these confirmations of the quality of Marula oil, the application of Marula oil in cosmetics continues to grow (Prince 2012).

Marula oil has not only been considered for its application in food and cosmetics, but also as a potential biofuel resource. Gandure & Kelogetswe (2011) and Robinson *et al.* (2012) assessed the applicability of Marula oil for biodiesel production. Separate studies recommend that Marula oil has some favourable properties which make it a suitable substrate (stock) for biodiesel production. Gandure & Kelogetswe (2011) found that Marula oil has lower ester content than is desirable for it to be used as a bio-fuel directly; namely 93.7 %, compared to the desired value of 96.5 % by the European Standards for Biodiesel (EN) 14214. Robinson *et al.* (2012) found that due to the properties of the oil, it may be used as a biodiesel after reducing the viscosity, as the higher oil viscosity may compromise engine durability. Although the octane number of Marula oil is comparable to diesel fuel, and the oil has high oxidative stability, the high viscosity of the oil and the lower heating value of Marula oil may affect engine performance and power output, respectively (Robinson *et al.* 2012). While the direct use of Marula oil as a biofuel may be limited, Robinson *et al.* (2012) recommended that due to the high viscosity, saponification value, and oleic acid content, it can be used as a bio-lubricant.

Table 2.2: Summary of some published physico-chemical properties of Marula oil extracted with solvent extraction.

Parameter	Gandure & Kelogetswe 2011	Robinson *et al.* 2012	Vermaak *et al.* 2011*	Zharare & Dhlamini 2000
Colour	-	Light Yellow	Clear Pale, Yellowish Brown	-
Odour	-	Nuttish	Nutty Aroma	-
Refractive Index	-	0.88	0.9	-
Viscosity, $mm^2 s^{-1}$	-	41	-	-
Ester Content, %	93.7	-	-	-
Acid Value, mg KOH g^{-1}	1.4	4.4	5.1-33.7	3.5-3.7
Free Fatty Acid Value, %	0.7	20.7	-	-
Unsaponified, %	-	3.06	0.7-3.10	-
Saponification Value (SP), mg KOH g^{-1}	-	178.6	162.7-193.5	180.1-188.2
Iodine Value , (g Iodine 100 g^{-1})	-	100.34	64.2-100.3	66.8-69.0
Oil Yield, %	58.6	-	-	50.8-64.9
***Vermaak *et al.* (2011) reviewed and summarised values from Mariod *et al.* (2004) and Ogbobe (1998).**				

Gandure and Kelogetswe (2011), Robinson *et al.* (2012), and Vermaak *et al.* (2011) evaluated several physico-chemical characteristics of Marula oil. These values are summarised in Table 2.2. The sensory

characteristics of the oil are light yellow colour, clear, with a nutty aroma. The acid value, the free fatty acid value, saponification, and iodine values provide information about the quality and fatty acid composition of the oil. Acid and free fatty acid values indicate the level of degradation of the oil, and the saponification and iodine values indicate the levels of unsaturation in the oil. The free fatty acid value measures levels of lipolysis in the oil and is used to assess the quality of the oil for food and cosmetic applications. The acid value and free fatty acid values for Marula oil range from 1.4-33.7 mg KOH g^{-1} and 0.7-20.7 %, respectively (Gandure & Kelogetswe 2011; Vermaak *et al.* 2011). The typical acceptable FFA value for nut oils is less than 5 % with greater values indicating a high level of degradation, hydrolytic rancidity, of the oil (CODEX STAN) (Belitz *et al.* 2009:654-655).

The quality of Marula oil has been investigated by several research groups. The antioxidant stability of the oil, the fatty acid analysis of Marula oil and the effects of refining techniques, such as deodorization and bleaching on the final composition, have been assessed. Some published summaries of the fatty acid profile of Marula oil have been presented in Table 2.3 (Salama 1973; Zharare & Dhlamini 2000; Mariod *et al.* 2006; Kleiman *et al.* 2008; Mariod & Abdelwahab 2012; Robinson *et al.* 2012).

Table 2.3: Summary of the fatty acid composition of Marula oil as reported in published literature.

Reference	Palmitic (C16:0)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	(γ and α) Linolenic (C18:3)
	Hexadecanoic	Octadecanoic	Octadecenoic	Octade adie noic	O tadecatri enoic
Salama 1973	17.1	10.9	67.0	4.3	-
Zharare & Dhlamini 2000	10.1-11.3	6.3-0.2	71.8-72.4	8.4-9.5	0.4-0.0
Glew *et al.* 2004	15.6	11.9	63.2	5.2	-
Vermaak *et al.* 2011*	9. -12.0	5.0-8.0	70.0-78.0	4.0-7.0	0.1-0.7
Mariod *et al.* 2012	14.2	8.8	67.3	5.9	-
Robinson *et al.* 2012	12.8	7.2	73.6	6.1	0.3
***Vermaak *et al.* (2011) summarised values from Mariod *et al.* (2004), Zimba *et al.* (2005), and Ojewole *et al.* (2010).**					

The antioxidant value of Marula oil was assessed by several researchers and a patent was filed by Charlier *et al.* (2006) for antioxidants based on Marula oil (Mariod *et al.* 2010; Kleiman *et al.* 2008). Published information indicates that Marula oil has high oxidative stability with an induction period ranging from to 37

to 43 hours at 110 °C (Kleiman *et al.* 2008; Mariod *et al.* 2008). This supports the commercial value of the Marula oil.

While Marula oil quality and possible applications have been evaluated, the literature search indicated no research results for optimising the recovery of the oil to have been published. Stemming from private research, improvements to the processing and pressing of Marula kernels have been made; however, these are not readily available in open literature. While claims are made of up to 85 % oil recovery in some instances, no technology recovers 100 % of the oil and some refining is thought to be required (Personal Communication, Mr. J. Visser 2014; Personal Communication, Mr. A. Joubert 2014 (AH)). The currently utilized technology for Marula oil recovery on a production scale is cold pressing. There are a number of issues with this technology, which in some instances affect the quality of the products adversely. These include: low yield, unrefined and denatured oilcake, contamination of the oil, potential thermal degradation due to exposure to heat generated in the press, and poor processing after extraction (Personal Communication, Mr. J. Visser 2014; Personal Communication, Mr. A. Joubert 2014 (AH); Personal Communication, Mr. A. Brink 2014 (MCP)).

After pressing, the cake contains very high oil content. Analyses of kernel cake samples in the Limpopo province in South Africa in 2012 indicate that the oil yield obtained with pressing may be considerably low, as the oil content of the kernel press cake was found to be 54 wt. % (Personal Communication, Dr. E. Buis, 2014 (DST)). The high oil content remaining in the cake is indicative of an inefficient extraction. Yields by subsidised establishments such as those found in rural areas, may have yields as low 7 wt. % (Personal Communication, Mr. A. Joubert 2014 (AH)); Personal Communication, Mr. A. Brink 2014 (MCP)). Some successful processing facilities claim to be able to obtain up to 47 wt. % yield with pressing but their technology is proprietary (Personal Communication, Mr. A. Joubert 2014 (AH)).

As a result of the inefficient extraction, the residual kernel cake, which remains after pressing, is not utilized sufficiently, primarily due to the high oil content as well as microbial issues (Personal Communication, Mr. V. Dhlamini, NMPT; Mostert 2012). The kernel cake is currently utilised as livestock feed. As indicated by Mlambo *et al.* (2011), the cake can be a valuable protein source for cattle feed. Prior to extraction, the kernels contain high protein content, 28-37 %, which is further concentrated in the oil cake after the oil extraction (Mostert 2012; Glew *et al.* 2004; Mariod & Abdelwahab 2012). This protein, however, is not utilised sufficiently because the oil is not effectively separated from the raw material, and the quality of the cake is not suitable for human consumption (Personal Communication, Dr. E. Buis 2014 (DST); Personal Communication, Mr. A. Joubert 2014 (AH); Personal Communication, Mr. A. Brink 2014 (MCP)).

Once the oil is expelled with a press, the oil is then filtered to remove any solids which may be extracted with the oil during the pressing process. However, in some instances, fine particles remain in the oil even after

completion of the filtering step. These particles deposit on the bottom of the containers used to store the oils and may compromise the quality of the oil. In some processing facilities pressure filtration and centrifugation are used to separate the solids and the water from the oil. However, this is not always available and does not adequately remove the powdered kernel from the oil unless an ultrafine filter is used (Personal Communication, Dr. E. Buis 2014 (DST); Personal Communication, Mr. A. Brink 2014 (MCP)).

Microorganisms, such as bacteria, fungi, viruses, protozoans and parasites, are a concern when found in foodstuff and cosmetics as they produce toxins and are often carriers for diseases. Microbial toxins are produced by bacterial and fungal metabolism. Common groups of bacteria producing toxins found in foodstuff and cosmetics, include the *Bacillus cereus*, *Clostridium botulinum* and *Staphylococcus aureaus*. Mycotoxins produced can contribute to the degradation of foodstuff, and are harmful to health. The fungi genera, *Aspergillus, Fusarium* and *Penicillium, and Rhizopus,* are also of concern when found in foodstuff as they also contribute to degradation of foodstuff and subsequently may cause sickness (Davidson & Critzer 2012:154; Kollanoor-Johny *et al.* 2012:33-35).

Due to handling and potential contamination of the presses used in rural areas there are often quality concerns (Davidson & Critzer, 2012; Personal Communication, Dr. E. Buis 2014 (DST); Personal Communication, Mr. A. Brink 2014 (AH)). A sample of a pressed Marula seed cake was analysed for *Clostridium botulinum, Escherichia coli, Coliforms*, *Lysteria monocytogenes* and *Staphylococcus aureaus.* The colony forming units per gram (cfu g^{-1}) for each of the bacteria tested for, were less than 10, with exception for the *Coliforms*, which were present at 880 000 cfu g^{-1} (Mostert 2012). Marula oil was analysed for microbial growth and the *Aspergillus*, *Penicillium* and *Rhizopus spp* were present. The content of *Rhizopus* increased with storage, from 10 to 1000 microbial counts per mL over a three month period, while the content of other bacteria and fungi in the oil decreased with storage (Mostert 2012). Mostert (2012) noted that *Rhizopus* was of a concern for oil as it contains lipase enzymes which increase the free fatty acid (FFA) content in the oil by cleaving the fatty acids from the glycerol backbone.

Data available from local South African processing facilities indicates that over and above yield, the quality of the extracted oil is a concern as well. The nutty oil obtained from the Marula kernels has traditionally been enjoyed in cooking. Recent reviews of the Marula oil indicate that it has a good fatty acid profile, high in oleic acid, 65-74 %, which is similar to olive oil (Mariod & Abdelwahab 2012). However, the commercial sales of the oil as a gourmet food product may have been limited due to the high free fatty acid values (FFA), as this requires further processing, and also due to the low oil yield with cold pressing.

While research on the uses of Marula is substantial, covering wide range of applications, studies focusing on the processing of Marula, are limited (Mariod *et al.* 2006; Mariod *et al.* 2010; Mariod *et al.* 2011; Mariod *et al.* 2012). Therefore it is evidence in literature that the optimisation of the yield of the oil has not been completed. While some private development has taken place in improving the oil yield from the kernels, no

studies have been published and information on modification of processing equipment is proprietary. Personal communication indicates that a hydraulic press with a micro filter is used to improve the yield and the purity of the oil (Personal communication, Mr. A. Brink 2014 (MCP); Personal communication, Mr. A. Joubert 2014 (AH)). However, the yield is less than the potential, 40-47 %, compared to a 50-65 % potential oil yield. Overall, for the intended use of the finished Marula oil product, a green processing technique is required, such that the oils may be considered organically processed. The optimal recovery would involve recovery of 90 % or above of the available oil, and recovery of the oilcake such that it may be applied in various commercial applications, including foodstuff. The optimal yield would only be significant if the optimal quality of the oil is also retained. Thus, the problem of optimizing the oil extraction of Marula oil, without the use of toxic chemicals, remains unanswered.

2.2 SEPARATION TECHNIQUES

In separation principles, there are five basic separation techniques, separation by phase creation, by phase addition, barrier, solid agent, and force field or gradient (Seader *et al.* 2006:7-16). The various separation techniques are used in industry based on legislation, materials being processed, and, most importantly, effectiveness and cost.

Oil extraction is a generalized category which includes extractions of edible oil, specialty oils used in cosmetics, essential oils and oleoresins, as well as various nutraceutical and pharmaceutical compounds. The following steps are generally applied in processing edible oils: preserving by drying (removing moisture), cleaning (removing trash and hulls), freeing the oil by grinding, heating to release oil trapped in cells (this also denatures proteins as well as enzymes) and separation of the oil from the biomass (Lusas *et al.* 2012:1349-1351, 2012). The last step can be done by one of three general methods: (i) pressing, (ii) solvent extraction, or (iii) a combination of the two (Lusas *et al.* 2012:1349-1357).

2.2.1 PRESSING

Expeller pressing (i.e. cold pressing) is a separation technique which uses a rotating screw to press out the oil from oilseeds and fleshy oil fruits like olives. Several types of presses exist, with modifications to improve upon the yield. The manual operated technique is useful in rural areas where access to technology is limited. Expeller pressing is particularly favoured as the technique of choice for extraction of virgin oils and organic processing of oils. Oil following cold pressing requires minimal processing beyond filtering (Belitz *et al.* 2009:662).

There are several drawbacks of this method: (i) it is very labour intensive, (ii) has a low yield, and in the case where heat is introduced to assist in freeing the oil, (iii) the oil cake protein becomes denatured. This subsequently limits the applications of the cake as animal feed. The cake is used for human consumption only when the pressing is done by individual households for personal use. Additionally, the introduced heat or heat generated from the pressing may compromise the quality of the oil by contributing to degradation (Lusas *et al.* 2012:1386-1387; Belitz *et al.* 2009:668-669).

2.2.2 Solvent Extraction

Solvent extraction is the process of transferring a substance from any matrix by the use of a liquid in which the substance is soluble. Solvent extraction provides a significant improvement in the extraction yield of oils significantly compared to pressing. Depending on the solvent used, it can extract all desirable substances from the starting material; however, it is not selective. The commonly used solvents in industry are low cost, low boiling solvents such as hexane, a by-product of petroleum processing. Hexane, due to its low boiling point and high solubility, is the industry standard for solvent extractions (Lusas *et al.* 2012:1360; Reverchon *et al.* 2000).

There are several drawbacks of standard solvent extraction, including: (i) solvent residue, (ii) the cost of the solvent removal process (desolventising), and (iii) extraction of undesirable components due to the high selectivity of the solvent. While solvent extraction is one of the most efficient processes for oil extraction, solvents used are inherently toxic and subsequently require a solvent removal step. This increases the cost of the processing. In addition, due to growing understanding of the effects of various chemicals on the human body, there are now legal limits on the amount of residual solvent allowed in foodstuffs, cosmetics and other consumer products. Regulatory agencies, such as the United States Food and Drug Administration (FDA) (21CFR173.270), and the European Parliament and Council of the European Union (2009/32/EC), have set limits as to the amounts of hexane permitted in the finished products, both for oils and residues. According to the Council of the European Union (2009/32/EC), the maximum permitted hexane content in oils and butters is 1 mg kg^{-1} whereas for the defatted material it is 10 mg kg^{-1}. Therefore, the industrial solvent of choice, hexane is gradually being phased out as industry searches for alternative feasible technologies (Lusas *et al.* 2012:160-162).

Due to rising concerns with the quality and purity of ingredients and objectionable solvents with inherent toxicity, there is drive for identification of alternative means of extraction.

2.2.3 Combined Expeller and Solvent Extraction Method

In industry the optimal process is the one that is most economical as well as most efficient. In oil extraction, the combined expeller pressing and solvent extraction process is such a method. In industrial oil extraction the majority of the oil is removed by (i) expeller pressing, followed by (ii) solvent extraction to remove the remaining lipids. This technique is also applied in the extraction of soybean oil and production of defatted soy flakes (Hammond *et al.* 2005; Lusas *et al.* 2012:1355-1362).

2.2.4 Novel Methods: Possible Solutions to Drawbacks of Traditional Techniques

Alternative processing techniques, such modified traditional expeller processing, and novel technologies such as supercritical fluid extraction (SFE), have been the focus of research and development in various industry fields using lipids (Reverchon & De Marco 2006; Sahena *et al.* 2009; Temelli 2009; Pereda *et al.* 2008:17-19; Srinivas & King 2010:39-72). SFE is a high pressure extraction technique based on separation by phase addition, which utilizes solvents in their supercritical state, at or above the critical pressure and temperature point. Such that supercritical fluid extraction with carbon dioxide (SFE-CO_2) utilizes CO_2 at or above its critical pressure (P_c), namely 73.8 bar, and above the critical temperature (T_c), 31.06 °C (Brunner 1994:5). As a result of its properties and recent research, SFE-CO_2 is slowly being introduced to the industry as an alternative process to replace typical solvent extraction using hexane (Srinivas & King 2010:68-72).

Supercritical fluid extraction is still in its infancy compared to older separation techniques such as steam distillation (Seader *et al.* 2006:17). SFE is primarily performed on laboratory and pilot plant scale. However, multiple commercial processing facilities in Europe and Asia dealing with variety of applications ranging from for caffeine extraction from tea and coffee to wood impregnation and cork purification do exist (Temelli 2009; Srinivas & King 2010:68-72; Natural Process Technologies, (Pty) Ltd. (NATEX)). SFE-CO_2 has various applications in the pharmaceutical, cosmetic, and food industries. SFE can be used to extract, deodorize, fractionate and concentrate. The extraction chamber may be used as a reaction vessel (Lebovka *et al.* 2012:519). Brunner (2005) noted that the presence of the SFE-CO_2 processed products present in everyday life continues to grow; their presence may be noticed from breakfast to dinner products, ranging from coffee, tea, and alcohols, to spices, vitamins and oils. There is still great potential to explore the applications of SFE on a commercial scale and improve the technology to decrease the cost of operation, the latter being one of the major drawbacks of the technology (Martinez & Vance 2008:24-48; Temelli 2009; Srinivas & King 2010:72). There are several industrial-size plants operating worldwide, mainly for decaffeination of tea and coffee, and purification and preservation of materials (Temelli 2009; Srinivas & King 2010:68-72).

2.3 SUPERCRITICAL FLUID EXTRACTION (SFE)

Supercritical Fluid Extraction (SFE) is a relatively new technique, with most of the research related to it being completed over the course of the past 40 years, starting from the 1980s (Srinivas & King 2010:39-41).

The basic principle behind SFE is that the supercritical fluid (scFluid) diffuses through the feed matrix, dissolves the compounds of interest and the mix of scFluid and extract gets displaced out of the matrix by oncoming scFluid. SFE can utilize any of the commonly used solvents, such as hexane and methanol, but the most commonly used solvent in supercritical extraction processing is CO_2 (Brunner 1994:305-307; Pereda *et al.* 2008:3; King & Srinivas 2010). Research has been done with ethylene, ethane, propane, pentane, hexane, benzene, p-xylene, ethanol, methanol, including, but, not limited to, water (Brunner 1994:5; Srinivas & King 2010:41; Knez *et al.* 2010). While there are benefits to using each of these fluids as supercritical solvents, after comparing safety, viability, cost, and energy requirements, it is agreed that carbon dioxide remains the best choice.

Carbon dioxide is accepted as the most practical solvent to use in supercritical extraction because of its properties. These include its, (i) low critical temperature and pressure, (ii) non-toxicity, (iii) non-flammability, (iv) low cost, (v) GRAS recognition from the FDA, (vi) anti-bacterial and anti-fungal character, (vii) easy removal from the extract, and can be used to (viii) extract lipophilic compounds (King & Srinivas 2010:41-43; Brunner 1994:231-237; FDA). When used in SFE, its properties -- density and diffusivity -- can be easily manipulated by simply changing the temperature and/or pressure (Green & Perry 2008). The density changes as the pressure or temperature are changed, thus also changing its solvent properties. This permits for selective extraction of components from the raw material. Benefits of SFE include (i) high quality extracts and products, (ii) preserved functional activity, (iii) high purity of concentrated extracts, (iv) no thermal damage, (v) non-flammability, and (vi) non-toxicity (Knez *et al.* 2010). CO_2 therefore, is a well-recognized, safe solvent to use in supercritical processing, as it is natural and does not remain in the final products beyond what is normally found in the environment.

2.3.1 THE SFE PROCESS

Laboratory scale extractions using supercritical carbon dioxide ($scCO_2$) typically do not include recycling of the solvent, as it is not energy efficient. However, on a pilot plant and commercial scale, the solvent is recycled, with only minute quantities being lost during sampling and preparation. A typical pilot plant extraction process with recyclable CO_2 is presented in Figure 2.3**.**

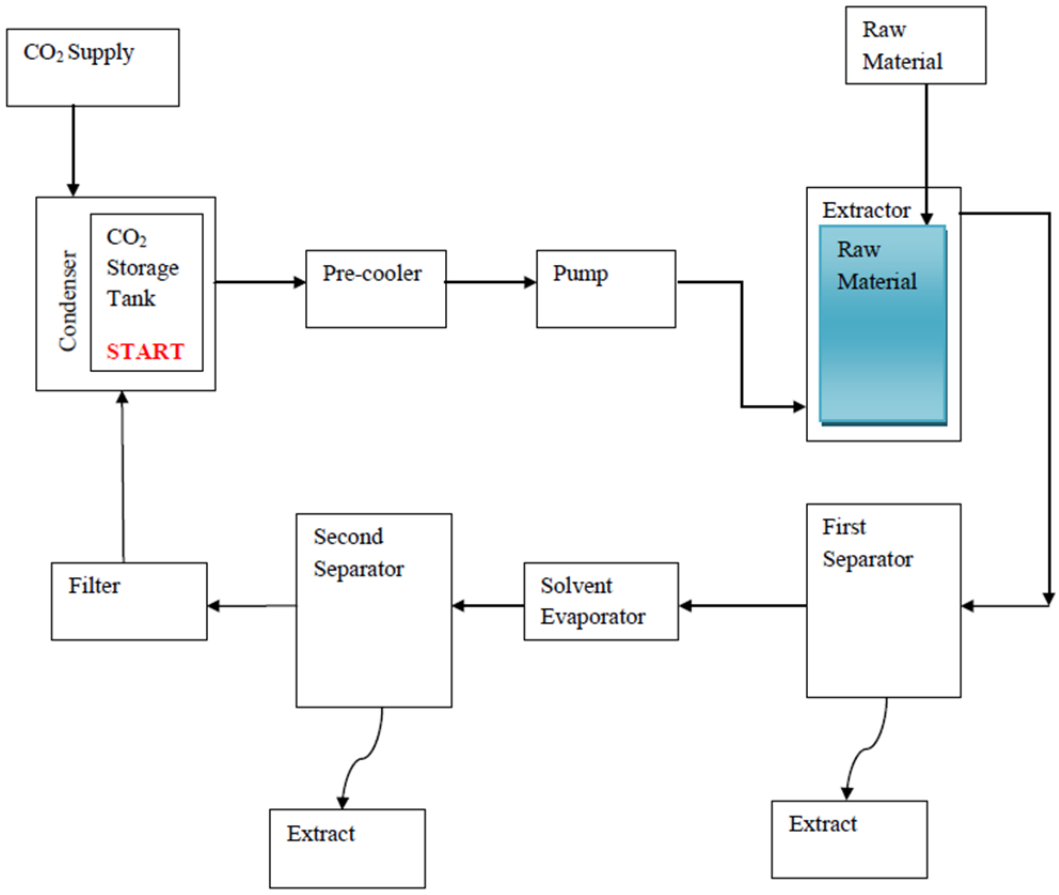

Figure 2.3: Typical SFE-CO_2 batch process with two separators.

In a general pilot plant SFE system, as illustrated in Figure 2.3, liquid CO_2 is stored in a high pressure vessel, labelled *CO_2 storage tank*, out of which it flows into a *pre-cooler* before being compressed by a *pump* to extraction pressure (74-900 bar), remaining in fluid state. The fluid CO_2 is then pumped forward through a *heating coil* inside the extractor, where it is heated to extraction temperature (31-85 °C). Once heated, the CO_2 transitions into its supercritical state, where there is no distinct phase; rather it has the diffusivity of a gas, and the density of a liquid. The scCO_2 then flows through the feed matrix (*raw material*), diffuses into the particles and in combination with solubility leaches out the compounds of interest. The solvent and the solute then flow through a pressure reduction valve into the *first separator*, where pressure is reduced to change the affinity of the CO_2 for the solute (60-120 bar). By reducing the pressure and temperature the *extract* precipitates towards the bottom of the separator. In a typical apparatus the *extract* can be drained off periodically through a valve at the bottom of the separator. After the first separator, the CO_2 passes through another pressure reduction valve, where the pressure is reduced below the supercritical state.The CO_2 is now in two phases, liquid and gas. After the pressure reduction valve, the CO_2 is taken through *a solvent vaporizer*, where the rest of the liquid CO_2 is vaporized. The CO_2 and any remaining extract then enter the *second separator*, where any remaining extract is deposited before the CO_2 goes through the *filter trap*. A *mass counter* records the mass of CO_2 used at a given time and the quantity returning to the storage tank. The CO_2

flows through a condenser where it is cooled to a fluid state, prior to entering into the working tank. From the working tank, the CO_2 can be cycled through continuously (Brunner 1994:180-181; Appendix A).

2.3.2 PRINCIPLES OF OPERATION

Two main principles from separation govern the extraction during SFE processing, these include mass transfer and thermodynamic equilibrium, the first guides the rate and the latter the extent of the extraction. These two principles are influenced by changes in density (influenced by temperature and pressure), matrix characteristics (i.e. pre-processing: moisture, grinding, drying etc.), including but, not limited to solvent flow rate. Thus the parameters to be modified to improve or optimize an extraction include (i) particle size, (ii) moisture, (iii) pressure, (iv) temperature, and the (v) solvent flow rate (Brunner 1994:199-200; Seader *et al.* 2006:66-68).

2.3.3 MATHEMATICAL MODELLING AND EXPERIMENTAL RESULTS EVALUATION

According to Reverchon and De Marco (2006), there are three different approaches in studying the applicability of SFE to the extraction of specific compounds: (i) empirical, (ii) heat and mass transfer, and (iii) differential mass balance integration. Experimental studies of SFE extraction of various materials continue to be the primary means of evaluating applicability. Mathematical modelling, taking into consideration transfer and mass balance integration, can be used to make sense and relate the data from an experiment of similar materials, or to evaluate the applicability of an extraction prior to empirical studies (Reverchon & De Marco 2006; Tabernero *et al.* 2013). Because SFE-CO_2 experimental work is costly, mathematical modelling is an approach that has received much attention.

These theoretical approaches are limited, as they require individualized models due to matrix differences (particles shape, availability of solute, location of solute within matrix, etc.) and interactions between solute, matrix and solvent. With regards to plant-based matrices, cell structure can play a tremendous role in influencing the extent and rate of the extractions. According to Brunner (1994:199), simple models and equations may be used to gain a superficial understanding of an extraction; however, in order make these theoretical approaches more reliable, more factors need to be taken into consideration, which subsequently complicate the modelling process. Some factors that need to be factored into the equation are not always measurable and need to be individually considered for different plant-based materials. Regardless of the limits, theoretical solubility determinations are considered to be of value to establish potential operating conditions and may assist in improving efficiency of the extraction. One of the most commonly used equations for estimating the solubility of various solutes in CO_2 is Chrastil's equation. The Chrastil Equation was proposed by Chrastil in 1982 and provides a linear connection between solubility and the CO_2 density. A

log-log plot of experimentally determined solubility of pure compounds in CO_2 on the y axis and the CO_2 density on the x axis yields the slope of k. Equation [1] is the proposed equation and Tables 2.4 and 2.5 consist of the definitions of the parameters and the experimentally determined values for constants a, b and k for oleic acid, stearic acid, α-tocopherol and water as published by Chrastil (1982).

$$C = D^k \exp(\frac{a}{T} + b) \quad [1]$$

Table 2.4: Variables in the Chrastil equation for solubility determination of pure compounds in $scCO_2$ (Chrastil 1982).

Value	Units	Explanation
C	g/L	Concentration of a solute in a gas
D	g/L	Density of a gas
k	Dimensionless	Association number
a	Dimensionless	Total reaction heat (ΔH)/ R value
b	Dimensionless	$\ln(M_a + k\ M_b) + q - k \ln M_b$ where M_a =the molecular weight of the solute M_b =the molecular weight of the gas
T	K	temperature

Because natural extracts are comprised of various compounds, the solute system would be classified as a multi-component system. Predictability calculations become complicated when the effect of all the components in the solute must be taken into account. In order to simplify the prediction the solubility of the extract in the supercritical solvent is calculated based on one main constituent in the solute, such that the solvent-solute system is considered as a binary system. In determining potential solubility of Marula oil in $scCO_2$, where the main constituent of the oil is oleic acid, 65-73 % oleic (Vermaak *et al.* 2011), the solubility of Marula oil would be calculated based on oleic acid. Thus the experimentally determined values, for the constants a, b, and k, used in the Chrastil equation, may be used to calculate the solubility of oleic acid in $scCO_2$ at various pressure and temperature conditions (Chrastil, 1982). For the determination of the Marula oil solubility the values for k, a, and b for oleic acid presented in Table 2.5 will be used in the Chrastil equation.

Table 2.5: Experimentally derived constants (k, a, and b) for solubility calculation of oleic and stearic acid, α-tocopherol, and water in $scCO_2$, Chrastil (1982).

Compound	k	a	b
Oleic acid	1.821	-10664.5	22.320
Stearic acid	7.922	-15360.	-2.499
α-tocopherol	8.231	-17353.5	0.646
Water	1.549	-2826.4	-0.807

In the Chrastil equation the density of the CO_2 plays an important role. Determining behaviour and density of non-ideal gasses, and in particular supercritical fluids, is typically done with the assistance of equations of state (EOS). Several models exist, these are built upon the ideal gas law (PV=nRT) and each new equation provides corrections and additional factors, which account for variations in the system, such that one can get as close as possible to predicting and correlating to experimental results. The primary function of EOS in modelling SFE extractions is to determine the density of the supercritical gas in given conditions of the extraction. The Peng-Robinson (PR) equation is the one most commonly used in determining the density of CO_2 for modelling supercritical extractions using CO_2 (Stahl *et al.* 2006; Chrastil 1982). Peng-Robinson (PG) EOS, Equation [2], may be used to calculate the density used in the Chrastil equation (Equation 1). Values for the PG equation are summarised in Table 2.6.

$$P = \frac{RT}{(V-bi)} - \frac{ai}{(V \times (V+bi)+bi \times (V-bi)} \qquad [2]$$

Table 2.6: Peng-Robinson EOS variables (Seader *et al.* 2006).

Variable	Definition	Units
P	Pressure	bar
R	Universal gas constant	(bar cm^3) (mol $K)^{-1}$
T	Temperature	K
V	Volume	cm^3
$\mathbf{a_i}$	Peng-Robinson constant a_i= 0.45724 x(RT_c)2/P_c x [1+ k (1-Tr1/2)]2 Tr=reduced temperature Tc=critical temperature	-
$\mathbf{b_i}$	Peng-Robinson constant b_i=0.07780 x (RT/P_c)	-

Beyond estimating the solubility of solutes in the supercritical carbon dioxide, over the years with growing interest in SFE, new kinetic models which aim to explain, predict and fit SFE empirical results, continue to be evolved and evaluated. The four most commonly cited and applied models include, (i) Sovova's broken-intact cell (BIC) model, (ii) Goto's shrinking core (SC), (iii) Glueckauf's linear driving force model, and (iv) Reverchon and Marrone's combined BIC-SC model. These models employ mass balances, equilibrium relations and kinetics laws to describe the process (Oliveira *et al.* 2011). Ajchariyapagorn *et al.* (2009), studied extraction from neem seeds, and did a predictive study using contribution methods, EOS and a shrinking core extraction model. The combined model results compared well with the experimental values and the model was able to predict the optimal pressure and temperature conditions for the optimal yield. However,

for modelling extractions from plant matrices the Sovova's Broken-Intact-Cell model remains the most commonly used.

Sovova (1994) proposed the Broken-Intact Cell (BIC) model, which maintains that the extraction curve has two parts: first part, where the extraction proceeds quite fast, being guided by the solubility of the solutes and the second part, slowed down, being guided by the diffusivity of the solutes to the surface of the particles. The BIC model assumes that the solubility is faster in the beginning as the solutes available at the surface where cells may be broken will proceed faster whereas the intact cells further inside will take longer to extract because of diffusivity. Figure 2.4 is a representation of the particles in Sovova's BIC model, where the intact cells are in the centre of the particle and the broken cells are found on the surface.

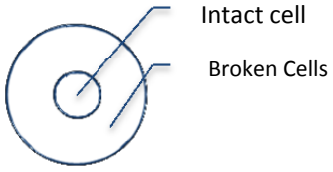

Figure 2.4: A representation of the particles in Sovova's Broken-Intact Cell model.

Sovova's model (Sovova, 1994) is the most commonly used one as it has been found to be the best fit when modelling data from plant matrices and in particular empirical data of seed and kernels oils such as those from walnuts, almonds, hazelnuts and pine kernels (Marrone *et al.* 1998; Bernardo-Gil & Casquilho, 2007; Mezzomo *et al.* 2009; Salgin & Salgin 2013). Marrone *et al.* (1998), Mezzomo *et al.* (2009), and Bernardo-Gil and Casquilho (2007), fitted results of almond oil, peach almond oil, hazelnut and walnut oils scCO_2 extraction to the Sovova Model (1994). While the Sovova Model originally indicates two stages, Mezommo *et al.* (2009), Bernardo-Gil and Casqulho (2007), and Salgin & Salgin (2013), agree that there are three periods of extraction observed: (i) constant, (ii) falling, and (iii) diffusion controlled. It is claimed that extractions from the vegetable matrices consist of three periods, (i) constant extraction rate (solute is easily accessible, and equilibrium controlled (between solid and fluid phase) mass resistance is at play), (ii) decreasing extraction rate (easily accessible oil is reaching a minimum and the diffusion controlled oil extraction period is beginning, therefore, the rate starts to slow down), and a (iii) final extraction rate (mass transfer is diffusion controlled; extraction of the less accessible oil). These three stages are presented in Figure 2.5.

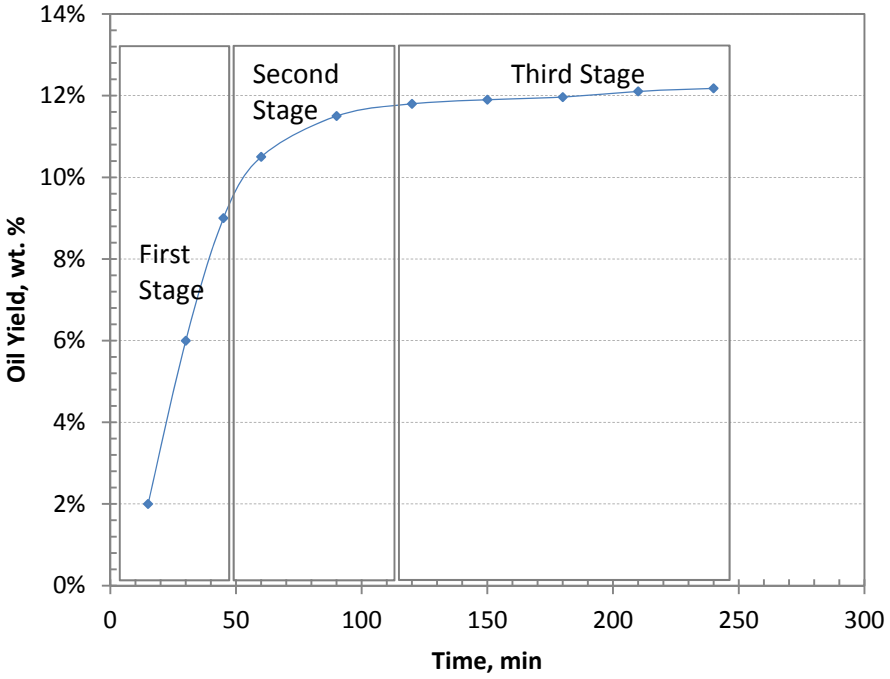

Figure 2.5: A representation of a typical three stage extraction curve of oilseeds (soybeans) extraction using SFE-CO_2, consisting of: (a) first stage: solubility controlled period, (b) second stage: transition period and (c) third stage: diffusion controlled period.

Mezzomo *et al.* (2009), investigated extraction of peach almond kernels. The goal was to assess the effects of pressure, particle size and flow on the kinetics of the extraction and define scale up parameters (extractor volume and flow). Sovova's model Broken-Intact Cells (BIC) was the best fit because it included information on the different mass transfer mechanisms. The extraction process for the peach almond kernels was mainly diffusion controlled. Out of the four models evaluated by Mezzomo *et al.* (2009) the models guided by diffusion, such as Sovova's, best fit the models of scale up. Bernardo-Gil & Casquilho (2007) completed modelling of extraction of both hazelnut and walnut oils, and compared the models with actual experimental data. The study evaluated the effect of the flow rate on the hazelnut oil extraction rate and particle size effects for walnut oil extraction. The experimental data for both walnut and hazelnut oil extraction were found to agree well with the Sovova model. Salgin & Salgin (2013) studied the effects of extraction conditions on the pine kernel oil yield. Salgin & Salgin also reported a similar extraction mechanism consisting of three stages: solubility, transition, and diffusion controlled regimes. An example of a typical SFE-CO_2 of soybeans three-stage extraction curve is presented in Figure 2.5. This information will be used to relate the results of the Marula oil extractions completed in this study.

2.3.4 SFE-CO_2 PROCESSING

Amongst its many applications, SFE-CO_2 has been applied to the extraction of a variety of specialty oils from seeds, cereals, nuts, legumes, vegetables and fruits (Reverchon & De Marco, 2006). These specialty oils are considered high value products due to their composition. These oils include bioactive compounds such as tocopherols, sterols, carotenoids, squalene, and poly-unsaturated fatty acids (PUFA's) (Reverchon & De Marco 2006). Raw materials from which oils have been extracted with SFE-CO_2 include but are not limited to amaranth, grape seeds, soybeans, palm kernels, pecans, cashews nut shell, apricot kernel, pistachios, walnuts, hazelnuts, macadamia, almonds, and peach almond kernels (Alexander *et al.* 1997; Marrone *et al.* 1998; Özkal *et al.* 2005a; Özkal *et al.* 2005b; Patel *et al.* 2006; Zaidul *et al.* 2006; Westerman *et al.* 2006; Fiori *et al.* 2007; Bernardo-Gil & Casquilho 2007; Martinez *et al.* 2008; Silva *et al.* 2009; Mezzomo *et al.* 2009; Senyaya-Oncel *et al.* 2011; Jokic *et al.* 2012; Akanda *et al.* 2012; Salgin & Salgin 2013). Additionally, extraction of oils from some exotic seeds and kernels such as, adlay seed, daphne seed, nimbin seeds bayberry kernels, moringa kernels, and white pitaya kernels, have been completed with SFE-CO_2 (Beis and Dunford, 2006; Ajchariyapagorn *et al.* 2009; Rui *et al.* 2009; Nguyen *et al.* 2011; Hu *et al.* 2012; Xia *et al.* 2013).

Studies on the optimizing extraction of the aforementioned oils have assessed several parameters, including pressure, temperature, particle size, and flow rate. The application of SFE-CO_2 to kernel oil extraction such as that of walnuts, hazelnuts, pistachios, and palm kernels, indicate that it is possible to use this technology for the extraction of Marula kernel oil. Extraction conditions such as pressure, temperature, flow rate, particle size, yields (where available), and notable techniques and results, for extractions of oils from kernel, nut and seed matrices, are summarised in Table 2.7. The overall findings relevant to the study of Marula oil extraction using SFE-CO_2 are subsequently reviewed in this section.

- **Temperature and Pressure**

One of the motivating factors for growing interest in SFE-CO_2 is due to the low critical pressure and temperature of CO_2; therefore, most extractions are carried out at a low temperature (31-80 °C). Depending on the material to be extracted, typical pressures used a range from 80 bar to 200 bar for essential oils to about 250-600 bar for heavier compounds and fatty acids. The density of carbon dioxide is considered easily manipulated by adjusting temperature and pressure. The solvating power of CO_2 is directly related to its density.

The results of published studies on SFE-CO_2 oil extraction agree that the solubility of the oils increases with increasing pressure. Özkal *et al.* (2005a) found that solubility of apricot oil increased with increasing pressure from 300-600 bar. Özkal *et al.* (2005a) obtained the greatest oil yield at 600 bar and 70 °C in the shortest amount of time. Alexander *et al.* (1997) found that pressure considerably increases the oil yield from pecans,

such that raising the pressure from 413 to 668 bar results in a more drastic change and faster rate of extraction. Martinez *et al.* (2008) found that greater yield of walnut oil was obtained at 400 bar, compared to extractions completed at 200 bar. Silva *et al.* (2008) extracted macadamia nut oil but at very low pressure conditions and relatively high temperature (80 °C) for the low pressure used (198 bar). It was reported that a yield of less than 1 % (0.76 %) was obtained. Salgin & Salgin (2013) used pressures ranging between 200 and 500 bar for palm kernel oil extraction, and reported that at higher pressures, 400 and 500 bar, the extraction proceeds faster and in a shorter amount of time. Senyay-Oncel *et al.* (2011) studied the SFE extraction of pistachios; the selected ideal conditions according to this study were 240 bar and 60 °C. Nguyen *et al.* (2011) studied extraction of oil from *Moringa oleifera* kernels, extractions of the kernel oil were completed on a pilot plant scale and optimal conditions for the extraction of the oil were recommended, 300 bar and 44 °C.

Temperature effects on extractions are divided depending on the operating pressure. Alexander *et al.* (1997), found that increasing temperature from 45 to 75 °C at a constant pressure doubled the pecan oil yield at all of the pressures tested (413 bar, 551 bar, 668 bar). Özkal *et al.* (2005a) found that solubility of apricot oil increased with increasing temperature from 40 to 70 °C, at pressures 300 to 600 bar. However, while these two studies found positive influence of temperature on yield at the pressures tested between 300-700 bar, Martinez *et al.* (2008) found that for walnuts, a higher yield was obtained at a lower temperature of 50 °C at 400 bar, compared to 70 °C. A well noted phenomenon describing this variance is the cross-over pressure. Cross-over pressure is where the effects of temperature on extraction conditions switch. Different cross-over pressures have been reported for different materials (Salgin & Salgin, 2013). This means that at lower pressures, typically below 200 bar, at a constant pressure, increasing the temperature reduces the solubility of the oil, and above 200 bar, increasing temperature increases the solubility of the oil. The cross-over pressure for walnuts may be higher than for other materials.

- **Modifier (co-solvent)**

In cases where solubility in carbon dioxide is limited, a co-solvent can be incorporated to improve the selectivity of the CO_2. According to King *et al.* (2010), low polarity and non-polar compounds are easily dissolved in CO_2, however, high molecular weight and highly polar compounds (sugars, inorganic salts, flavonoid compounds, polysaccharides, and amino acids) are not easily solvated by CO_2. Recommended co-solvents; must have intermediate volatility of the SC-CO_2 as well as the compound to be extracted. Good co-solvents include methanol, acetone, octane, ethanol and water. Studies have shown that a minimum of 3.5 mole percent addition of a co-solvent, such as ethanol, is sufficient to affect the solubility of the desired compounds in CO_2. The effects of the co-solvent addition may be attributed to the increase in hydrogen bonding solubility parameters with increasing solvent addition and subsequently resulting in an increased extraction yield of SC-CO_2 (King *et al.* 2010; Knez *et al.* 2010).

Table 2.7: Summary of operating conditions of oil extraction from various plant materials using SFE-CO_2.

Material	Botanical Name	Pressure (bar)	Temperature (°C)	Particle size	Flow Rate	Moisture	Size of extraction	Yield (%)	Special Note	Reference
Adlay Seed oil	*Coix lachrymal-jobi*	100-250	35, 40, 45, 50	n/a	2.5-4.0 L hr^{-1}	n/a	Lab Scale 100 g loaded	-	Ultrasound Assisted Extrac ion	Hu *et al.* 2012
Al onds	*Prunus dulcis*	350	40	0.3, 0.7, 1.9 mm	0.72, 1.43 kg hr^{-1}	n/a	Lab Scale 200 mL vessel 72-160 g loaded	-	Investigated particle size and solvent flow only. 200 mL extractor.	Marrone *et al.* 1998
Apricot kernels	*Prunus armeniaca*	300, 375, 450, 525, 600	40-70	0.425-1.5 mm	1-5 g min^{-1}	3.9 %	Lab scale, 10 mL vessel	99 % oil recovery 48 % oil	600 and 70 bar fastest extraction; Recommended 3g min^{-1} flow	Özkal *et al.* 2005a
Bayberry kernel	*Myrica rubra Sieb. et Zucc*	350	45	n/a	4.0 L min^{-1}	n/a	Lab Scale 100 mL vessel 5.0 g loaded	N/a	Static and dynamic extraction periods	Xia *et al.* 2013
Daphne Seed	*Laurus nobilis L.*	140-680	35-65	0.35-1.25 mm	1.5 mL min-1	7.5 %	Lab Scale 10 mL vessel	14-28 %	Used petroleum ether as a co-solvent; also assessed the value of the oils	Beis & Dunford 2006
Hazelnuts	*Corylus avellana*	150-600	40-60	1-2 mm	2 mL min^{-1}	3 %	Lab Scale 10 mL vessel	59 %	Agglomeration due to sieving; high oil content	Özkal *et al.* 2005b
		180-234	34.85-47.85	0.7 mm	4.42-7.10x10^{-4} m^3 s^{-1}	n/a	N/a	N/a	Modelling	Bernardo-Gil & Casquilho 2007
Macadamia	*Macadamia integrifolia*	100, 150, 180	40-80	n/a	1.64x10^{-7} m^3 s^{-1}	n/a	Lab Scale 42 mL vessel 12 g loaded	0.74 % oil recovery	Extraction completed on laboratory scale. Used a simple model based on the Langmuir model.	Silva *et al.* 2008
Mango seed kernel	*Mangifera indica*	350, 422	60, 72	n/a	3.4 mL min^{-1}	6.1-7.5 %	Lab Scale 2.5 mL vessel	6.4-13.5 %	FFA value could be reduced by fractionation of the oil using SFE.	Jahurul *et al.* 2014

Material	Botanical Name	Pressure (bar)	Temperature (˚C)	Particle Size	Flow Rate	Moisture	Size of Extraction	Yield (%)	Special Note	Reference
Moringa kernels	*Moringa oleifera*	150-300	35-60	0.16-1. 2 mm	0.5 m^3 hr^{-1}	8.7 %	Pilot Plant 500 mL vessel 70 g loaded	37.82 % at 0.63 mm particle/ 300 bar and 47.5 °C.	Used EtOH at 10 %	Nguyen *et al.* 2011
Nimbin Seeds	*Azadirachta indica A. Juss*	100-260	35-60	600 µm	0.24-1.24 mL min^{-1}	n/a	Lab Scale 1.0-2.5 g loaded	n/a	Used group contribution methods, EOS and SC model to predict the extraction yield from neem seeds	Ajchariyapagorn *et al.* 2009
Palm Kernel	*E. guinensis*	276-483	80	<2 mm	Varied	n/a	Lab Scale 42.5 mL vessel 20 g loaded	44.75	SFE can be used to separate short from long-chain fatty acids in palm kernel oil.	Norulaini *et al.* 2004
		198	51	1-2 mm	Varied	n/a	Lab Scale: 100 g loaded	5.9 % oil extracted, best yield obtained with 100 mL EtOH addition.	0-100 mL ethanol addition Initially Static extraction followed by a dynamic extraction period.	Khrishnaiah *et al.* 2012
		276, 345, 414	40-70	106-450 µm	1.0-3.0 mL min^{-1}		Lab Scale 10 mL vessel 9.0 g	9.26 % yield	Recovery of oil from palm kernel cake; Particle size had the greatest effect on the yield.	Ab-Rahman *et al.* 2012
		20.7-48.3	40, 80	<2 mm	Varied	4.87 %	Lab Scale, 20 g loaded	99.6 % recovered at 48.3 and 80 °C	Fractionation of the fatty acids for different applications.	Zaidul *et al.* 2006
Peach Almond	*Pruns persica*	150, 250	40	0.883, 3.3 mm	3.3, 10.0 g min^{-1}	50.2 %	Lab Scale 12.5 g loaded	19 %	Extraction from w*et alm*ond peach kernels; and scale up evaluation.	Mezzomo *et al.* 2009

Material	Botanical Name	Pressure (bar)	Temperature (˚C)	Particle Size	Flow Rate	Moisture	Size of Extraction	Yield (%)	Special Note	Reference
Pecans		413, 551, 668	45, 62, 75	Pecan halves	2.5 slp	4.8 %	Lab Scale 300 mL vessel 70 g loaded	95 % of total oil recovered	Studied oil recovery	Alexander *et al.* 1997
		620	75	Pecan halves	3.0 *L* min^{-1}	3.5-11.6 %	Lab Scale 300 mL vessel, 20 g loaded	14-21 %	Depressurization contributed to pecan breakage. Greatest oil yield occurred at 6.7% water content.	Li *et al.* 1999
Pistachios	*Pistacia terebinthus*	100-300	30, 55, 80	0.5 mm	10,15,20 g min^{-1}	n/a	Lab Scale 100 mL vessel 30 g loaded	n/a	Focus on optimizing unsaturated fatty acid extraction conditions	Senyay-Oncel *et al.* 2011
Pine Kernel	*Pinus pinea*	200-500	40-60	0.362 mm	0.061-0.259 kg hr^{-1}	5.06 %	Lab Scale 10 mL vessel 3.2 g loaded	47.7-48.8 % oil	Lipids contained more unsaturated FA's than those extracted by solvents. 0-5% Ethanol addition.	Salgin & Salgin 2013
Walnut	*Juglans regia*	200, 400	50, 70	1.2-2.4 mm	10.5 kg hr^{-1}	7.9 %	Pilot Plant 400 g loaded	92 % recovered from 17 % residual oil in the cake	400 g of pre-pressed cake used.	Martinez *et al.* 2008
		180-234	34.85-47.85	0.01-0.5 mm	$6.8x10^{-4}$ m^3 s^{-1}	n/a	Lab Scale 10 mL vessel	n/a	Modelling	Bernardo-Gil & Casquilho 2007
		300, 400, 500	40, 50, 60	0.178-0.9 mm	0.0167-0.133 mL s^{-1}	3.8 %	Lab Scale	65 % yield (kg oil/kg dry sample)	Ethanol addition at 2, 4, 8, and 12 vol %.	Salgin & Salgin 2006
White Pitaya kernels	*Hylocereus undatus Britt and Rose*	250	35	n/a	N/a	n/a	Lab Scale 100 mL vessel	5.54 % (g/100g)	Compared 4 methods of extraction: Microwave assisted (MAE); Supercritical fluid (SFE); Aqueous enzymatic (AEE); Combined AEE and MAE (MAEE)	Rui *et al.* 2009

Özkal *et al.* (2005a) and Krishnaiah *et al.* (2012) found that adding a co-solvent to the extraction improved oil yield. Özkal *et al.* (2005a) noted that an addition of ethanol of up to 3.0 wt % at 450 bar can increase the rate of extraction to the same rate as that obtained at 600 bar. Krishnaiah *et al.* (2012) found that ethanol addition at pressure of 198 bar increased the oil recovery from kernel cake. Salgin & Salgin (2013) reported an increase in pine kernel oil solubility and a faster extraction rate by addition of 2.5 % and 5.0 % ethanol, with an increase from 520 to 716 and to 840 g oil kg^{-1} kernels, respectively.

- **Particle Size**

It is well established that the smaller the particle size, the greater the surface area, and the faster the extraction rate, and consequently greater yield, in a given time. Maximizing the surface area maximizes access to the solutes of interest such that the extraction is less influenced by mass transfer resistance, and thus less diffusion controlled. However, the particle size has lower limits, which may result in problems with the extraction. Problems encountered from a fine particle size include, fluidization of the particles, which may clog up the filter of the basket (Martinez & Vance 2008:35-37).

Several studies dealing with extraction of oils from kernels have reported that smaller particle size improved the yield and the rate of the extraction. Özkal *et al.* (2005a) noted that, with regards to apricot kernels, the apricot oils that are not unrestricted during grinding contribute to the slow rate of extraction during the second period of the extraction. Marrone *et al.* (1998) observed a faster rate of extraction for almond kernel oil, during extraction completed with a kernel particle size less than 0.3 mm. Alexander *et al.* (1997) tested the effects of pressure and temperature on pecan halves; as a result it took 7.5 days to extract 95 % of the pecan oil. Khrishnaiah *et al.* (2012) recovered oil from palm kernel cake and found that pressure and temperature had an effect on the yield; however, the effects of the particle size were quite pronounced with particle size of less than 150 μm providing the best oil recovery. Salgin & Salgin (2013) compared 362.5 μm and 725 μm particle sizes, and found that while the extraction rate during the first hour was the same for both sizes, the difference was observed during the following three hours, and in particular during the diffusion controlled stage of the extraction, such that the yield was doubled for the smaller particle size. The effects of longer extraction periods have been noted for extractions where larger particle sizes are used and this has been attributed to mass transfer resistance (Marrone *et al.* 1998).

- **Moisture**

Raw materials vary in moisture content. This can assist in an extraction or inhibit it. In some cases, such as caffeine extraction, high moisture content has a positive effect on the solubility of the components (Brunner 1994:185; Temelli *et al.* 2010:66). In other extractions where the extract is sensitive to moisture, not only is

the extraction inhibited, but it may have some adverse effects on the extracts. Brunner (1994:183-184) explained that in a plant cell structure, an elementary membrane surrounds the cell components, which consist of the starch proteins, sugars and fats. The membrane consists of molecules with hydrophobic and hydrophilic ends, and subsequently has specialised pores which allow transport of water and lipids, hydrophilic and hydrophobic pores respectively. In some instances, if there isn't enough water and the pores close and water cannot escape, the effect of the moisture content on yield is attributed to the behaviour of these pores (Brunner 1994:184-185). Martinez *et al.* (2008), in extracting walnut oil, found that a higher moisture content (4.5 wt. %) of the raw material improved the oil yield, compared to 2.5 wt. % as the lower moisture content.

Carbon dioxide and water are at equilibrium with the following equation representing this relationship:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^{\oplus} + HCO_3^{\ominus}$$

No adverse effects have been reported of the CO_2 and H_2O equilibrium during SFE processing. However it has been noted by some studies that the presence of water can improve oil recovery. Li *et al.* (1999) applied the SFE system to de-fat pecan halves; it was found that breakage of the pecan halves can be minimized by water addition and oil recovery can be increased; the optimal moisture was found to be 6.7 wt. % with a 22 wt. % oil yield.

- **Solvent Flow**

Solvent flow rate plays an important role in SFE because it affects the duration of an extraction, overall, the faster the flow rate, typically the faster the extraction. The faster the solvent flow rate, the more solvent the particles are exposed to.

In a reported study for pine kernel oil extraction by Salgin & Salgin (2013), the recommended/optimal flow rate was 0.194 kg h^{-1}. According to their reported results, there is a point up to which increasing the flow rate increases the yield. This is the state at which a full saturation of the solute in the solvent is achieved, and thereafter increasing the flow rate does not increase the yield (Salgin & Salgin 2013).

- **Further Improving SFE Extraction**

In recent years, modifications, such as ultrasound assistance, have been applied to SFE in order to improve the extraction. For seed matrices, such as adlay seeds, Hu *et al.* (2012) compared supercritical extraction with and without ultrasound assistance and found that recovery of the oil was considerably increased at milder

extraction conditions due to the ultrasound assistance. Thus, indicating that agitation of the material inside the extractor can improve the yield of the system.

- **Comparing Oil Quality: Traditional Methods vs. SFE**

Benefits to quality of oils extracted with SFE-CO_2 have been noted by several research groups (Temelli, 2009). Martinez *et al.* (2008) extracted walnut oil using a continuous screw press and SFE to recover as much of the walnut oil as possible; their study reported that the oils extracted at 200 bar and 70 °C had the greatest tocopherol content compared to oils extracted at 400 bar and 50 °C and 70 °C, respectively, and 200 bar and 50 °C. Oliveira *et al.* (2011), indicated that the oils extracted with SFE are already considerably refined compared to other methods, thus indicating a potential use of the oils without further processing. Additionally, Martinez *et al.* (2008) noted that oils extracted with SFE had higher carotenoid content than those extracted with screw pressing.

Senyay-Oncel *et al.* (2011), found that the SFE extracted pistachio oils had higher content of unsaturated fatty acids similar to cold pressing, whereas the opposite is true of hexane extracted oils. Jahurul *et al.* (2014), extracted oil from mango seed kernel and reported yields comparable to solvent extraction. Nguyen *et al.* (2011) found that one moringa kernel oil sample extracted using hexane and the other extracted with SFE-CO_2, were not significantly different.

Martinez *et al.* (2008) found that the lowest acid value of walnut oil, 0.34 % oleic, was obtained at a pressure of 400 bar and 50 °C. Additionally, findings by Jahurul *et al.* (2014), show the FFA value of mango kernel oil to be reduced using SFE-CO_2, which means a further study for the Marula would be to look at fractionating in order to reduce the FFA value. Xia *et al.* (2013) studied the effects of SC-CO_2 extraction on the fatty acid composition of the bayberry kernel oil and to evaluate the oxidative stability of the oil. Their goal was to evaluate the extracted oil for commercialization. It was found that the SFE extracted oil had a greater oxidative stability.

- **Fractionating**

Norulaini *et al.* (2004), and Zaidul *et al.* (2006), found that the palm kernel oil could be fractionated into short and long fatty acids by removing fractions throughout the extraction process. Norulaini *et al.* (2004) reported the oil fractions taken out during the first portion of the extraction contained the shorter FA's C8, C10, C12, whereas the latter fractions contained higher concentration of the longer fatty acids, C16, C18. The only fatty acid which remained relatively constant in all the fractions was C14. Zaidul *et al.* (2006) varied pressure and temperature and collected four fractions at 10 minute time intervals. The study found that the lower chain fatty

acids, C8, C10, and C12, were extracted in the first fraction and the longer chains, C16, C18, fatty acids in the fourth fraction. Additionally, the study noted that if one fractionates palm kernel oil using SFE, the oil could be used as a cocoa butter replacer. Jahurul *et al.* (2014) found that the quality of mango oil extracted by fractionating with SFE can be used as a cocoa butter replacer in various confectionary applications.

2.3.5 CONCLUSIONS FROM LITERATURE

From the reviewed literature of similar materials to Marula, it may be deduced that the oil extraction using SFE is anticipated to yield favourable results. Overall, pressures higher than 200 bar, ranging up to 670 bar, with temperature ranges from 40-80 °C, as noted in Table 2.7, are applied for oil extraction from kernel and seed matrices. Thus, that would be the recommended range of operating pressures and temperatures for optimizing the oil extraction conditions for Marula kernel oil during this study.

Overall, pre-processing of the Marula kernels is necessary to obtain a faster rate of extraction and to improve yield. In the case of high oil content oilseeds, it is difficult to adequately separate particle sizes without the use of liquid nitrogen to freeze the seeds before milling and sieving to separate the different particle size fractions. Therefore, the Marula kernels will be ground, but only sieved to determine the average particle size after the oil extraction process.

While Nguyen *et al.* (2011), Salgin & Salgin, (2013), and Khrishnaiah *et al.* (2012) found that ethanol addition improves the yield of an extraction, adding a co-solvent diminishes the benefit of using $scCO_2$ because of the subsequent solvent removal step. For the purposes of oil extraction from Marula kernels, co-solvent addition will be avoided.

Martinez *et al.* (2008), in extraction of walnut oil, found that a moisture content of 4.5 wt. % compared to 2.5 wt. % resulted in a higher oil yield, and Li *et al.* (1999) found that 6.7 % moisture gave the best yield for pecan oil recovery. Depending on the moisture content of the Marula kernels, a standard of 4 to 7 wt. % will be accepted as favourable and additional drying of the seeds will be considered only if the results indicate that the moisture of the Marula kernels is interfering with the oil yield.

With regards to quality of the oils extracted with SFE-CO_2, the reported greater oxidative stability and reduced FFA values compared to oils obtained with traditional methods, indicate that there is potential that Marula oil with an improved quality may be obtained with SFE compared to pressing (Martinez *et al.* 2008; Xia *et al.* 2013; Jahurul *et al.* 2014). Reported fractionation of palm kernel oil by Noruliani *et al.* (2004) and Zaidul *et al.* (2006) into short and long chain fatty acids, by sampling at designated times through the extraction process, is an indicator that evaluating the fatty acid composition of the fractions obtained

throughout the extraction period of Marula oil may be of value. Overall, assessing the quality of the oils is necessary in order to have an understanding of the value of the SFE system for oil extraction and the potential it holds for fractionation of fats. In order to further explain and understand the significance of the quality of the oils that may be obtained with SFE-CO_2 it is necessary to have a fundamental understanding of fats and oils. The following section will provide a background on fats and oils and their characterisation.

2.4 Fats and Oils

2.4.1 Origin and Classification

Oils and fats fall into one generally defined class, lipids, characterised by being insoluble in water but soluble in organic solvents. The distinction between oils and fats (composed of lipids) is made based on their physical appearance at room temperature; fats are solid at room temperature and oils are typically characterised as being liquid at room temperature, with typical temperature between 21 and 25 °C (Pike 2003:229-230). Fats and oils may be of animal or plant origin. The origin of the oils affects their composition and consequently their classification and applications. Lipids are classified based on two different characters, either on their acyl residue or their polarity (Belitz *et al.* 2009: 158-159). The most commonly dealt with edible lipids are triacylglycerols, which are composed of glycerol and three fatty acids (Figure 2.6). The fatty acids can be classified as saturated (even and odd numbered straight and branched chains), unsaturated (-*cis* or -*trans* conjugated and non-conjugated double bonds) or substituted (hydroxy, furan, and oxo fatty acids). Table 2.8 summarizes some of the most common fatty acids found in lipids (Belitz *et al.* 2009:158-171).

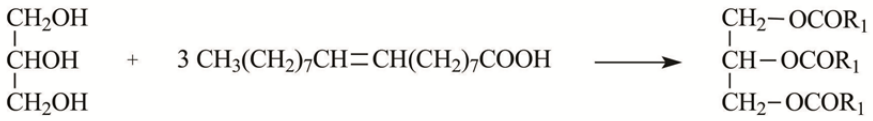

Figure 2.6: Glycerol and three fatty acids (oleic acids) yield a trioleic tiacylglycerol: R1= $(CH_2)_7CH=CH(CH_2)_7CH_3$. The fatty acids can be any of the ones noted in Table 2.8.

Fats and oils consist not only of triglycerides (fatty acids and glycerol) but also of other minor constituents, such as free fatty acids, esters, tocopherols, carotenoids, sterols (phytosterols), ketones, phospholipids, trace metals, and several other compounds. The composition depends on origin, and consequently affects the value of the oils and their applications. The different fatty acids affect the reactivity of the lipids, and therefore the stability of the oils (Belitz *et al.* 2009:158-178). Lipids are insoluble in water (depending on the chain length, there are exceptions), and soluble in organic solvents.

Table 2.8: The typical fatty acids found in oils and fats are listed, including their structural formula, systematic name, common name and abbreviation, number of carbons (C) vs. number of double bonds (D) (Belitz *et al.* 2009: 161-163).

Abbreviation C:D	Systematic Name	Common Name	Structural Formula
4:0	Butanoic Acid	Butyric	$CH_3(CH_2)_2COOH$
5:0	Pentanoic Acid	Valeric	$CH_3(CH_2)_3COOH$
6:0	Hexanoic Acid	Caproic	$CH_3(CH_2)_4COOH$
7:0	Heptanoic Acid	Enanthic	$CH_3(CH_2)_5COOH$
8:0	Octanoic Acid	Capryllic	$CH_3(CH_2)_6COOH$
9:0	Nonanoic Acid	Pelargonic	$CH_3(CH_2)_7COOH$
10:0	Decanoic Acid	Capric	$CH_3(CH_2)_8COOH$
12:0	Dodecanoic Acid	Lauric	$CH_3(CH_2)_{10}COOH$
14:0	Tetradecanoic Acid	Myristic	$CH_3(CH_2)_{12}COOH$
14:1 (9)	-	Myristoleic	CH_3-$(CH_2)_3$-CH=CH-CH_2-$(CH_2)_7$ COOH
15:0	Pentadecanoic Acid	-	$CH_3(CH_2)_{13}COOH$
16:0	Hexadecanoic Acid	Palmitic	$CH_3(CH_2)_{14}COOH$
16:1 (9)	-	Palmitoleic	CH_3-$(CH_2)_5$-CH=CH-CH_2-$(CH_2)_7$COOH
17:0	Heptadecanoic Acid	Margaric	$CH_3(CH_2)_{15}COOH$
18:0	Octadecanoic Acid	Stearic	$CH_3(CH_2)_{16}COOH$
18:1 (9)	-	Oleic	CH_3-$(CH_2)_7$-CH=CH-CH_2-$(CH_2)_7$ COOH
18:1 (tr9)	-	Elaidic	CH_3-$(CH_2)_7$-CH=CH-$(CH_2)_7$COOH
18:2 (9,12)	-	Linoleic	CH_3-$(CH_2)_4$-$(CH$=CH-$CH_2)_2$-$(CH_2)_6$ COOH
18:2 (9, tr11)	-	-	CH_3-$(CH_2)_4$-CH=CH-CH_2-CH=CH-$(CH_2)_7$ COOH
18:2 (tr9, tr12)	-	Linolelaidic	CH_3-$(CH_2)_4$-CH=CH-CH_2-CH=CH-$(CH_2)_7$ COOH
18:3 (6,9,12)	-	γ-Li olenic	CH_3-$(CH_2)_4$-$(CH$=CH-$CH_2)_3$-$(CH_2)_3$COOH
18:3 (9,12,15)	-	α- Linolenic	CH_3-CH_2-$(CH$=CH-$CH_2)_3$-$(CH_2)_6$ COOH
18:3 (9,tr11,tr13)	-	α-Eleostearic	-
18 3 (tr9, tr11, tr13)	-	β-Eleostearic	-
18:4 (9,11,13,15)	-	Parinaric	-
20:0	Eicosanoic Acid	Arach dic	$CH_3(CH_2)_{18}CO$ H
20:4 (5,8,11,14)	-	Arachidonic	-
20:5 (5,8,11,14,17)	Eicosapentanoic Acid	EPA	-
22:0	Docosanoic Acid	Behenic	$CH_3(CH_2)_{20}COOH$
22:1 (13)	-	Erucic	-
24:0	Tetracosanoic Acid	Lignoceric	$CH_3(CH_2)_{22}COOH$
24:1 (15)	-	Nervonic	-
26:0	Hexacosanoic Acid	Cerotic	$CH_3(CH_2)_{24}COOH$

Lipids are susceptible to the following reactions: enzymatic hydrolysis, peroxidation, polymerization and radiolysis. Depending on their composition, these reactions may be used to modify fatty acids for a specific application (i.e cosmetics, food, industry etc.). These reactions may also be used to do quality tests on the oils, such as determining adulteration and contamination (Belitz *et al.* 2009:656-669).

Main concern with foods is rancidification, a chemical decomposition of fats, oils and other lipids. Three pathways exist: (i) hydrolytic, (ii) oxidative, and (iii) microbial (Belitz *et al.* 2009:188, 203; O'Brien 2009:217-220). The hydrolytic pathway is caused by the presence of water, which promotes hydrolysis, i.e splitting the fatty acid chains from the glycerol backbone. This is measured by the amount of free fatty acids. The oxidative pathway is caused by oxygen in the air, it's a free radical process; the double bonds of an unsaturated fatty acid undergo cleavage releasing volatile aldehydes and ketones, and thus contributing to an unpleasant scent of the oils. The peroxide value measures the primary products of an oxidation reaction and the anisidine value measures secondary products of an oxidation reaction. Therefore, the peroxide value is primarily used for analysis of freshly extracted products and the anisidine value is used to evaluate oxidative degradation after storage. To get the best idea of the quality of the oil using both analyses may be beneficial in some instances. Presence of natural anti-oxidants inhibits oxidation. Microbial degradation is caused by microorganisms and enzymes, which breakdown the fat (Belitz *et al.* 2009:187-208; O'Brien 2009:217-220).

2.4.2 Oil Characterization

Oils have a wide variety of applications as edible oils, moisturizers for skin, leather treatment, cosmetic formulations, including, but not limited to, spa therapy treatments. Based on the application, the oils undergo further processing after the initial extraction process in order to meet certain quality, composition, and physico-chemical standards. The quality requirements for measuring the oil or fat quality are established by the application. There are a number of parameters used to determine the quality of oils. The most commonly used parameters for evaluation of oils are summarised in Table 2.9. These values are of primary interest in assessing quality of oil for consumption and industrial applications; however, they are not comprehensive.

The main physicochemical parameters for assessing oil quality include iodine value, refractive index, specific gravity, saponification value, and unsaponifiable content. The quality of oils is evaluated by the peroxide value and the free fatty acid value. How these values are measured and evaluated is summarised in Table 2.9. Standard values used to assess oils may be obtained from the CODEX Alimentarius (CODEX STAN).

While these values provide critical information, the physicochemical parameters, such as the iodine value, saponification value, and refractive index can be derived from the fatty acid composition. The fatty acid composition provides the fatty acids composition and quantities of the fatty acids, which subsequently can be

used to determine quantitative values for levels of saturation and unsaturation in the oil, and depending on the method used to identify and quantify the unsaturated fatty acid isomers present (O'Brien 2009:213-214).

Table 2.9: Commonly used quality and physico-chemical characteristics for evaluation of oils (Belitz *et al.* 2009:662-669; Pike 2003:227-240; O'Brien 2009:210-217).

Parameter	Units	Theory	Application
Iodine Number	% Iodine	The procedure is done by a titration with Iodine. It is a halogen addition reaction of a halogen to the carbon double bonds. Therefore it measures the amount of halogens a 100 g of oil is able to absorb.	This value indicates the degree of unsaturated fatty acids in the fat; it is used in identification of adulterated oils. The iodine number (IV) is used to determine the content of oleic, linoleic, and linolenic acids present in oil.
Refractive Index (RI)	N/a	Using a Refractometer (equipment for measuring refractive index) a value is obtained for the material which is then divided by the RI value for water (speed of light in air divided by the speed of light in given medium).	Oils have specific RI and this value may be used to determine the purity and confirm the identity of oil. It can be related to fatty acid composition, chain length, degrees of unsaturation and conjugation and the molecular weight. It is affected by temperature; therefore, a correction is used to account for temperature when measuring it. It can be also used as a control point in hydrogenation reactions.
Saponifica--tion Value	mg KOH g^{-1}	The procedure is done by a titration with a base, typically using potassium hydroxide (KOH). Therefore, the amount of base in mg needed to hydrolyse 1g of fat is measured.	The saponification value (SP) is used to determine the types of fatty acids present in the triacylglycerol's. Higher SP indicates that there is a higher concentration of lower molecular weight fatty acids present in the oil.
Peroxide Value	mEq of active oxygen/ kg oil	The peroxides in the oil are reacted with excess potassium iodide (KI). The peroxides and iodide ion react to form iodine, which is then measured by a titration with sodium thiosulphate ($Na_2S_2O_3$). A calculation is done to obtain the mEq of peroxides.	Unsaturated fats upon oxidation form primary products, peroxides, which at a certain point trigger formation of secondary oxidation products: ketones, aldehydes, hydroxyl groups. The peroxide value indicates the extent of primary oxidative degradation of the oil (rancidity).
Free Fatty Acid Value	mg g^{-1}	It is done by titration reaction with sodium hydroxide, NaOH, and it is based on the quantity of milligrams needed to neutralize the organic acids present in 1 g of fat.	Lipolysis is a reaction which involves the breakdown of triglycerides into glycerol and free fatty acids, also known as hydrolysis. Free fatty acid value and acid value both measure the extent of lipolysis of a sample, and are used to determine if the oil can be directly consumed. This value indicates the extent of hydrolytic rancidity. It can be related to the Acid Value by multiplying the FFA % value by 1.99.

Fatty acids may be characterized by using either Gas Chromatography (GC) or High Performance Liquid Chromatography (HPLC). Gas chromatography (GC) is the primary method of analysis for quantitative and qualitative characterization of the fatty acid profile of oils. GC analysis after derivatization of the oils is primarily used for oils and fats which contain low content of aromatic compounds. Oils which contain large quantities of ketones, aldehydes, and rings need to be analysed with a different method which does not destroy them. Prior to analysis with either GC or HPLC, the oil sample typically undergoes a direct transesterification (i.e. derivatization). There are two general reasons for the derivatization procedure, either to enable the analysis or to improve the quality of the analysis (Pike 2003:241-245; Poole 2012:530).

Through transesterification, the hydrogen bonding forces, which hinder the volatility of the molecules, can be increased. The polar groups (NH, OH, SH) of a fatty acid are substituted with an application-specific group. Therefore, the transesterification aids in the analysis process in the following ways: enhances separation by reducing the volatility, improves separation and peak shape by stabilising highly volatile oils, and reduces adsorption of the polar components onto the column (Knapp 1979:2-4).

There are various procedures for derivatization, each varying in degree of involvement and cost, as well as application. Depending on the compounds of interest and the composition of the solution to be analysed, one can select a suitable reaction. Derivatization reactions are classified into (i) alkylation, (ii) silylation, (iii) acylation, and (iv) condensation (Knapp 1979:151-152). The standard transesterification involves a catalyst (this may be an acid or a base) and a higher alcohol (such as methanol). The reaction essentially works on the basis that the catalyst saponifies the lipids, thus cleaving the ester bond and releasing the glycerol and the fatty acid salts; subsequently, the fatty acids can be esterified by the alcohol to create fatty acid methyl esters (FAMEs) (Poole 2012: 530-535).

CHAPTER 3: EXPERIMENTAL PROCEDURE AND RESULTS

SCOPE

The current chapter covers the experimental procedures used to determine the quality of the Marula kernels used for the extraction, the processing of the Marula kernels, the SFE conditions applied, the analysis of the extracted oils, and the physico-chemical analysis of SFE extracted Marula oil acquired at the optimal operating conditions and one obtained by pressing (for comparison). The quality of the extracts and their composition was evaluated using GC-FID analysis of the fatty acid profile at various extraction conditions (completed by the SABS). Additionally, the free fatty acid (FFA), peroxide value (PV) and iodine value (IV) of the oil obtained at the recommended conditions were evaluated and compared to the values of a cold pressed sample of Marula oil (analyses completed by the SABS). These values are used to make a recommendation on the applications and quality of the oil. Additionally, the raw material for the extractions as well as the residue were analysed for crude fat, protein, fibre, and ash content (analyses completed by Quantum Laboratories).

3.1 MATERIAL PREPARATION

Medical grade, liquid CO_2, with a purity of 99.0 % was used. The cleaned raw Marula kernels, 75 kg, were sourced from Marula Products (Pty) Ltd., Palaborwa, South Africa; collected during February through to March, 2013. The kernels were stored at regulated room temperature, 21 °C, until the day of use. The kernels were prepared immediately prior to extraction by grinding with a food processor. The average particle size distribution was 850 µm.

The moisture content of the kernels was tested prior to every extraction and was tested using a 103 °C isocratic program on a Citizen Moisture Analyzer (ISO 9001:2000 certified) with a halogen lamp. The average moisture of the kernels was 4.4 wt. %.

To evaluate the quality of the cleaned, raw Marula kernels, a nutritional analysis was completed. The fat, protein, fibre and ash content were analysed in duplicate using AOCS, AOAC and ALASA standard procedures (analyses were completed by Quantum Laboratories).

The crude fat content of the Marula kernels was determined as described in method AOCS Am 5-04, using the ANKOM XT15 solvent extraction system. A representative sample of marula kernels was loaded into a filter bag and encapsulated. The sample was exposed to petroleum ether in order to extract the oil, and the percent of oil was calculated by measuring the loss in mass after the extraction period.

The crude protein was determined as indicated in method AOAC 992.23/990.03, using the Dumatherm Combustion System. A representative marula sample was combusted to nitrogen, carbon dioxide and water in the presence of pure oxygen and at temperature of 900 °C. The carbon dioxde and water were selectively removed and the nitrogen content was measured by a thermal conductivity detector. A factor based on sample type was used to convert the nitrogen to crude protein. The factor used for the Marula kernels was 6.25.

The crude fibre was analysed as per method AOCS Ba 6a-05. The protein, fat, starch and other digestible carbohydrates were hydrolysed using hot acid and alkali, and subsequently removed. The residue was dried and ashed to determine the loss on ignition of the crude fibre.

The total ash content of the sample was determined as described in methods AOAC 942.05 and ALASA 2.5.1. The organic material was removed from the sample by heating it in a muffle furnace, and the mineral ash content was determined by determining the loss in mass after heating.

3.2 SUPERCRITICAL FLUID EXTRACTION

3.2.1 SYSTEM INFORMATION

The plant used for the extractions is a standard Research and Development (R&D) high pressure extraction unit, Natural Process Technology (NATEX), Austria (Figure 3.1). The extraction system is designed as 1000 bar pressure unit, however, the maximum operating pressure and temperature are 925 bar and 93 °C, respectively. The system includes a CO_2 storage tank (a), which is filled prior to extraction and is used to recycle the carbon dioxide. The CO_2 goes through and evaporator (f) and a mass filter (h) prior to returning to the storage tank(s) to be reused. The extractor vessel (d) is vertically mounted and is equipped with a removable basket with an internal volume of 5.5 *L* (10 cm diameter, 70 cm height). The extractor may be used without the basket, and has an 8 *L* internal volume. The system has the option for a two-step separation, as it is equipped with two separators (e, f). The amount of CO_2 consumed throughout an extraction is measured by a digital mass flow meter (h). The temperatures of the extractor and the two separators are manipulated by independently controlled heating circuits for each vessel. The system is equipped with a pre-cooler (b) to cool off the CO_2 to the adequate temperature before being compressed by the diaphragm pump (c) to reach the desired extraction pressure. The flow rate of CO_2 may be varied between 5 and 40 kg hr^{-1}, however recommended maximum operational flow rate is 30 kg hr^{-1}. During extraction, the system parameters and the system itself are controlled by a programmable logic control system (PLC).

Figure 3.1: The 5 L Supercritical extraction pilot plant unit, (Natex, Austria): (a) CO_2 Storage Tank, (b) Pre-cooler, (c) Pump, (d) Extractor (within which the extractor basket is stored), (e) Separator 1, (f) Evaporator, (g) Separator 2, (h) Filter Trap and (i) Flow Meter.

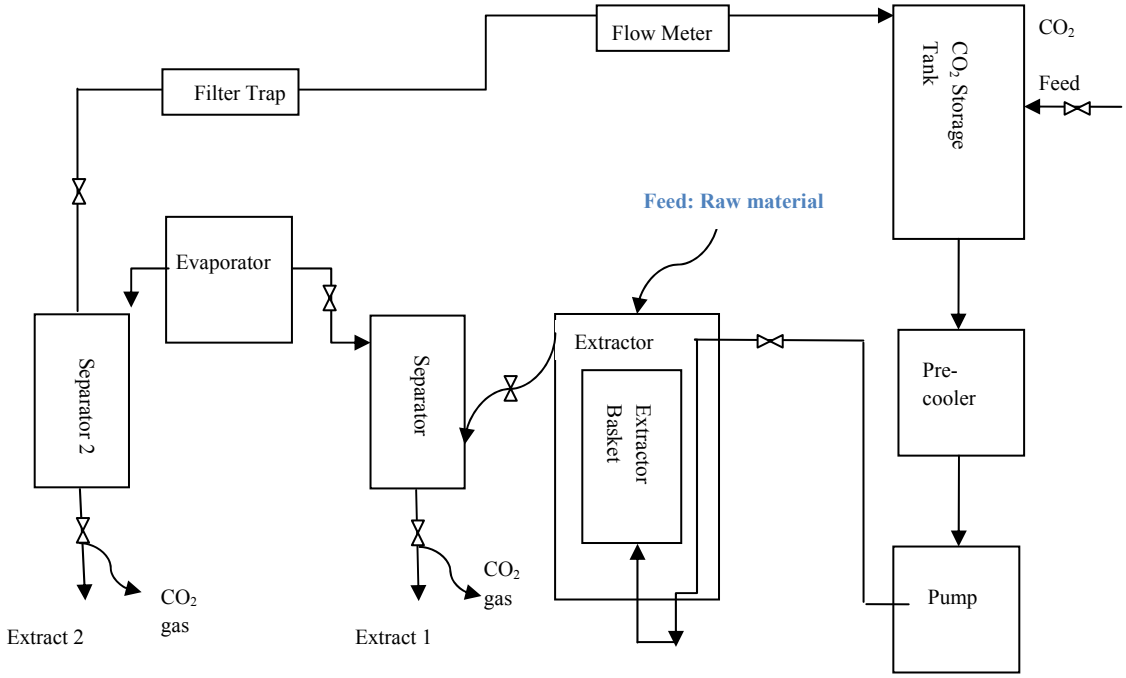

Figure 3.2: A schematic of the subsystems of the 5L SFE pilot plant presented in Figure 3.1.

3.2.2 Extraction Conditions

The equipment was prepared in advance, prior to extraction, as described in the manual provided with the equipment (Appendix A). For each run, 2500 g of ground Marula kernels were loaded into the basket, leaving out a 15 cm headspace to account for expansion of the material during extraction. The material loaded was purged once with CO_2 before filling up to pressure (48-60 bar).

Liquid CO_2 (99.0 % purity) was passed through the pre-cooler (Figure 3.2) before being compressed to operating pressure by the diaphragm pump (Figure 3.2), after which it was passed through the heated jacket (inside the extractor, Figure 3.2) to reach the desired extraction temperature. The pressure was controlled by a back-pressure regulator. Compressed and heated CO_2 flowed into a vertically mounted extraction vessel containing an extraction basket (Figure 3.2) with an inner diameter of 10 cm and height of 70 cm, with an internal volume of 5.5 *L*. The temperatures of the extraction vessel and the two separators were controlled by the surrounding jacket water bath; the temperature is detected by a thermocouple in the water bath and regulated by a digital controller within an accuracy of 0.1 °C. The extraction was kept static until the extraction pressure was reached; this took an average of 15 min per run to reach extraction conditions. Once the extraction pressure and temperature were reached, the timer on the extraction run was commenced and sampling initiated. After the timer was started the entire extraction process was dynamic. The extract was collected in the first separator. The phase of the CO_2 was changed from supercritical to gaseous by reducing the pressure and temperature; this subsequently reduced the CO_2 affinity for the extract and the extract deposited to the bottom of the separator (Figure 3.2). The samples were drained from the bottom valve at prescribed time intervals; no samples were collected in the second separator (Figure 3.2). The volume of CO_2 consumed was measured using the mass flow meter (Figure 3.2).

Table 3.1: The supercritical extraction variables tested in this study: pressure, temperature, and CO_2 density.

Run No.	Pressure (bar)	Temperature (°C)	CO_2 Density (kg m^{-3})
NWU 50, 59, 68	250	75	712
NWU 51, 60, 69	250	60	787
NWU 52, 61, 70	250	40	880
NWU 53, 62, 71	450	75	867
NWU 54, 63, 72	450	60	913
NWU 55, 64,73	450	40	970
NWU 56, 65, 74	350	75	808
NWU 57, 66, 75	350	60	863
NWU 49, 67, 76	350	40	935

The extraction conditions, pressure and temperature, were varied and are summarised in Table 3.1. The experimental temperatures were 40, 60 and 75 °C, and pressures were 250, 350 and 450 bar. The flow rate, particle size and duration of extraction were kept constant. Each extraction lasted for 270 min with a constant flow rate of 30.0 kg CO_2 hr^{-1}. Each set of extraction conditions was repeated in triplicate. Samples were taken out at set time intervals in order to track the total oil extracted per given time; samples were taken at start, 15, 30, 60, 90, 120, 150, 180, 210, 240 and 270 min. Samples were individually weighted to determine the quantity of oil extracted. Oil extracts were combined and stored at 4 °C until analysis; the fractions taken out at the various times were combined into a single sample. The accumulated yield was calculated as per Equation 3. The weights of the samples and accumulated percent yields are summarized in Table 3.3.

$$Accumulated\ yield = \left(\frac{total\ extract\ weight\ of\ oil\ at\ a\ given\ time\ point,\ g}{weight\ of\ substrate,\ g} \times\ 100\%\right) \quad [3]$$

Throughout each run, the quantity of carbon dioxide consumed was recorded at each of the sampling periods. Subsequent to the completed extractions, the accumulated amount of oil per kg of carbon dioxide was calculated as per Equation 4. The results are summarised in Table 3.2.

$$\frac{oil,\ g}{CO2,\ kg} = \frac{accumulated\ oil,\ g}{acumulated\ solvent\ consumed,\ kg} \quad [4]$$

Table 3.2: The quantity of oil extracted per kg of solvent consumed over time for the extractions completed at 250, 350, and 450 bar and 40, 60, and 75 °C.

P, bar	250	250	250	350	350	35	450	450	450
T, °C	75	60	40	75	60	40	75	60	40
Time, min	g oil kg^{-1} CO_2								
15	1.8	5.4	8.1	15.9	19.6	15.7	37.5	41.5	21.9
30	2.7	6.4	8.9	14.6	17.6	14.3	33.5	34.3	19.0
60	3.3	6.2	8.9	14.3	16.8	13.3	29.1	29.9	17.3
90	3.7	6.3	8.5	13.1	15.1	12.7	26.6	25.9	16.4
120	3.8	5.8	8.0	12.2	14.0	12.2	22.1	21.4	15.4
150	3.4	5.5	7.6	11.5	13.2	11.8	18.0	17.6	13.7
180	3.2	5.2	7.3	11.1	12.4	11.2	15.2	14.8	12.1
210	2.9	4.9	7.0	10.8	11.3	10.5	13.2	12.8	10.8
240	2.7	4.7	6.8	10.3	10.2	9.5	11.6	11.3	9.6
270	2.6	4.5	6.6	9.6	9.1	8.7	10.3	10.1	8.7

Table 3.3: Extraction yields and extracts weights obtained at extraction conditions shown.

P, bar	250		250		250		350		350		350		450		450		450	
T, °C	75		60		40		75		60		40		75		60		40	
Density, kg m^{-3}	712		787		880		808		863		935		867		913		975	
Time, min	Oil, g	Cumulative Yield, %	Oil, g	Cumulative Yield, %	Oil, g	Cumulative Yield, %	Oil, g	Cumulative Yield, %	Oil, g	Cumulative Yield, %	Oil, g	Cumulative Yield, %	Oil, g	Cumulative Yield, %	Oil, g	Cumulative Yield, %	Oil, g	Cumulative Yield, %
15	12	0	39	2	57	2	115	5	136	5	99	4	245	10	285	11	166	7
30	26	2	55	4	72	5	101	9	130	11	102	8	230	19	197	19	142	12
60	58	4	89	7	136	11	210	17	250	21	189	16	379	34	390	35	275	23
90	69	7	96	11	114	15	163	24	172	27	174	23	299	46	266	46	240	33
120	62	9	69	14	98	19	148	29	155	34	159	29	131	51	117	50	202	41
150	28	10	62	16	93	23	132	35	150	40	153	35	36	53	43	52	82	44
180	26	11	54	19	84	26	136	40	117	44	126	40	23	54	18	53	25	45
210	24	12	51	21	82	29	124	45	72	47	99	44	17	54	13	53	18	46
240	21	13	51	23	80	33	105	49	32	49	55	46	12	55	13	54	12	47
270	21	14	47	25	77	35	66	52	17	49	22	47	9	55	9	54	8	47
Average Yield, %		14		25		35		52		49		47		55		54		47

3.3 Characterisation of Extracts

3.3.1 Fatty Acid Analysis

Following the extractions, in order to evaluate the oils obtained, the extracts' fatty acid composition was analysed. All of the extracts were analysed using a Agilent 6890A gas chromatograph fitted with an FID detector (Agilent Technologies). The instrument was equipped with an HP-88 column (L *x* ID *x* df: 100 m *x* 0.25 mm *x* 0.20 µm). The carrier gas used was helium with a 10:1 split, and a flow rate (26.5 mL min^{-1}). The temperatures of the injector and detector were 250 °C and 280 °C, respectively. The program used was as follows: starting temperature at 140 °C, with a linear temperature ramp to 200 °C at 6 °C min^{-1}, and final ramp 2 °C min^{-1} to 240 °C (hold 15 min). The total time of analysis was 45 minutes. The system was pre-calibrated to identify 37 fatty acids, using a Supelco 37-component FAME mix standard, 10 mg mL^{-1} in CH_2Cl_2, (Catalog no. 47885-U). All oils were derivatised prior to analysis to prevent degradation of the column, using the AOCS C2-65 procedure. The injection volume of sample was 1 μL. For the determination of the fatty acid in the oil, the area normalization procedure was followed, using the area on the chromatograph; the solvent peak was excluded. The results of the analyses are presented in Table 3.4. A typical GC chromatogram obtained of the Marula fatty acid methyl esters is presented in Figure 3.3.

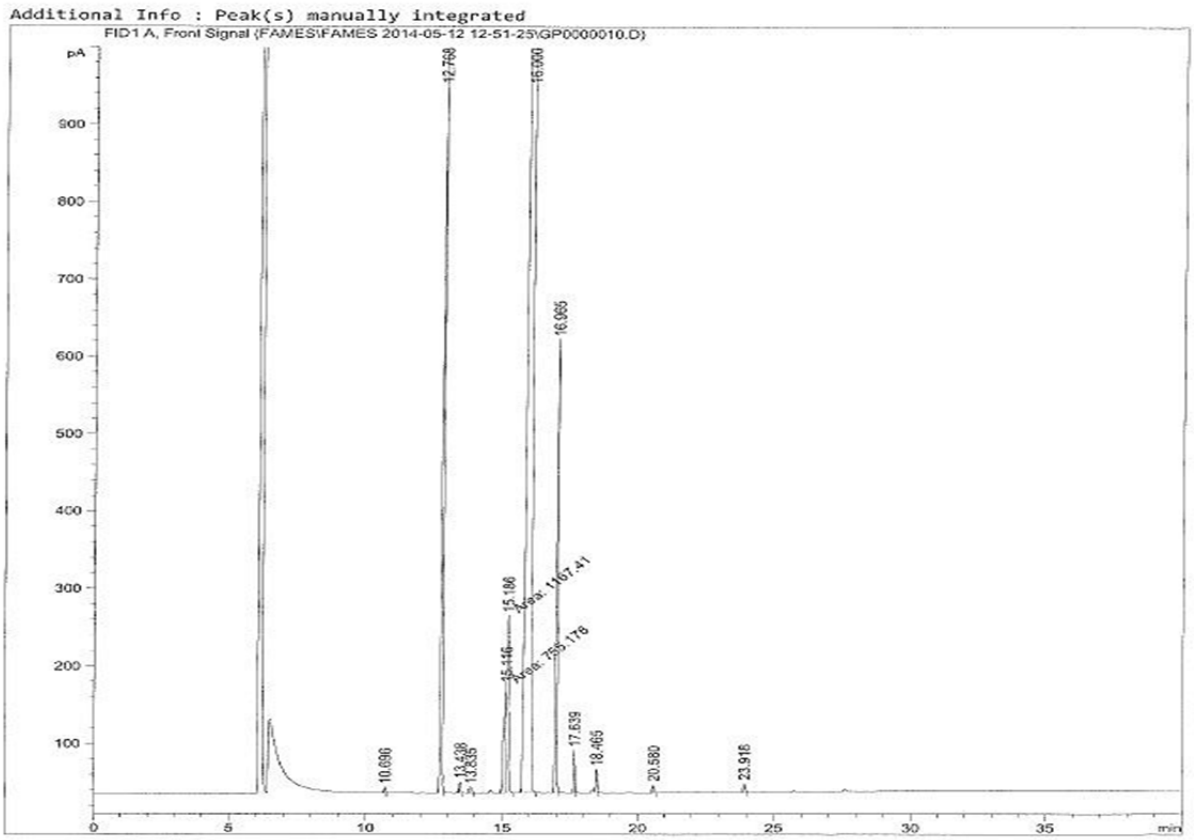

Figure 3.3: Typical Marula FAMEs chromatogram.

Table 3.4: The fatty acid composition of SFE extracted Marula oil.

Sample Information		Fatty Acids, %							Saturation, %		
T (° C)	P (bar)	Palmitic (C16:0)	Margaric (C17:0)	Stearic (C18:0)	Palmitoleic (C16:1)	Oleic (C18:1)	Linoleic (C18:2)	(γ and α) linolenic (C18:3)	Saturated	Mono-unsaturated	Poly-unsaturated
40	**250**	**13.0**	**0.1**	**5.6**	**0.2**	**72.6**	**7.5**	**0.7**	**18.9**	**72.9**	**8.2**
40	**350**	**12.1**	**0.1**	**6.7**	**0.1**	**72.4**	**7.3**	**0.9**	**19.1**	**72.6**	**8.3**
60	**250**	**13.1**	**0.1**	**5.3**	**0.2**	**72.7**	**7.7**	**0.7**	**18.7**	**72.9**	**8.5**
60	**350**	**12.6**	**0 1**	**6.3**	**0.1**	**72.4**	**7.1**	**0.9**	**19.2**	**72.6**	**8.2**
60	**450**	**12.7**	**0.1**	**6.3**	**0.1**	**72.2**	**7.2**	**0.9**	**19.3**	**72.5**	**8.2**
80	**250**	**13.4**	**0.1**	**5.5**	**0.2**	**72.6**	**7.2**	**0.7**	**19.2**	**72.9**	**7.9**
80	**350**	**12.4**	**0.1**	**6.4**	**0.1**	**72.3**	**7.4**	**0.9**	**19.1**	**72.5**	**8.4**
80	**450**	**12.7**	**0.1**	**6.6**	**0.1**	**72.3**	**6.8**	**0.9**	**19.6**	**72.5**	**7.9**
Cold pressed Marula oil*		**12.3**	**0.1**	**6.5**	**0.1**	**72.9**	**6.7**	**0.9**	**19.1**	**73.1**	**7.8**

***Cold pressed Marula oil sample obtained from Natural Marula Products (Pty) Ltd, Palaborwa, South Africa.**

3.3.2 Free Fatty Acid, Peroxide, and Iodine Values

The sample of oil obtained at the optimised extraction conditions, 450 bar and 60 °C, and the cold pressed Marula oil sample were analysed for quality by determining the Free Fatty Acid (FFA), Peroxide (PV), and Iodine Values (IV) via standard AOCS methods.

The FFA value was determined as described in method AOCS Ca 5a-40. The method is based on a neutralisation reaction, such that the amount of base needed to neutralize the organic acids present in 1 g of fat is measured. The value was obtained by titration with sodium hydroxide (NaOH).

The PV was determined as described in method AOCS Cd 8-54. The value was measured by dissolving the oil in glacial acetic acid, and subsequently, reacting the peroxides or other oxidation compounds within the oil with potassium iodide (KI). The peroxide determination relies on the assumption that the compounds reacting with the KI are peroxides or similar products of lipid oxidation. The peroxides and iodide react to form iodine, which is subsequently quantified by titration with sodium thiosulfate ($Na_2S_2O_3$). Using the quantity of $Na_2S_2O_3$ needed to react with iodine, the miliequivalents (mEq) of peroxide present in one kg of sample were calculated (1 mEq is one thousandth of the equivalent weight of an element, radical, or compound).

The iodine value was determined as described in method AOCS Cd 1-25. The iodine measurement is obtained by determining the number of grams of iodine, which bind to 100 g of fat. The oil samples were reacted with excess Wijs reagent (Iodine monochloride (ICl)) for 30 min at 25 °C; subsequently, the excess reagent was reacted with KI to convert to iodine, and titrated with $Na_2S_2O_3$ and a starch indicator. The results obtained for the FFA, PV and Iodine parameters for the cold pressed and the SC-CO_2 extracted Marula oil are summarised in Table 3.5.

Table 3.5: Quality characteristics of the cold pressed and SC-CO_2 extracted Marula oil.

Parameter	Unit	CP	450 bar, 60 °C
Iodine Value (IV)	**Iodine Absorbed**	78	77.3
Peroxide Value (PV)	**mEq kg^{-1}**	1.08	1.84
Free Fatty Acid (as oleic acid), FFA	**g 100g^{-1}**	9.01	14.8

If the fatty acid profile is available, the iodine value is calculated using the values from the fatty acid profile of the oil using Equation 4. The calculation sums up the percent of unsaturated fatty acids multiplied times their respective constants (O'Brien 2009:214; Pike 2003:233).

IV= (% C16:1*0.95) + (% C18:1*0.86) + (% C18:2*1.732) + (% C18:3*2.616) + (C20:1*0.786) + (% C22:1*0.723) [4]

3.3.3 *Vitamin Analyses*

The Marula oil samples obtained by SFE and by cold pressing were analysed for carotenoid (vitamin A) and tocopherol (vitamin E) content using a combination of two methods, one described in the procedure for HPLC analysis of tocopherols by the European Committee for Standardisation, EN12822 (2003), and the other for HPLC analysis of tocopherols and carotenoids, by Franke *et al.* (2013). The determined vitamin values for the Marula oils analysed are presented in Table 3.6.

Table 3.6: Vitamin content of the cold pressed and SC-CO_2 extracted Marula oil.

Parameter	Unit	CP	450 bar, 60 °C
Δ-tocopherol	**mg 100 g^{-1}**	0.64	0.77
γ-tocopherol	**mg 100 g^{-1}**	18.8	17.0
β-carotene	**mg 100 g^{-1}**	ND	ND

3.4 Extract Residue Analysis

The residue (defatted flour) of the raw material exposed to the extraction conditions which provided the highest oil yield was evaluated for proximate nutritional composition. The sample was analysed for fat, protein, fibre, and ash content using AOCS, AOAC and ALASA procedures as described in Section 3.1. The results of the nutritional composition analyses of the residue and the raw material prior to extraction are summarised in Table 3.7.

Table 3.7: Nutritional composition of the Marula kernels and the defatted flour after SFE-CO_2 extraction.

Parameter	Unit	Defatted Flour	Marula Kernels (Full Fat)
Ash	**%**	8.1	3.4
Crude Fat	**%**	12.2	52.1
Crude Fibre	**%**	7.5	6.4
Moisture (103 °C)	**%**	-	4.4
Protein Crude	**%**	69.4	28.1

3.5 Marula Kernel Cell Structure

The Marula kernels (endosperms) are composed mainly of parenchyma (storage) cells, packed with nutritional components (fats, proteins, starches, fibre) (Salunkhe *et al.* 1992:506). Parenchyma cells typically have thin

walls which include minor quantities of polysaccharides, glycoproteins and phenolic compounds (Aguilera & Stanley 1999:180). To gain an understanding of the Marula kernel cell structure, slides with stained Marula kernels slices were prepared. The proteins and fats in the cell were stained with a polyethylene glycol based solution for identification and scanned with a light microscope equipped with a Nikon camera. Two selected images depicting Marula cell structure and components are presented in Figure 3.4.

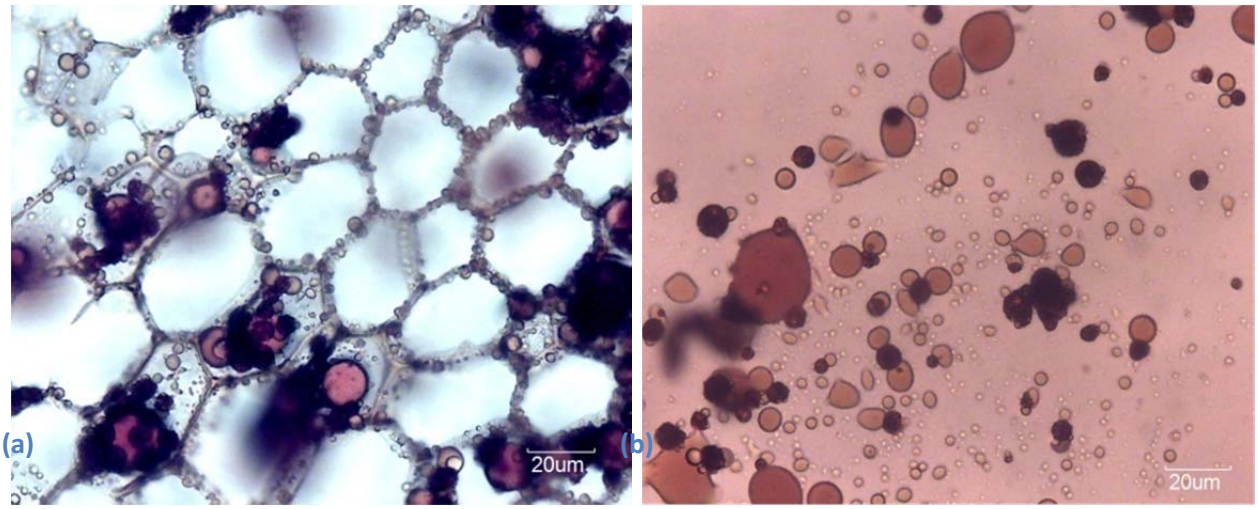

Figure 3.4: Light microscope images of an (a) intact Marula endosperm cell layer and (b) burst cell components dispersed; oil droplets (light) and protein bodies (dark) are visible.

In Figures 3.4 (a) and (b) the lipids are the light spheres and the dark spheres are protein bodies. In Figure 3.4 (a), the interconnections between the cells and the cell walls can be seen. There are some intact cells with the protein and lipid bodies filling out the entire cell centre, and there are empty cells where only the oil droplets aligning the cell walls are visible. Overall, it can be concluded that Marula oil is attached to the cell wall and loosely dispersed within the cells. As discussed for other plant-based materials breaking the cells is required in order to improve the extraction yield (Brunner 1994:185). The cells in Figure 3.4 (b) look hollow due to the oil falling out of the sliced cell layer during sample preparation. The cells may be classified as isodiametric in shape and vary in size from cell to cell, with a range of 20-50 µm in diameter. A three dimensional visual would be needed to obtain a correct cell shape and dimensions.

Scanning Electron Microscope (SEM) images were taken of the defatted Marula obtained from the extractions at 450 bar and 75 °C using the FEI Quanta 250 FEG SEM. To prepare for the scan the samples were loaded onto double sided carbon conductive tape, followed by a Gold/ Palladium (66:33 wt. %) coating. The sample was then placed under 5 kV and high vacuum mode. Several images were taken at various magnifications; selected images are presented in Figures 3.5 (a) and (b).

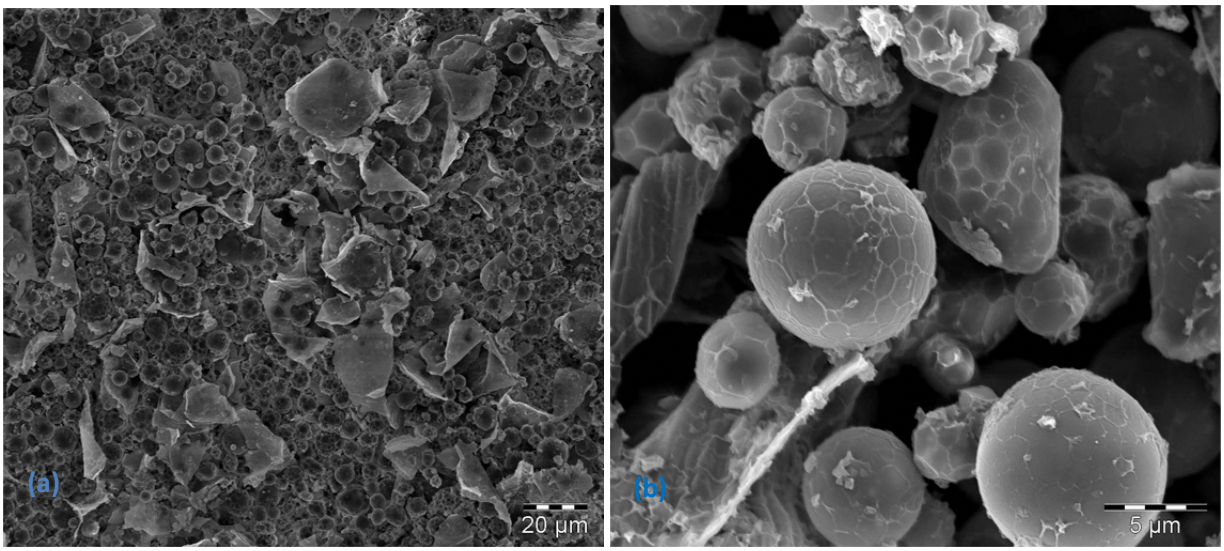

Figure 3.5: SEM images of the surface of a Marula kernel particle extracted with supercritical CO_2. (a) Broken vegetable structures (cells) that contained the free oil, the connections between the cells, the fragmented membranes, some oil droplets and protein bodies dispersed within. (b) Starch globules with indentations caused by the compression within the cells, and broken cell membrane fragments.

In Figure 3.5 (a), at 20 µm magnification, the broken cell walls and the loosely found protein bodies and remaining oil droplets may be observed. In Figure 3.5 (b), at 5 µm magnification, closer look of the protein bodies (globular), cell wall fragments, and oil droplets (spherical) may be observed with indentations on their surface where they may have been attached to the cell wall or to each other.

CHAPTER 4: DISCUSSION

In order to evaluate SFE-CO_2 as a possible extraction method for Marula kernel oil production, the effects of pressure and temperature on oil yield were evaluated and the oils were characterised. To determine the optimal safe operating conditions and study the effects of pressure and temperature on oil yield, the selected conditions tested were 250, 350, and 450 bar and 40, 60, and 75 °C. The flow rate was maintained at the maximum recommended operational flow rate of 30 kg CO_2 hr^{-1}. The particle size effects were not studied; however, the material was ground prior to extraction and the average particle size was determined post extraction, 850 μm. The extraction period was dynamic and lasted 270 min.

4.1 EXTRACTION PARAMETER OPTIMISATION

The results of the extractions, weight yield at a given time, and accumulated percent yield over time (grams of oil extracted per grams of substrate), at the varying conditions are summarised in Table 3.3. The yields as a function of solvent consumed are presented in Table 3.2. Out of the conditions tested in the range of pressures (250, 350, and 450 bar) and range of temperatures (40, 60, and 75 °C), the greatest yield was attained at extraction conditions of 450 bar, 75 and 60 °C, and 350 bar and 75 °C, with average percent yields of 55, 54, and 52 %, respectively. Because using lower temperatures is more favourable and because the oil yield per kg solvent within the first hour of extraction is greatest at 450 bar and 60 °C, 41.48 g oil kg^{-1} CO_2, the recommended conditions for oil extraction of Marula using SFE-CO_2 are 450 bar and 60 °C.

4.1.1 INFLUENCE OF EXTRACTION TEMPERATURE

The effects of the temperature on the extraction yield and rate of the extractions at a constant pressure are presented in Figure 4.1. During the extractions, the effects of temperature on extraction yield and rate at a constant pressure were not consistent between those observed at the lower pressure, 250 bar and the 350 and 450 bar.

The effect of temperature on the extraction rate at 250 bar may be visualised in Figure 4.1 (a). It can be deduced that the rate of extraction is equilibrium controlled at all temperature set points as indicated by the linear extraction curves. At constant pressure of 250 bar, the extraction yields as well as the rates of extraction were greater at 40 °C, 35 % oil yield, and considerably lower at 60 °C, 25 % yield, and 75 °C, 14 % yield, the lowest yield obtained.

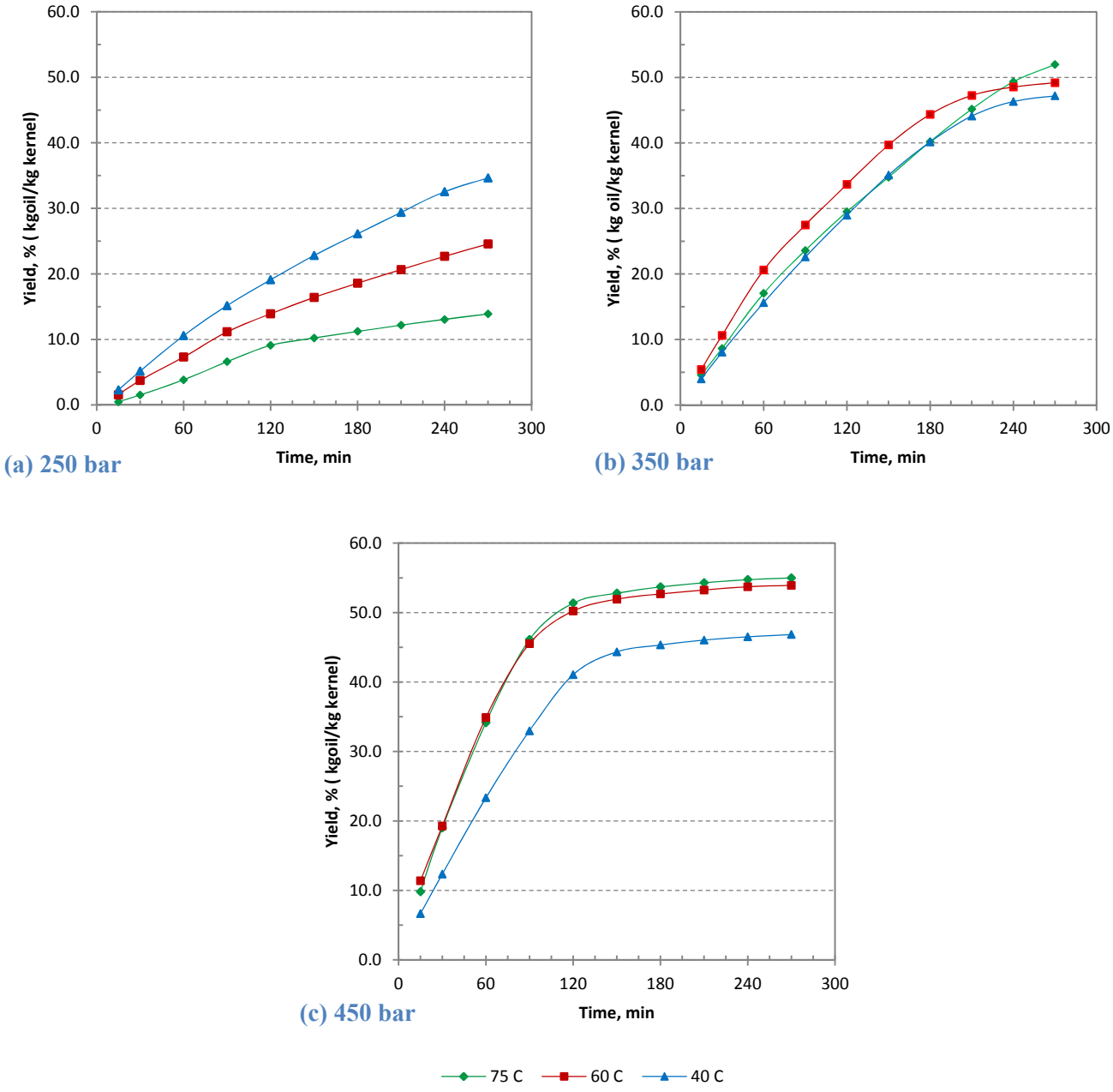

Figure 4.1: The effect of temperature on the extraction rate of Marula kernel oil at a constant pressure: (a) 250 bar, (b) 350 bar, and (c) 450 bar.

In Figure 4.1 (b), at a constant pressure of 350 bar, the effects of temperature on the yield are not drastic. It can be perceived that during the first 210 min the extraction rate and yield are nearly identical between those at 75 and at 40 °C (Figure 4.1b); by the end of the extraction between 210 min and 270 min, the yield attained at 75 °C increases by 5 %, compared to the yield at 40 °C. During the beginning of the extraction period, at an operating temperature of 60 °C, the extraction proceeds at a faster rate than the extractions at 75 or 40 °C.

However, at the end the extraction, the yield at 60 °C is 3 % lower than that at 75 °C. Therefore, it may be concluded that using 60 °C , compared to 40 °C and 75 °C, in order to increase the rate of the extraction at 350 bar and shorten the extraction time, 350 bar and 60 °C are satisfactory operating conditions.

In Figure 4.1(c), at constant pressure of 450 bar, the extraction curves at 60 and 75 °C were almost identical (Figure 4.1c). However, the extraction curve at 40 °C indicates a slower rate of extraction compared to the curves at 60 and 75 °C. It may be deduced that at a constant pressure of 450 bar, the increase in temperature from 40 to 60 °C increases the rate of extraction. The increase from 60 to 75 °C does not significantly affect the rate of the extraction, as the difference between the yields obtained at the two temperatures is 1 %.

Overall, the observed effects of temperature at 250 bar are opposite from the effects observed at 350 bar and 450 bar. The effects of temperature are more drastic at 250 bar than at the higher pressures.

The observed differences of the influence of temperature on solubility at 250 bar, compared to the effects of temperature at higher pressures, is a result of the large decrease in CO_2 density and subsequently a reduced solute solubility with an increase in temperature from 40 to 60 and 75 °C (Brunner 1994:192-195; Jokic *et al.* 2011a). Therefore, it can be concluded that at 450 bar, the increase in solid sublimation pressure with increasing temperature overcomes the density reduction effect observed at 250 bar, such that the solubility increases with increasing temperature.

4.1.2 Influence of Extraction Pressure

From Figure 4.2, the increase in pressure had the most drastic effect on the yield and the rate of extraction at a constant temperature. The yield and rate of extraction increased with the increase in pressure from 250 to 350 and to 450 bar. A difference in yield of 20 % between the maximum yields obtained at 250 and 450 bar was observed to be 35 % and 55 % respectively. Additionally, while two different extraction periods may be observed in the extractions completed at 450 bar, at 250 bar the extraction curves are close to linear, indicating equilibrium controlled extraction throughout the entire extraction period of 270 min. At 450 bar, there are two stages of the curve; (i) the equilibrium controlled stage and the (ii) diffusion controlled stage. The equilibrium guided stage is marked by the steep linear slope, followed by a short transition period, and a subsequent reduced linear slope indicating a slow extraction rate. The results obtained at 450 bar are similar to published curves using the Broken-Intact Cell model published by Sovova (1994) to model extractions of kernels (Bernardo-Gil & Casqulho 2007; Mezommo *et al.* 2009; and Salgin & Salgin 2013).

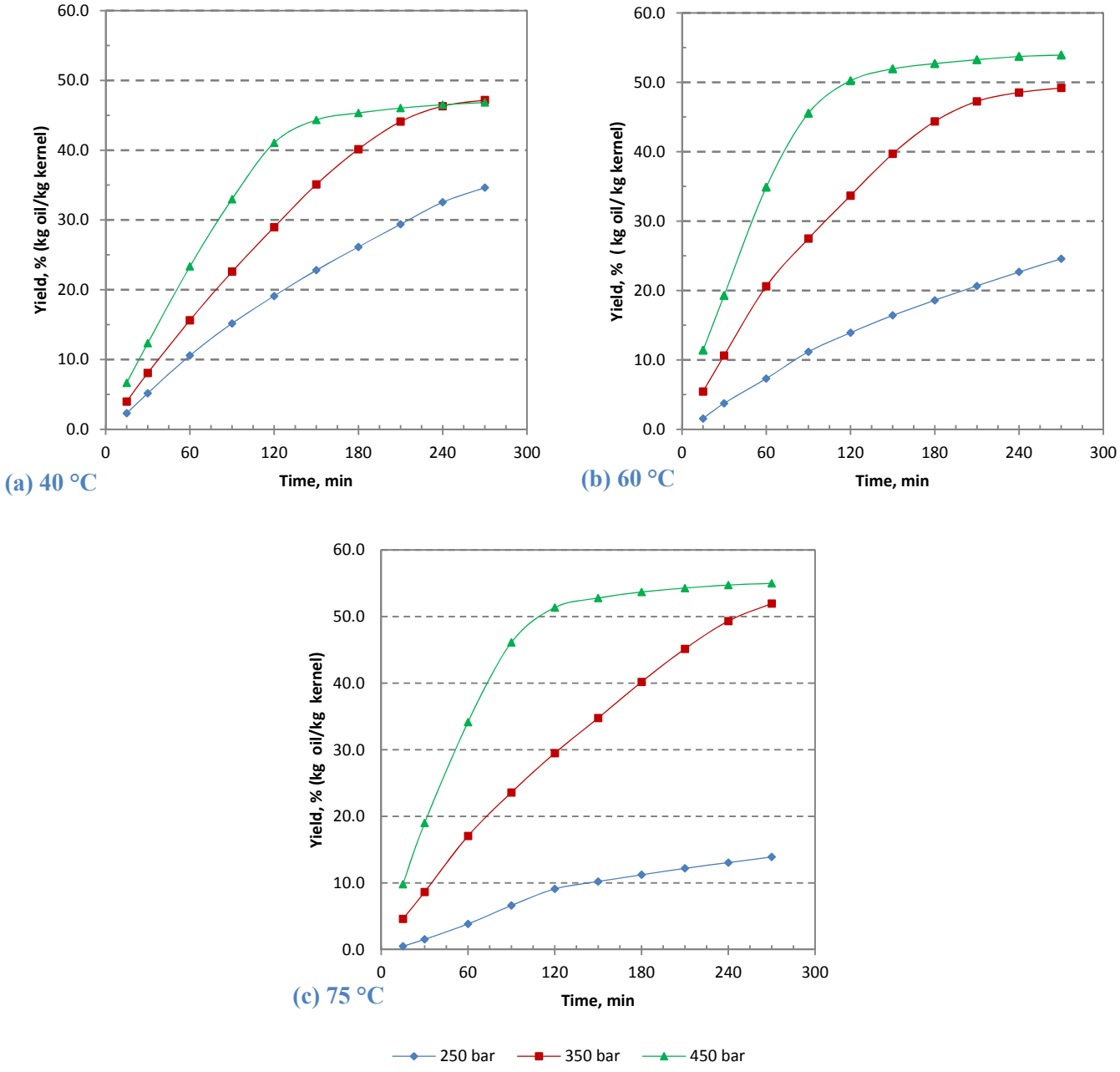

Figure 4.2: The effect of pressure on the extraction rate of Marula kernel oil, at constant temperature: (a) 40 °C, (b) 60 °C, and (c) 75 °C.

The rate of extraction at 350 bar, at all temperatures tested, appears linear, indicating a primarily equilibrium controlled extraction. When compared to the extraction rate at 250 bar and 450 bar, the rate is linear, much like that observed at 250 bar, however, the results are similar to those observed at 450 bar. The yields obtained at 350 bar, 47, 49, and 52 %, are close to those obtained at 450 bar, 47, 54, and 55 %, at the tested temperatures 40, 60, and 75 °C, respectively. Overall, the final yield at 80 °C and 350 bar compared to that obtained at 450 bar and 60 °C and 80 °C, is only 2 % and 3 % respectively lower.

From the graph presented in Figure 4.3 one can deduce that the cross-over pressure for Marula is around 330 bar. Therefore, for Marula oil, below 320 bar increasing the temperature decreases the yield, whereas above 330 bar, increasing the temperature increases the yield. Hazelnut oil is composed of nearly 75 % oleic acid; therefore, it is similar in composition to Marula oil whose major fatty acid, as reported in literature, is oleic acid (65-73%). Özkal *et al.* (2005b) reported a cross-over pressure between 150 bar and 300 bar for hazelnut and compared it to the cross-over phenomena reported by King and Bott, (1995). Salgin & Salgin (2013) reported a cross-over pressure of 230 bar for palm kernel oil. This cross-over phenomenon was explained to arise from the competing effects between increasing solvent density and the increase of volatility of the fatty acids, which increases with temperature (Özkal *et al.* 2005b). Overall, the findings of Özkal *et al.* (2005b) and Salgin & Salgin (2013) support the possible cross over pressure for Marula around 320 and 330 bar due to the similar behaviour observed for hazelnut and palm kernel oil. The higher cross-over pressure for Marula compared to hazelnut and palm kernel may be attributed to natural matrix differences between the materials. Such that, while there are compositional similarities between the materials, the accessibility of the oil can account for the differences in cross-over pressure for similar materials to Marula.

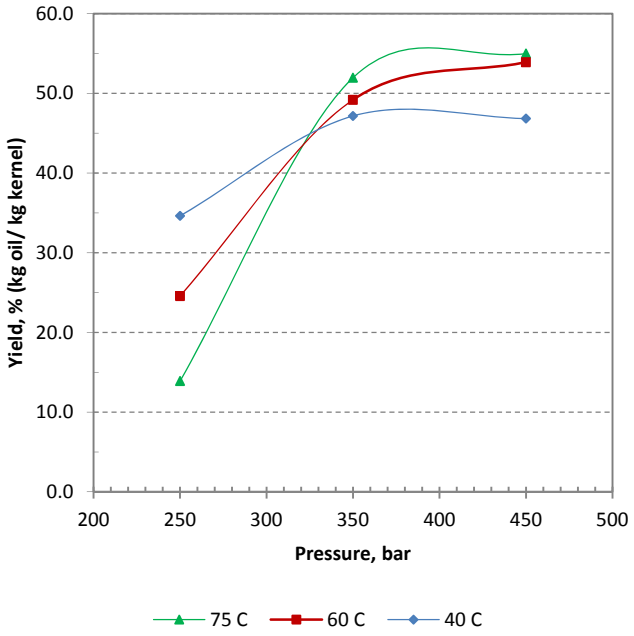

Figure 4.3: Effect of operating pressure on solubility of Marula kernel oil in CO_2 under different operating temperatures (Operating conditions: particle size, 750 µm, flow rate, 30 kg CO_2 hr^{-1}).

The observed differences in behaviour of the effects of pressure and temperature on the extraction are referred to as a cross-over pressure (Özkal *et al.* 2005b). The well-established cross-over pressure concept is marked

by the reduced density of CO_2 at higher temperatures at the lower pressures and subsequently lower extraction yields (Jokic *et al.* 2011a).

4.1.3 Yield vs. Solvent

The yield of oil per kg $scCO_2$ is dependent on the extraction conditions, as can be seen in Figure 4.4, and Table 3.2. The greatest yield of Marula oil per kg solvent is obtained at 450 bar and 60 °C, with a 41.5 g oil kg^{-1} CO_2 in the first fifteen minutes of extraction. The results of this extraction show that at a constant flow rate, over time, the amount of solvent that goes through the extraction matrix does not increase the yield, such that the extraction is primarily guided by the effects of the pressure and temperature.

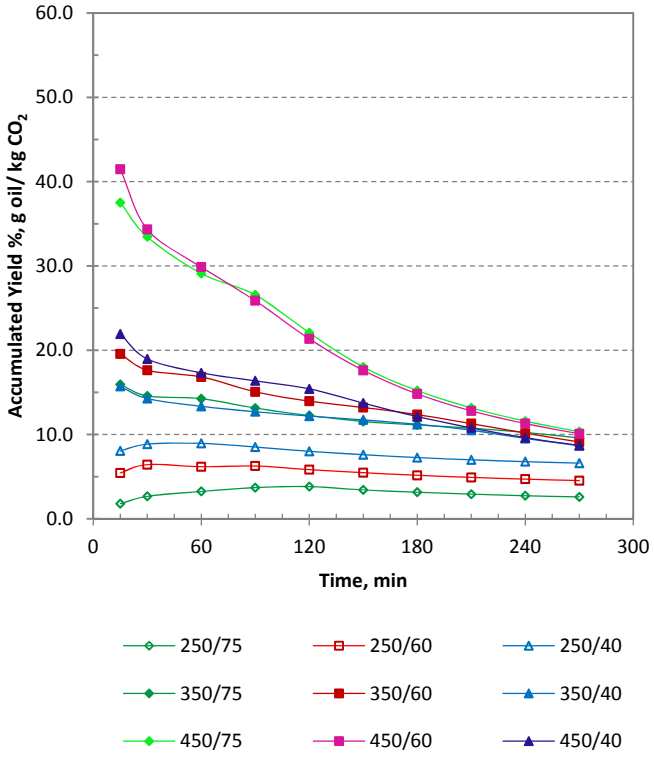

Figure 4.4: Accumulated weight of oil extracted per kg of $scCO_2$ over time.

In evaluating the cost of the extractions, although the yield may increase over time, there is a point where the solvent consumption is greater than the increase, such that the differences between g oil per kg solvent at 270 min is minor for the extractions completed at 350 bar and 450 bar. The differences in yield per solvent for 250 bar at the chosen extraction temperatures are more pronounced compared to the differences between samples obtained at the higher pressures.

4.5 Solubility Prediction vs. Empirical Data

Using the equation by Chrastil (1982), predicted solubility values were determined for the extraction conditions tested (Table 4.1) and are represented in Figure 4.5. The predicted solubility trends correspond well to the obtained extraction results for 350 and 450 bar, but not to the results obtained at 250 bar. Such that, at 250 bar and 40 °C the prediction indicates that the solubility of oleic acid is smaller than at 60 and 75 °C. Additionally, the indication that the greatest yield is obtained at 450 bar and 80 °C also agrees with the experimental conditions.

Table 4.1: The densities and the Chrastil solubility values for the extraction conditions evaluated in this study.

Temperature, °C	40		60		75	
Pressure, bar	Solubility g L^{-1}	Density kg m^{-3}	Solubility g L^{-1}	Density kg m^{-3}	Solubility g L^{-1}	Density kg m^{-3}
250	1.8	880	116	787	38.4	712
350	2.1	935	13.7	863	47.6	808
450	2.2	975	15.2	913	54.3	867

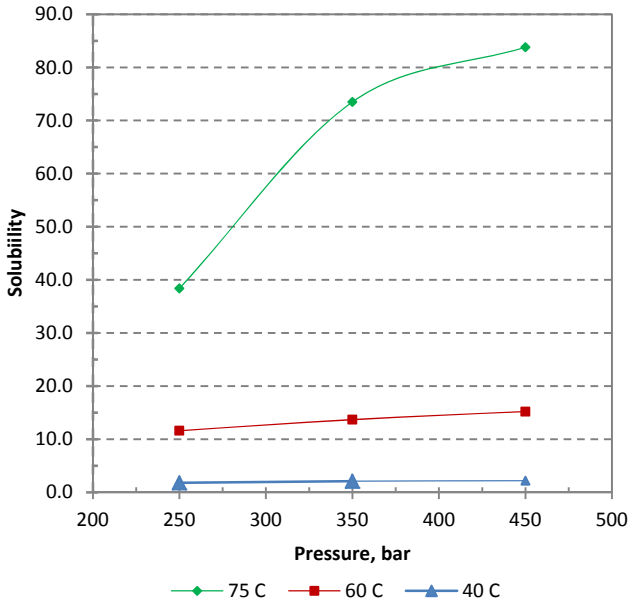

Figure: 4.5: The Chrastil predicted solubility values for oleic acid in $scCO_2$, at the conditions tested in this study.

The effects of the CO_2 density are different at different temperatures because of mass transfer (Brunner 1994:185-189). Mass transfer resistance overcomes the solubility effect of the CO_2 such that the extraction is primarily influenced by diffusion of solute and solvent through the plant matrix cell structure.

The density range of supercritical CO_2 from 860 to 880 kg m^{-3} enables the best yield for extraction of Marula oil. Increase in density above 860 kg m^{-3} does not improve the extraction yield significantly as the raise in yield is just 2 to 3%. However, visual differences in the oil may be observed between the samples obtained at different pressure set points achieving the chosen CO_2 density. Beyond yield, oil quality characteristics must be taken into account and the oil must be evaluated in comparison to traditionally extracted oil. The typical behaviour of CO_2 is summarised in the literature review presented in Chapter 2.

4.2 Oil Quality and Composition

The composition and physical properties of oils determine their applications, further processing requirements, adulteration and quality. Therefore, the fatty acid profile, the free fatty acid value (FFA), peroxide value (PV), and iodine value (IV) were determined for the Marula oil sample extracted with the optimal SFE conditions and a cold pressed oil sample. Additionally, the content of tocopherols and carotenoids in the two aforementioned samples was evaluated.

4.2.1 Oil Characterisation

The profile for the oil obtained at 450 bar and 60 °C and a summary of previously published results, where the Marula oil has been extracted with Sohxlet solvent extraction and characterized, are summarized in Table 4.2.

Table 4.2: Fatty acid composition of Marula oil as summarised in published literature and values from present study.

Reference	Fatty Acids, %					
	Palmitic (C16:0)	Stearic (C18:O)	Palmitoleic (C16:1)	Oleic (C18:1)	Linoleic (C18:2)	(γ and α) Linolenic (C18:3)
Salama 1973	**17.1**	**10.9**	**-**	**67**	**4.3**	**-**
Zharare & Dhlamini 2000	**10.7**	**6.9**	**-**	**72**	**-**	**-**
Glew *et al.* 2004	**15.63**	**11.9**	**-**	**63.19**	**5.21**	**-**
Mariod *et al.* 2012	**14.16**	**8.84**	**-**	**67.25**	**5.93**	**-**
Robinson *et al.* 2012	**12.8**	**7.2**	**-**	**73.6**	**6.1**	**-**
Present Study, (450 bar, 60 °C)	**12.8**	**6.3**	**0.2**	**72.2**	**7.2**	**0.9**

From Table 4.2, it can be seen that Marula oil consists mainly of oleic acid (C18:1), palmitic acid (C16:0), stearic acid (C18:0), and linoleic acid (C18:2). The results of the fatty acid analyses of the oils extracted at various supercritical extraction conditions is summarised in Table 3.3. Overall the fatty acid profile of the SFE obtained oil agrees with results published by Robinson *et al.* (2012) and Zharare & Dhlamini (2000).

According to the analyses obtained of the SFE extracted oils and the cold pressed sample, the SFE extracted oils have a similar composition to the cold pressed oil. Slight differences from the cold pressed sample FA profile were observed in the extracts obtained at 250 bar such that a higher content of palmitic acid, 13.4 % compared to 12.3 %, and a slightly lower gamma and alpha linolenic fatty acid was obtained (0.7 %, vs. 0.9 %). For the oils extracted at 350 bar and 450 bar, the values are very similar to those of the cold pressed sample. This is supported by studies comparing cold pressed and SFE samples, such as pistachio oil, where higher content of unsaturated fatty acids similar to cold pressing was reportedly found (Özkal *et al.* 2005b). Observed differences in the fatty acid composition, compared to results obtained by Glew *et al.* (2004), Salama (1973), and Mariod *et al.* (2012), may also exist due to the variety of kernels and seasons, regional differences, and methods of analysis.

Visual differences may also be observed in the samples extracted at the different extraction conditions (Figure 4.6). The oils extracted at 250 bar are clear with an ivory undertone, whereas the oils extracted at 350 bar have a clear to cloudy light-yellow colour, and the oils obtained at 450 bar are a clear golden-yellow colour. Therefore in selecting optimal operating conditions, the visual appearance and choice of colour will also influence the choice.

Figure 4.6: Visual differences between the oils extracted at the various SFE operating conditions tested; left to right: NWU61 (250 bar, 40 °C), NWU69 (250 bar, 60 °C), NWU59 (250 bar, 75 °C); NWU49 (350 bar, 40 °C), NWU57 (350 bar, 60 °C), NWU56 (350 bar, 75 °C); NWU71 (450 bar, 75 °C), NWU72 (450 bar, 60 °C), NWU53 (450 bar, 75 °C).

4.2.2 COMPARING COLD PRESSED OILS VS. SFE-EXTRACTED OILS

A sample of Marula oil extracted by cold pressing was evaluated for fatty acid composition and physico-chemical characteristics, which may be used to describe the quality of the oil and assess the potential application. The physico-chemical values determined were peroxide value (PV), free fatty acid (FFA), and iodine value (IV). Additionally, the vitamin content of the oil was determined. The same procedures were repeated for a Marula oil sample obtained at the optimal recommended conditions for SFE-extraction of Marula oil, 450 bar and 60 °C. The two oils are compared in this section. Results are summarised in Tables 3.5 and 3.6.

The peroxide value indicates the level of oxidative rancidity and values range from zero for high quality freshly deodorized oils to greater than 20 mEq for very low quality and highly oxidized oils or fats (Pike 2003:237-239). Values 1-5 mEq indicate low rancidity, 5-10 mEq indicate medium levels, and 10-20 mEq indicates high levels of rancidity. These are typically not acceptable to be used as is, and require further processing (CODEX STAN). The PV value of the cold pressed Marula oil was 1.08 mEq and that of the SFE oil was 1.84 mEq (Table 3.5). Both values are in the acceptable range of good quality oils with low levels of rancidity.

The free fatty acid values are relative and depend on the stage of processing of the oil, such that the FFA of a crude fat indicates the amount of oil to be lost during refining steps used to remove fatty acids. For refined fats, it indicates a poorly refined fat or breakdown during storage. If the fatty acids are volatile, the FFA value indicates the level of hydrolytic rancidity. The FFA is important for quick characterization of the quality of a fat.

The free fatty acid value of the cold pressed sample was 9.01 g per 100 g and the SFE extracted oil had a value of 14.8 g per 100 g (Table 3.5). However, the higher FFA values may be accounted for through several explanations. According to Mostert (2012), the treatment of the kernels during handling prior to extraction contributes to the growth of fungi, such as Rhizopus. These fungi contain a high content of lypase enzymes, which break fat down and subsequently contribute to elevated FFA values (O'Brien 2009:221). O'Brien (2009:607) suggests that the presence of carbon dioxide may contribute to elevated FFA values. It is not likely that the oils would have been hydrolysed during processing with SFE because reported studies which have evaluated the effect of SFE on FFA value, indicate that the oils obtained via SFE-CO_2 have lower FFA values compared to traditional extraction methods (Martinez *et al.* 2008; Jahurul *et al.* 2014). Jahurul *et al.* (2014) noted that the FFA values of mango kernel oil extracted with SFE-CO_2 were lower compared to values of oils obtained with solvent extraction. Similarly, Martinez *et al. (*2008) also reported low FFA values for walnut oils. Without eliminating other factors, which would have contributed to the high FFA value of the SFE

obtained Marula oil; the elevated FFA values cannot be attributed to SFE-CO_2 processing. Additionally, according to Pike (2003:235), the acidity of oils may also be influenced by acid phosphates and amino acids present in the oil.

The iodine value indicates the degree of unsaturation number of double bonds in relation to the amount of fat. It is defined as the weight of iodine absorbed per 100 g sample. This value is affected by content of oleic, linoleic, and linolenic acids. The higher iodine value, the greater the degree of unsaturation present in the oil (Pike 2003:229). Alternatively, the degree of unsaturation is derived from the free fatty acid profile, if available. The cold pressed and SFE extracted oil samples have values that are very similar, with 78.0 and 77.3 % Iodine absorbed, respectively.

Tocopherols are natural antioxidants found in vegetable oils that prevent the oxidation of oils. The tocopherols react with the peroxides and impede the free-radical chain reactions. Tocopherol analysis can aid in understanding an oxidative stability problem with oils (O'Brien 2009: 217). The Marula oil and the cold pressed sample were evaluated for tocopherol and carotenoid content. The analyses indicate that the Marula oils do not contain any carotenoids; however, they do contain tocopherols, mainly Δ- tocopherol, 0.67 mg 100 g^{-1} and 0.77 mg 100 g^{-1}, and γ-tocopherol, 18.8 mg 100 g^{-1} and 17.0 mg 100 g^{-1} for the cold pressed and SFE extracted oils, respectively. The contents of the two tocopherols found in the Marula oil derived by both extraction methods are very similar. This indicates that both methods adequately recover the antioxidants present in the Marula kernel oil.

4.3 Extract Residue

The residual defatted kernels consist of a clean fine powder after the extraction. It was sent for nutritional analysis with the data presented in Table 3.7. The analyses completed were of crude fibre, protein and fat, and are proximate analyses of the content, such that they do not distinguish between types of protein, fibre, and fat and provide information about the major components which constitute the foodstuff. The crude fat is defined as the total fat, which includes all lipid based components soluble in lipid dissolving solvents. Crude protein indicates that the total nitrogen content is measured and multiplied by a factor (6.25) to determine the total protein value. Crude fibre indicates there is no distinction made between the types of fibre. (Greenfield & Southgate 2003).

The results of the defatted Marula were compared with a full fat Marula kernel sample. The defatted Marula powder contains 69.4 % protein, and 12.2 % fat, while the samples of the full fat Marula kernels, an average of three analyses, indicate 28.1 % crude protein and 52.1 % crude fat in the starting sample. There is a

discrepancy with the data as the analysed residue was from an extraction with a 54 % oil yield, 450 bar and 60 °C. Therefore, it may be the case that the solvent extraction does not completely extract all the oil. The images of the Marula kernel cells taken with the light microscope (Figure 3.4) prior to extraction indicate that there are oil droplets which are attached to the cell walls and ones which are found loosely within the cytoplasm. It may be stipulated that the oil droplets found loosely are easily extracted and those bound to the wall may not be soluble in the SFE conditions tested or the petroleum ether solvent extraction. The images of the Marula kernel flour taken with the SEM (Figure 3.5) post extraction indicate there are oil droplets still trapped in the flour which confirm the chemical analysis of fat content in the kernels. Preliminary extractions of the Marula kernels, at 650 bar indicated at 59 % oil recovery which falls in line with the oil extraction results obtained with petroleum ether of the defatted Marula flour. The 12.2 % oil remaining in the defatted kernels indicates that 6 % of the oil in the original raw kernels still remains post extraction, and the maximum oil recovered at the conditions tested was 54 %. Therefore it may be stipulated that the true oil content in the Marula kernels is 59-60 % oil.

From the obtained analyses of protein, fat, ash, and fibre, of the Marula defatted kernels, it can be concluded that the residue may be valuable in food applications. The high protein concentration in the defatted Marula kernels, 69.35 %, is of value for production of high protein products. Mariod & Abdelwahib (2010), evaluated the protein content of the Marula kernels and found that while some of the essential amino acids (leucine, lysine, theronine and the pheylalnanine-tyrosine pair) are slightly lower than World Health Organization (WHO) protein standards, the Marula kernels do contain all of the amino acids and have a high content of protein, 28-36 % (Mariod & Abdelwahab 2012). Mariod (2005) assessed the digestibility of the Marula protein, and found it to be comparable to soybean concentrate, while less digestible than lupine. Therefore, Mariod & Abdelwahib, (2010) and Moyermane & Tshwenyane (2004) agree upon the nutritional value of Marula seeds and their potential in foodstuff applications. Yet, this application has been limited due to the high oil content of the kernels, as well as the oilcake, which at times is denatured due to the high temperature used during oil extraction. The quality of the SFE purified Marula kernels, is such that new applications in foodstuff may now be explored.

4.4 CONCLUSION

Pressure, temperature and particle size have the greatest influence on yield during extraction of kernel oils (Özkal *et al.* 2005a, Alexander *et al.* 1997, Marrone *et al.* 1998). Özkal *et al.* (2005b) found that while the preliminary analysis of the material indicated only 56% oil content, at a pressure of 600 bar and 60 °C, a 59 % maximum oil recovery from hazelnuts could be obtained. This is similar to the results obtained for Marula at

450 bar and 60 °C and 75 °C but preliminary oil analysis, found it to be 52 %. From this, one can conclude that higher oil yields may be obtained using SFE, compared to solvent extraction.

Overall, the yields and the extraction curves indicate that out of the tested conditions, the 450 bar and 60 °C are the best conditions to use for the extraction of the oil from the Marula kernels. The oil quality was assessed, and the cold pressed sample and the SFE extracted oil had very similar oil composition and quality characteristics. This further confirms that SFE-CO_2 is a possible solution to the optimisation of the extraction yield of Marula oil from the kernels.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

This study focused on identifying an extraction process which can be used to optimize the oil yield from Marula kernels. It was identified and consequently confirmed that using SFE-CO_2 as the process for oil extraction, a 100 % oil recovery can be achieved. In addition, another high value product could be created using the process, namely high protein powder. The oil extracted with SFE-CO_2 can be classified as organic, as no residual CO_2 remains in the oil beyond what is normally found naturally, and there is no introduction of solvents which may affect the nature of the raw material.

From assessing the quality of the extracted oils, the recommended conditions for the extraction of the Marula oil are pressure, 450 bar, temperature, 60 °C, flow rate, 30 kg CO_2 hr^{-1}, and pre-processing of the Marula kernels by grinding in order to break up the cell walls of the kernels. The oil obtained with SFE-CO_2 has similar composition to cold pressed oil. Therefore, the oil yield was optimised without compromising the quality of the oil.

According to the European Union regulations on organic processing (189/7/2007) a processing aid may be used. The approved processing aid is defined as "any substance not consumed as a food ingredient by itself, intentionally used in the processing of raw materials, foods or their ingredients, to fulfil a certain technological purpose during treatment or processing and which may result in the unintentional but technically unavoidable presence of residues of the substance or its derivatives in the final product, provided that these residues do not present any health risk and do not have any technological effect on the finished product (20.7.2007 EN Official Journal of the European Union L 189/7)." Therefore, as $scCO_2$ is actually removed from the material as a result of the change in pressure and temperature when collecting the oil sample, and when removing the residue from the extractor basket, as well as not "presenting any health risk" or "technological effect on the finished product", the SFE-CO_2 processed products can be qualified as organic processed products.

Further, the EU specifies that organic products should be "produced by the use of processing methods which guarantee that the organic integrity and vital qualities of the product are maintained through all stages of the production chain." Using SFE-CO_2 for oil extraction from Marula kernels, a high quality product whose integrity is not compromised is produced. In addition, what normally results as an animal feed, the oilcake, can now be used for human consumption as the quality of the oilcake is preserved. The raw material, as well as the two finished products, oil and protein powder, are not exposed to any mechanical or chemical

processing beyond solvent extraction with $scCO_2$. This solvent is quickly removed from the oil and solid residual material in the extractor by simply changing pressure and temperature to ambient conditions, such that the integrity of the natural raw ingredients is maintained.

The aim of the study was accomplished and the objectives of reviewing Marula oil and its applications, the different processing techniques for oil extraction from oilseeds, and identification of a potential solution to recover maximum oil were met. Additionally, the oil was characterised and compared to a cold pressed sample as set out to be done in the objectives of the study.

Through literature review, an additional topic was identified that could have been accomplished if adequate analytical equipment and funding was available to complete the analyses. It has been noted from literature that in SFE-Extraction, throughout the extraction period, the first several fractions obtained consist of the short chain fatty acids and the latter fractions of the long chain fatty acids. This is beneficial in the production of cocoa butter alternatives. Therefore, by analysing every fraction of the oils obtained at the different extraction conditions, the same would have been confirmed for Marula oil and a potentially new application may have been confirmed. However, nine sets of extraction conditions were tested and 10 samples per run were obtained and repeated in triplicate, for a total of 270 samples. A duplicate analysis of each these fractions would have resulted in 540 samples for oil analysis. However, while an attempt was made to do the analyses, due to the high mineral content of the oil the alternative affordable derivatisation procedure of choice was not feasible as two GC columns were destroyed in the process due to the consistency of the oil. Therefore, only the combined oil extracts were analysed for fatty acid content using a standard applied AOCS recommended procedure.

5.2 Further Research: Recommendations

While this study has covered the optimized extraction of Marula oil using SFE-CO_2, several additional research areas were identified. Due to time constraints and limited access to analytical equipment, some quality parameters of the SFE extracted oils were not evaluated. Therefore, further studies using freshly collected and processed kernels, evaluated for fungal and bacterial content, should be used to do additional extractions at the optimised conditions. These include: determining the optimal particle size for SFE extraction, effects of CO_2 on FFA value of the oil, recovery of the oil from the press cake, including but not limited to evaluating the quality of the SFE defatted Marula flour in comparison to the pressed cake.

Due to time constraints and limited access to necessary analytical equipment, the fractions of oil obtained throughout the extractions were not evaluated. This would have been of benefit for possible fractionation of the Marula oil.

Additionally, to evaluate the effect of the SFE CO_2 process on the FFA and peroxide values, freshly extracted oil obtained from freshly collected and processed kernels, evaluated for fungal and bacterial content prior to extraction, should be used to do additional extraction at the recommended conditions.

5.2.1 Effects of Particle Size

Özkal *et al.* (2005b) studied extraction of hazelnut oil at various particle sizes and noted that due to the high oil content the ground hazelnut could not be sieved, as agglomeration was occurring during sieving. Similar issues were faced with Marula kernels during this experiment. Therefore, the Marula kernels were sieved after the extraction process to determine the average particle size of the Marula in the raw material. However, due to the difficulty in sieving, it was difficult to work on the effects of particle size on oil extraction from Marula kernels.

While particle size was not studied during this project, previous studies have found that there are upper and lower limits for an optimal extraction before other issues arise during the process. Additionally, it has been noted that pre-processing of the material is essential; this is supported by the extractions completed on whole pecan halves by Alexander *et al.* (1997), taking 7.5 days to extract 95 % of the pecan oil.

Particle size effects may be studied by freezing the oilseeds with nitrogen and subsequently grinding down and separating the fractions by sieving to determine the effects of particle size effects on the yield and rate of extraction.

5.2.2 Effects of SFE-CO_2 Processing on the FFA Value of Marula *Oil*

The SFE extracted oils had a high FFA value; however, it has been noted that in analysing the FFA value of oils, the presence of nitrogen in the oil sample may give a higher FFA value reading than the true value of the oil. Therefore, a study to evaluate the effects of the presence of CO_2 in the final product on the FFA value and the quantity of CO_2 in the final extracts is recommended.

5.2.3 Recovery of Marula *oil from the* Marula *Expeller Cake Using SFE*

It has been noted by Abu-Arabi *et al.* (2000), in oil extraction from jojoba (*Simmondsidia chinensis*), that expeller pressing followed by solvent extraction can recover the majority of oil from the starting material. Therefore, it will be relevant to evaluate if oil from the Marula press cake can be recovered, and the quality of this oil evaluated.

5.3 Recommendations

With population growth, an insurgence of appraising available natural resources as food stuff is observed. In combination with contemporary trends for bio-beneficiation of resources to achieve sustainability, the recycling of natural components, as well as recovery of valuable constituents in foodstuffs, there is room for and need for beneficiation with SFE. Therefore, the potential commercial application of SFE to the bio-beneficiation of the kernel oil of the Marula tree, a traditionally valued resource available and harvested sustainably, is relevant to this global agenda of sustainability. Since two products are created using this technology as, compared to traditional processing, where efficient recovery of nutritional components is not achieved and oilcake with limited commercial value is produced, SFE is the ideal technology for efficient processing of this valuable resource, as has been found within this study.

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