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AQUEOUS ENZYMATIC EXTRACTION OF *Moringa oleifera* OIL WITH HIGH PRESSURE PROCESSING PRE-TREATMENT

Thesis submitted for the degree of Doctor of Philosophy

Department of Food and Nutritional Sciences

By

Masni Mat Yusoff

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'Declaration

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

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MASNI MAT YUSOFF

ABSTRACT

Moringa oleifera (MO) is a plant species of Indian subcontinent. Its pods contain kernels, which upon maturation consists of up to 35% protein and 41% oil (w/w). This oil is extractable using solvents such as hexane, but this is generally not looked upon favourably due to safety and environmental concerns. This thesis explores the use of aqueous enzymatic extraction (AEE) as an alternative, which involves incubating a mixture of MO kernels, water and enzymes under controlled conditions. Given that the oil globules are predominantly surrounded by proteins and cellulose, the enzymes used in this study were protease and cellulase. Centrifugation of the incubated mixture resulted in four distinct layers: free oil at the top, followed by an oil-in-water cream emulsion, an aqueous phase, and a residual solid meal at the bottom. The highest oil recovered in the free layer at the top following AEE was approximately 73% (w/w), measured in relation to the use of hexane. Another disadvantage of AEE in relation to organic solvent extraction is that a significant part of oil released from the cell ends up in the emulsified layer. Therefore, high pressure processing (HPP) pre-treatment was applied to the kernels prior to AEE, which resulted in increase in free oil quantity and a reduction in emulsion layer thickness. It is hypothesized that HPP modifies the MO protein structure into a form that lowers its emulsifying ability. The use of HPP considerably simplifies downstream oil separation steps. Storage tests showed that enzymatic extraction resulted in MO oil having better oxidative stability than the hexane-extracted oil. The MO oil also contained up to 75% oleic acid and high tocopherol contents, which contributed to its enhanced oxidative stability.

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To My Family

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ABBREVIATIONS

α	=	alpha
γ	=	gamma
ð	=	delta
ºC	=	degree Celsius
g	=	gram
hr	=	hour
M	=	molarity
min	=	minute
ml	=	millilitre
MPa	=	Mega Pascal
N	=	Normality
nm	=	nanometre
ppm	=	parts per million
rpm	=	revolution per minute
v	=	volume
w	=	weight
X	=	variable of the model
Y	=	response variable of the model
ANOVA	=	analysis of variance
AV	=	acid value
AEE	=	aqueous enzymatic extraction

B-AEE	=	aqueous enzymatic extraction with boiling pre-treatment
CLSM	=	confocal laser scanning microscopy
FAC	=	fatty acid composition
FAME	=	fatty acid methyl esters
FFA	=	free fatty acid
GC-FID	=	gas chromatography with flame ionization detector
HPLC-UV	=	high performance liquid chromatography with ultraviolet detector
HPP	=	high pressure processing
HPP-AEE	=	aqueous enzymatic extraction with high pressure processing pre-treatment
IV	=	iodine value
KI	=	potassium iodide
KOH	=	potassium hydroxide
MO	=	*Moringa oleifera*
$NaHSO_3$	=	sodium bisulphite
PV	=	peroxide value
p-AV	=	*p*-Anisidine value
SE	=	solvent extraction
SEM	=	scanning electron microscopy
TOTOX	=	total oxidation value

CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1 Introduction

Moringa oleifera (MO) mature seeds consist of kernels which contain edible MO oil, also known as ben or behen oil. A number of techniques are available for oil extraction from MO kernels, which include solvent extraction and aqueous enzymatic extraction (AEE) methods, among others. The AEE of oil from oil-bearing materials was reviewed by Mat Yusoff et al. (2015) and Rosenthal et al. (1996), the former reference is a part of this thesis, Chapter 2. This process involves addition of selected enzymes into a mixture of oleaginous material with pre-determined amount of water at a given pH value, followed by incubation of the mixture for a given time at pre-set temperature and agitation conditions. The added enzymes function in hydrolyzing and breaking the cotyledon cell walls of the material, thus making the structure more permeable and further expose the oil component. The water-soluble components diffuse into the aqueous phase, while the released oil distributes itself between two phases: i) an oil-in-water cream emulsion in the aqueous phase and ii) a superficial oil layer on top of the emulsion (Rosenthal et al.1998).

The advantages of AEE over solvent extraction were also pointed out by Rosenthal et al (1998), which include lower environmental impact and lower process costs. In the case of *Moringa oleifera* (MO) kernels, the use of AEE was reported by Abdulkarim et al. (2005, 2006) and Latif et al. (2011) which highlighted the importance of protein hydrolysis in the MO kernels for higher oil recovery. Hence, the main enzyme used in earlier studies was protease. Despite its obvious advantages, the MO oil recoveries resulting from AEE

are significantly lower than the recoveries observed by solvent extraction, ranging between 69-73% g oil / g solvent-extracted oil (Latif et al., 2011; Abdulkarim et al., 2006, 2005). Latif et al. (2011) found that the MO cream emulsion formed at the end of the AEE process required further de-emulsification in order to enhance oil recovery, which was particularly difficult because of the added emulsion stability imparted by the proteins. Formation of creamy emulsion following an AEE process was also reported in the case of *Isatis indigocita* seeds (Gai et al., 2013), bayberry kernels (Zhang et al., 2012), and soybean seeds (Lamsal and Johnson, 2007).

These findings therefore suggest that oil recoveries can be enhanced by decreasing the emulsifying capacity of the proteins prior to AEE, possibly by altering their structures. The alteration of protein structure may be induced by both hydrolysis and denaturation, which can be promoted by subjecting the kernels to treatments such as high pressure processing (HPP). Upon denaturation, the peptide bonds of the protein become more available or more susceptible to hydrolysis by proteolytic enzymes, besides lowering the protein solubility (Anglemier & Montgomery, 1976). In addition, HPP also disrupts electrostatic and hydrophobic interactions in proteins (Messens et al., 1997).

It is noteworthy that MO oil exhibits high oxidative stability due to the presence of high oleic acid and tocopherols which also reduce the risk of developing coronary heart disease, and many studies are available on the physicochemical properties of MO oil (Noakes et al., 1996; Tsaknis et al., 1999; Tsaknis and Lalas, 2002; Lalas and Tsaknis, 2002; Anwar and Bhanger, 2003; Abdulkarim et al., 2005, 2006; Rahman et al., 2009; Latif et al., 2011; Nguyen et al., 2011; Ogbunugafor et al., 2011; Zhao and Zhang, 2013).

However, the quality attributes of MO oil upon storage at different temperatures had never been reported, which is of great importance in determining the shelf life of the oil.

1.2 Objectives

Given the above background, the main objectives of this study were:

i. To observe the microstructure of MO kernel cell.

ii. To determine the effect of different AEE parameters on MO oil recovery based on statistical optimization, besides the effect of selected parameters on the formation of its cream emulsions.

iii. To test the re-usability of the enzymes in successive AEE process.

iv. To explore the effect of HPP as a pre-treatment prior to AEE of MO oil.

v. To determine the effect of different particle sizes of the MO kernels for the HPP pre-treatment (i.e. the whole and ground-sieved kernels) on the oil recovery after the AEE process.

vi. To indicate the effect of different extraction methods on the oxidative stability of MO oil during storage at different temperatures.

The literature review is reported in Chapter 2, followed by studies on the microstructure of MO kernels, AEE of MO oil, and re-usability of the enzymes in Chapter 3. The use of HPP pre-treatment prior to the AEE of MO oil is discussed in Chapter 4. In Chapter 5, the effect of extraction methods conducted in Chapter 3 and Chapter 4 on the oxidative properties of MO oil during storage at different temperatures are reported.

The literature review (Chapter 2) on AEE and enzymatic de-emulsification method has been published as: "Mat Yusoff, M., Gordon, M. H., Ezeh, O., and Niranjan, K. (2015). Aqueous enzyme assisted oil extraction from oilseeds and emulsion de-emulsifying methods: A review. *Trends in Food Science and Technology, 41(1)*, 60-82".

Chapter 3 has been published as: "Mat Yusoff, M., Gordon, M. H., Ezeh, O., and Niranjan, K. (2016). Aqueous enzymatic extraction of *Moringa oleifera* oil. *Food Chemistry, 211*, 400-408".

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to *Moringa oleifera* oil

Moringa oleifera (MO), also known as horse-radish, kelor, or drumstick tree, belongs to the Moringaceae family (Morton, 1991). In Nile valley, this tree is known as Shagara al Rauwaq which means the 'tree for purifying'. The trees are native of Indian subcontinents and of Pakistan and Afghanistan. They are also widely distributed in Kenya, Bangladesh, Malaysia, Nigeria, Carribean Island, Cambodia, and Phillipines. The height of the tree range from 5-15 m, and almost each part of the tree including its leaves, flowers, roots, and trunks are valuable for food, industrial, and traditional medical practices. Thus the MO tree is also acknowledged as 'Natural Nutrition of the Tropics'.

One of the important parts of the tree is its fruit pods which are approximately 25-50 cm in length (Fig. 2.1(a)). The young pods are soft and tender, and are always consumed as vegetables in certain countries such as in Malaysia and Indonesia. Each pod contains 10-50 MO seeds (Fig. 2.1(b) and 2.1(c)) which are yellowish-green in colour and exhibit triangular or globular shape, with three 'papery wings'. In certain areas of West Africa and India, the rural populations consume the MO seeds as sources of nutrients. Differently, mature pods are brown in colour and contain mature brown MO seeds (Fig. 2.2(a)). These mature seeds consist of light, dry shell which can be peeled off in order to obtain the MO kernels inside (Fig. 2.2(b)).

(a)

(b)

(c)

Figure 2.1 Immature *Moringa oleifera* pods with its seeds

(a)

(b)

Figure 2.2 (a) Mature *Moringa oleifera* seed; (b) De-hulled mature *Moringa oleifera* seed with its kernel

In contrast with the immature MO seeds, the kernels inside the mature seeds contain the edible MO oil, which is also known as Ben or Behen oil. This oil is applicable in perfume industry, hair-care products, in medicinal practices, and act as a good lubricant for fine machineries. As an edible oil, MO oil has great taste and is generally applied as culinary and salad oil in Haiti and other countries. Tsaknis and Lalas (2002) and Abdulkarim et al. (2007) concluded the suitability of MO oil for frying purpose. The fatty acid composition of MO oil resembles that of olive oil, with high oleic acid content in addition to the significant amount of tocopherols as reported by Rahman et al. (2009), Tsaknis and Lalas (2002), and Tsaknis et al. (1999). These properties contributed to the oil's oxidative stability (Lalas and Tsaknis, 2002), and consumption of oleic acids was always related to reduced risk of developing coronary heart disease (Abdulkarim et al., 2007; Anwar et al., 2007). The oxidative properties of MO oil are thoroughly discussed in section 2.5.3. Furthermore, the MO protein exhibits coagulant properties which is useful for low cost water and wastewater treatment (Ghebremichael et al., 2005; Katayon et al., 2006), and as a pre-treatment of palm oil mill effluent (Bhatia et al., 2007).

2.2 Aqueous enzymatic extraction method

Generally, cotyledon cells of an oilseed are surrounded by cell wall which is mainly composed of cellulose, hemicellulose, lignin, and sometimes pectin. Each cell consists of lipid and protein bodies which bears the oil and protein content, respectively. In the case of soybeans, peanuts, and some other oilseeds, the lipid bodies are enclosed by cytoplasm which is primarily composed of protein. These cell wall and cytoplasm compositions need to be ruptured in order to expose and further release the oil content of the material.

One of oil extraction processes is known as an aqueous extraction method, where water is used as the extraction medium at pre-determined incubating conditions. In this process, the cells are ruptured which allow the water-soluble components to dissolve into the water, while the oil is released in a separate layer or forms an oil-in-water emulsion. Addition of enzymes in an aqueous extraction process is known as an aqueous enzymatic extraction (AEE) method. The main role of the added enzymes such as cellulases, hemicellulaces, pectinase, and proteases is to break or hydrolyze the cellulase, hemicellulase, pectin, and protein, respectively. Rupturing these cell components allow easier and faster oil release, where the extent depends on the particle size of the material. Different oil-bearing materials exhibit different compositions which determine the type of enzymes to be added for the cells' rupture. Table 2.1 summarizes the type of enzymes used in AEE processes conducted in different studies based on the commercial names, while Table 2.2 shows the enhancement in oil yield from different oil-bearing materials due to the addition of enzymes, in comparison with aqueous extraction without enzymes.

Table 2.1 Commercial enzymes used for aqueous enzymatic extraction (AEE): descriptions and compositions

Enzymes commercial names	Description/Composition
Alcalase®	Protease
Alcalase 2.4L	
As1398	
Flavourzyme® 1000 L	
Papain	
Protamex	
Multifect Neutral®	
Celluclast 1.5L®	Cellulase
ROHALASE® OS	
Glucanex	Glucosidases
G-ZYME® G999	Lysophospholipase A1
Lipomod 699L	Phospholipase A2
LysoMax™	
Neutrase 0.8L	Bacterial neutral protease
Nutrase	Xylanase
Pectinase 1.06021	Pectinase
Pectinase Multieffect FE®	
Pectinex®	
Pectinex Ultra SP	
Pectinex Ultra SP-L	
ROHAPECT® PTE	
Promozyme	Pullulanase
Protex 6L	Alkaline serine endopeptidase
Protex 30L	
Protex 7L	Natural metallo endopeptidase
Protex 50FP	Acid fungal endopeptidase-exopeptidase complex
Protex 51FP	Neutral fungal endopeptidase-exopeptidase complex
Protex 89L	Endopeptidase
Termamyl 120L	α-amylase
Bioliva	Cellulase, hemicellulase, pectinase, other minor enzymes
Cytolase 0	
Maxoliva	

Table 2.1 (Continue)

Enzymes commercial names	Description/Composition
Kemzyme	Cellulase complex, hemi-cellulase complex, α-amylase, β-glucanase, protease, xylanase
Multifect CX 13L	Cellulase, hemicellulase, β-glucanase, arabinoxylans
Multifect Pectinase FE	Cellulase, hemicellulase, pectinase
Natuzyme	Cellulase, xylanase, phytase, α-amylase, pectinase
Olivex	Cellulase, hemicellulase, pectinase
Rapidase® Liq plus	
Olivex-Celluclast	50%: Cellulase, hemicellulase pectinase 50%: Cellulase, hemicellulase
Pectinex Ultra SP-L	Cellulase, pectinase, xylanase
Protizyme™	Three different proteases with pH optima 3-4, 5-7, 7-10
Viscozyme® Viscozyme L	(Carbohydrases): Cellulase, hemicellulase, arabinase, xylanase, amylase, β-glucanase

Table 2.2 The oil yield enhancement with the use of enzymes as compared to aqueous extraction without enzymes

Oil-bearing material	Type of enzyme	Difference in oil yield (%)	Reference
Ground sunflower seeds (0.75-1 mm)	Celluclast 1.5L	~35.0	Sineiro et al. (1998)
Heat-treated soybean flour	Alcalase 2.4L	16.9	Rosenthal et al. (2001)
Ground *Jatropha* seed kernels (inedible)	Protizyme™	26.0	Shah et al. (2005)
Crushed borage seeds (≤2.0 mm)	Olivex / Celluclast (1:1)	7.8	Soto et al. (2007)
Rapeseed slurry	Pectinase	38.1	Zhang et al. (2007)
	Cellulase	21.5	
	B-glucanase	16.2	
	Pectinase / Cellulase / β-glucanase (4:1:1)	43.8	
Kernel flour of bush mango	Alcalase®	7.6	Womeni et al. (2008)
	Pectinex®	14.8	
	Viscozyme®	40.6	
Extruded soybean flakes	Protease	20.0	Lamsal et al. (2006)
	Multifect Neutral®	13.4	Lamsal and Johnson (2007)
	Protex 7L	22.1	Jung and Mahfuz (2009)
	Protex 51FP	16.0[a]	Wu et al. (2009)
	Protex 6L	20.0[a]	
	Protex 7L	17.0[a]	
Ground peanuts	Alcalase	42.86	Jiang et al. (2010)
	As1398	35.77	
	Nutrase	29.49	
	Protizyme	24.43	
	Protamex	18.30	

Table 2.2 (Continue)

Oil-bearing material	Type of enzyme	Difference in oil yield (%)	Reference
Ground sesame seeds	Alcalase 2.4L	12.5	Latif and Anwar (2011)
	Natuzyme	4.5	
	Protex 7L	6.4	
	Viscozyme L	9.1	
	Kemzyme	4.2	
Olive paste	Bioliva	1.20	Ranalli et al. (2003)
	Maxoliva	1.37	
	Cytolase 0	1.44	
	A (pectinase, cellulase, hemicellulase) / B (pectinase, hemicellulase) / C (pectolytic enzyme) (1:1:1)	152.0 (30 min) 91.4 (150 min)	Aliakbarian et al. (2008)
	Pectinex Ultra SP-L	1.96[b]	Najafian et al. (2009)
	Pectinase 1.6021	1.41[b]	

All aqueous enzymatic extraction processes resulted in higher oil yield than that of aqueous extraction without enzymes

AEE is a promising method for simultaneous production of oil and protein with minimum damage and better quality for human consumption. This method allows separation of extracted components with unchanged properties, and lower amount of by-products which can potentially affect the final product in terms of taste and smell. Interests in this method among researchers and food industries have also increased due to safety and environmental regulatory issues. In comparison with solvent extraction method, the water used is much safer, environmental-friendly, and economical. In addition, it contributes to a much safer and flexible operation, lower energy consumption and operational costs, and lower capital investment. A variety of temporal crops can be processed, and the extracted oil does not need further refining. Non-toxic meal and value-added fiber and protein as co-products are also produced which is due to the milder operating conditions employed. In

addition, the aqueous medium allows simultaneous separation of phospholipids from the oil. Therefore, degumming step (in case of oilseeds) is not necessary and the overall cost of the oil processing can be reduced (Latif and Anwar, 2011; Yang Li et al., 2011; Chabrand and Glatz, 2009; Jung and Mahfuz, 2009; Wu et al., 2009; Soto et al., 2007; Santos and Ferrari, 2005; Gros et al., 2003; Hanmoungjai et al., 2001; Rosenthal et al., 2001; Sineiro et al., 1998, 1998a; Ksenija et al., 1997; Rosenthal et al., 1996)

Despite the advantages, the application of AEE was still limited due to long processing time and the high cost spent for the drying process after the enzyme treatment (Shah et al., 2005; Dominguez et al., 1996). The high cost was also due to the enzymes themselves, besides high amount of enzymes required (commonly >1% of the weight of the material). In addition, in some studies, the enzymes used were not commercially available which have limited other workers to apply the process reported (Rui et al., 2009; Shah et al., 2005). After the AEE, it was almost impossible to avoid emulsification of the extracted oil, thus further de-emulsification process is always required for higher total oil yield (Latif and Anwar, 2011; Long et al., 2011; Wu et al., 2009; Chabrand et al., 2008; Santos and Ferrari, 2005; Rosenthal et al., 1998; Sineiro et al., 1998, 1998a).

2.2.1 Factors affecting the process efficiency

The main factors affecting the oil yield in an AEE method are the types of oil-bearing materials, the types of enzymes used, and the incubating conditions employed as summarized in Table 2.3. In reference to the table, some studies had fixed the pH and temperature during the incubation according to the optimum pH and temperature of the enzymes added. The effect of different incubating conditions on the oil yield had also been studied, where some studies had concluded the most suitable conditions through statistical optimization.

Table 2.3 Maximum oil yields as affected by the selected and optimized incubating conditions of the aqueous enzymatic extraction methods

Oil-bearing material	Type of enzyme	Enzyme / Material ratio (w/w)	Water / Material ratio (w/w)	pH	Tempera-ture (°C)	Time (hr)	Agitation rate (rpm)	Oil yield (% w/w)	Reference
Selected incubating conditions (*) used for maximum oil yield									
Ground sunflower seeds (0.75-1 mm)	Celluclast 1.5L	2.0%*	5:1*	4.8***	50***	2*	150	35.65	Sineiro et al. (1998)
Ground rice bran (16-mesh sieved)	Alcalase 0.6L	1.0%*	-	9.0	60*	3*	1000	79.1	Hanmoungjai et al. (2001)
Heat-treated soybean flour	Alcalase 2.4L	3.0% (v/w)*	-	8.0 ***	50***	1	200	58.7	Rosenthal et al. (2001)
Crushed borage seeds (≤2.0 mm)	Olivex / Celluclast (1:1)	0.25%*	20%* (correspon-ded to 1:5)	-	45*	9*	-	85.5	Soto et al. (2007)
Rapeseed slurry	Pectinase / Cellulase / β-glucanase (4:1:1)	2.5% (v/w)*	5:1*	5.0	48	4*	200	92.7[a]	Zhang et al. (2007)
Olive paste	A (pectinase, cellulase, hemicellulase) / B (pectinase, hemicellulase) / C (pectolytic enzyme) (1:1:1)	0.25% (v/w)*	-	-	30	2 hr 30 min*	10 (kneading)	17.5	Aliakbarian et al. (2008)
Ground peanuts	Alcalase	1.5%*	5:1*	8.5*	60*	5*	-	73.45	Jiang et al. (2010)

Table 2.3 (Continue)

Oil-bearing material	Type of enzyme	Enzyme / Material ratio (w/w)	Water / Material ratio (w/w)	pH	Tempera-ture (^{o}C)	Time (hr)	Agitation rate (rpm)	Oil yield (% w/w)	Reference
Ground pitaya seeds (40-mesh sieved)	Pectinase / Cellulase / Acid protease (1:1:1)	-	8:1	7.0	50*	1	90	6.94	Rui et al. (2009)
Optimized incubating conditions (**) for maximum oil yield									
Moringa. oleifera seed powders	Neutrase 0.8L	2.0% (v/w)	6:1 (v/w)	6.8 ***	45**	24**	120	22.6	Abdulkarim et al. (2005)
Ground pine kernels	Alcalase endo-protease	1.97%**	5:1**	8.4**	51**	3**	-	89.12	Yang Li et al. (2011)
Ground watermelon kernels	Protex 6L	2.63%**	4.35:1**	7.89**	47.1**	4.29**	-	77.25	Xiaonan Sui et al. (2011); Shan Liu et al. (2011)
Shattered bayberry kernels	Cellulase / Neutral protease (1:2)	3.17%**	4.91:1 (v/w)**	-	51.6**	4**	-	31.15	Zhang et al. (2012)

Values without any notation are fixed incubating conditions.

[a] Type of enzymes used for aqueous enzymatic extraction

*selected incubating condition; the authors varied the level of each incubating condition and finalized the conditions which resulted in highest oil yield.

**optimized incubating condition; the authors varied the level of each incubating condition and optimized the conditions which resulted in highest oil yield based on an experimental design and statistical software used.

***optimum incubating condition of the enzyme used; different types of enzymes possess different optimum pH and temperature where the enzymes attain maximum activity

2.2.2 Pre-treatment methods prior to the process

Some studies have highlighted potential pre-treatment methods, which are not necessarily enzyme-based that could be followed up by AEE as summarized in Table 2.4. In the case of high pressure processing as reported by Jung and Mahfuz (2009), the use of high pressure induced protein aggregation yet it was further hydrolyzed by protease, thus facilitated oil removal, despite the inability of the high pressure in rupturing the seeds' cotyledon cells. On the other hand, Shan Liu *et al.* (2011) reported that ultrasound generated cavitations which accelerated the leaching out of cellular components including oil. The use of extrusion prior to AEE has been extensively studied by Jung and Mahfuz (2009), Jung *et al.* (2009), and Wu *et al.* (2009). According to these authors, protein aggregates are formed during extrusion but these entrap or interact with the oil. The interactions could then be disrupted by the use of protease, which result in increasing the oil and protein yields. These studies have shown the potential of AEE assisted by other pre-treatment methods to increase oil yields. The operating costs of a number of pre-treatments are also highlighted as shown in Table 2.5, yet each processing type is very much dependent on the parameters used, the types and amount of samples been processed, and the different parts of the machines or instruments applied, among others, besides differences in their capital costs. Therefore, comparing these processing methods in terms of their costs is not easy and requires thorough studies.

Table 2.4 The advantages of the use of pre-treatments (non-enzymatic) prior to aqueous enzymatic extraction method

Oil-bearing material	Pre-treatment	Type of enzyme	Advantages	Reference
Ground *Isatis indigotica* seeds	Microwave	Cellulase / Proteinase / Pectinase (1:1:1)	- In combination with AEE, the use of optimal microwave irradiation power increased the oil yield up to 59.27%, and the oil yield had greater antioxidant properties than solvent-extracted oil.	Gai *et al.* (2013)
Ground *Jatropha* seed kernels (inedible)	Ultrasonication (5 min)	Protizyme™	The enzyme treatment time was reduced from 18 hr to 6 hr for maximum of 74% oil yield	Shah *et al.* (2005)
Ground linseeds	Electrical discharge	-	Mucilage (stabilizing agent) is removed which caused easier oil separation from the resulted residue by using enzyme treatment	Gros *et al.* (2003)
Grounds peanuts	Alkaline extraction	Alcalase	Oil yield of 5.87% higher than AEE alone	Jiang *et al.* (2010)
Ground pitaya seeds (40-mesh sieved)	Microwave	Pectinase / Cellulase / Acid protease (1:1:1)	- Oil yield of 0.84% higher than AEE alone	Rui *et al.* (2009)
Ground watermelon kernels	Ultrasound	Protex 6L	-Under the fixed parameters of the ultrasound, the yield was 20.67% higher than AEE alone -Under the selected parameters of ultrasound for maximum oil yield, the yield was 21.39% higher than AEE alone	Xiaonan Sui *et al.* (2011), Shan Liu *et al.* (2011)

Table 2.4 (Continue)

Oil-bearing material	Pre-treatment	Type of enzyme	Advantages	Reference
Soybean flakes	High pressure processing (200 MPa)	Protex 7L	Oil yield of 3.20% higher than AEE alone	Jung and Mahfuz (2009)
	High pressure processing (500 MPa)		Oil yield of 1.30% higher than AEE alone	
	Extrusion		- Oil yield of 29.90% higher than AEE alone - Free oil yield of 17.00% higher than AEE alone	
	Extrusion	Protex 6L	- Oil yield of 35.52% higher than AEE alone - After de-emulsification: Free oil from cream emulsion of 62.00% higher than AEE alone	Jung *et al.* (2009)

AEE: aqueous enzymatic extraction.

Table 2.5 Operating costs of selected types of pre-treatment methods (with reference to Table 2.4). mt, material

Processing method	Operating cost	Reference
Microwave	1-12 cent/kW.hr	Sheppard (1988)
Extruder	USD 9-15/mt.hr	Wenger Manufacturing Co., Sabetha, KS (2000)
Ultrasound (15 min)	USD 0.014 per liter	Jyoti and Pandit (2001)

2.3 Extraction methods of *Moringa oleifera* oil

AEE is one of the methods conducted in extraction of MO oil of different origin, besides solvent treatment, cold pressing, and supercritical fluid extraction methods as shown in Table 2.6. According to the table, it is clearly revealed that the solvent treatment and supercritical fluid extraction method resulted in higher MO oil yield (30-42%) than cold-pressing (25-26%) and AEE (16-23%) methods.

Table 2.6 *Moringa oleifera* oil yield from different extraction methods

***M.oleifera* seeds: Origin**	**Oil yield (%)**						**References**
	Solvent treatment		**Cold-pressing**	**Supercritical fluid (CO_2)**	**Aqueous enzymatic extraction**		
Kenya (variety	35.7	*n*-hexane	25.8	*	*	*	Tsaknis et al. (1999)
Mblolo)	31.2	Chl : Meth (1:1)					
India (variety	38.3	*n*-hexane	25.1	*	*	*	Lalas and Tsaknis (2002)
Periyakulum 1)	41.4	Chl : Meth (1:1)					
	33.1	*n*-hexane	*	*	*	*	Mani et al. (2007)
	31.8	Petroleum ether					
	31.1	Acetone					
Pakistan	38.4	*n*-hexane	*	*	*	*	Anwar and Bhanger (2003)
	34.8		*	*	*	*	Anwar and Rashid (2007)
	30.0-39.0		*	*	*	*	Anwar et al. (2006)
	32.4		*	*	22.5	Protex 7L	Latif et al. (2011)
					21.8	Multifect CX 13L	
					20.9	Viscozyme L	
					18.1	Kemzyme	
					16.9	Natuzyme	
Malaysia	30.8	Petroleum ether	*	*	22.2	Neutrase 0.8L	Abdulkarim et al. (2005)
	*	*	*	*	22.2	Neutrase 0.8L	Abdulkarim et al. (2006)
					20.3	Termamyl 120L	
					20.3	Celluclast 1.5L FG	
					17.4	Pectinex Ultra SP-L	

Table 2.6 (Continue)

***M.oleifera* seeds: Origin**	**Oil yield (%)**						**References**
	Solvent treatment		**Cold-pressing**	**Supercritical fluid (CO_2)**	**Aqueous enzymatic extraction**		
Bangladesh	37.5	*n*-hexane	*	*	*	*	Rahman et al. (2009)
	40.2	Chl : Meth (1:1)					
	35.6	Petroleum ether					
Nigeria	41.5	*n*-hexane	*	*	*	*	Ogbunugafor et al. (2011)
Phillipines	*	*	*	37.8	*	*	Nguyen et al. (2011)
Provided by Dept. of Agricultural and Food of Western Australia	40.1	*n*-hexane	*	37.1	*	*	Zhao and Zhang (2013)

Chl, chlorofm; Meth, methanol

Refer Table 2.1 for the types of enzymes used in the aqueous enzymatic extraction methods

2.3.1 Aqueous enzymatic extraction of *Moringa oleifera* oil

In studies conducted by Abdulkarim et al. (2006) and Latif et al. (2011), the effect of different enzymes were evaluated, and the pH values of the mixtures were adjusted based on the optimal pH of the enzymes added. According to Abdulkarim et al. (2006), under the optimum conditions of all enzymes tested, the use combination of different enzymes: Neutrase 0.8L, Termamyl 120L, Pectinex Ultra SP-L, and Celluclast 1.5L resulted in highest MO oil yield of 24.72% (g oil / g material). This was followed by the use of individual protease i.e. Neutrase 0.8L which resulted in 24.02% (g oil / g material); slightly lower than the use of combination of enzymes. Therefore, Abdulkarim et al. (2006) have concluded that addition of individual protease is preferable economically. Similar observation was reported by Latif et al. (2011), where highest MO oil yield of 22.5% (g oil / g material) was obtained with the use of individual endopeptidase i.e. Protex 7L, followed by other enzymes which are mainly composed of carbohydrases and pectinase. These outcomes indicated the significance of protease in hydrolyzing the protein surrounding the oil in the MO kernel cells. The protein content of MO kernels of different origins were reported to range from 25-38% (g protein / g material) (Rahman et al., 2009; Anwar and Rashid, 2007; Anwar and Bhanger, 2003).

2.4 Introduction to high pressure processing method

Application of high pressure processing (HPP) in food industry is critically reviewed by Yaldagard et al. (2008), Rastogi et al. (2007), and Torres and Velazquez (2005). The use of HPP was demonstrated by Hite et al. since 1914 as a preservation method for milk, fruits, and vegetables, besides its potential in denaturation of food protein and polysaccharides. However, commercialization of this technology was delayed until the 1980s due to technical difficulties and costs related with HPP units and packaging materials required (Yaldagard et al., 2008; Mertens, 1995). The HPP is well known as an expensive method as compared to traditional processing technologies (e.g. high-temperature sterilization). However, there is growing development of HPP in food industry due to increasing consumer demand on safe, healthier, and shelf-stable food products exhibiting natural flavor and taste with fresh appearance, besides being minimally processed with no or minimally added additives (e.g. preservatives, humectants) (Yaldagard et al., 2008; Rastogi et al., 2007). These demands trigger the interests of researchers at universities and institutes, and the equipment suppliers for enhanced studies and exploration on the HPP technology in food industry (Hendrickx et al., 2005). A number of high pressure-treated food products are commercially available nowadays including fruit-based products (juices, jellies, and jams), dairy products (milk, yogurt, and cheese), meat-based products (pork, beef, sliced ham, ready-to-eat meats), seafoods (fish, raw squid), vegetables, and guacamole, i.e. avocado puree, among others (Yaldagard et al., 2008).

Listed below are the applications of HPP in food industry as summarized by Yaldagard et al. (2008), Rastogi et al. (2007), and Torres and Velazquez (2005):

a) Inactivation of pathogenic and spoilage microorganisms and enzymes in foods for preservation purpose in terms of the food's shelf life and quality attributes
b) Modification of food biopolymers which further induce changes in the food's physicochemical and functional properties
c) Creating ingredients with novel or desirable functional properties
d) Development of wide range of new or value-added food products
e) The use of extreme or increasing pressures may assist in lowering the freezing point of water (Kalichevsky et al., 1995; Knorr et al., 1998)

The advantages of HPP treatment are summarized as follow (Yaldagard et al., 2008; Rastogi et al., 2007; Torres and Velazquez, 2005):

a) The HPP is isostatic, where the pressure is rapidly and uniformly applied throughout the whole food. Thus it is independent on food's geometry i.e. size and shape. This unique advantage makes size reduction of the treated food to be optional, besides facilitates in scale-up from laboratory findings to full-scale production.
b) It is possible to carry out the HPP treatment at room or lower than room temperature which reduces the amount of thermal energy needed as in the conventional processing methods.
c) The process requires only electric energy and generates no waste products, thus it is also known as an environmental-friendly technology.

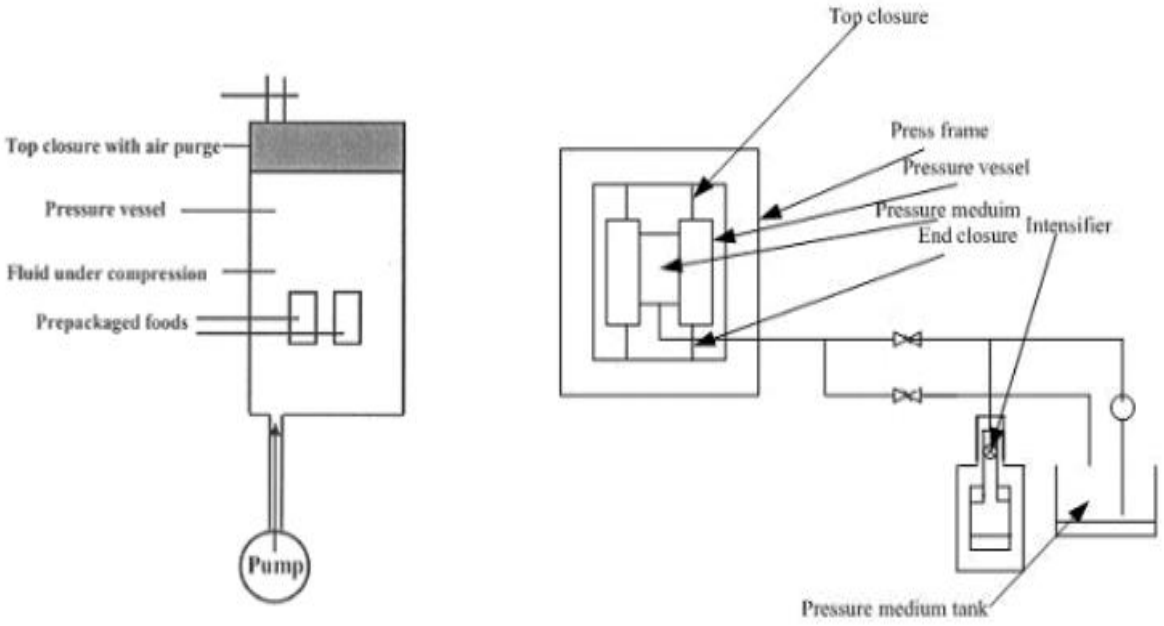
d) Combination of suitable pressure, temperature, and time during the HPP treatment induces less changes in the food's quality attributes such as texture, color, and flavor as compared to the use of conventional technologies, besides retaining the food's sensory characteristics (Shellhammer et al., 2003) and nutritional values.
e) The HPP treatment does not break covalent bonds, thus preventing development of undesirable or unknown flavor in the food product and maintains its natural flavor.

Despite the ability of the HPP as a preservation method, there are types of food enzymes and bacterial spores which can only be inactivated at very high pressure. Additionally, certain food components may undergo degradation due to enzymatic and oxidative reactions in the presence of residual enzyme and dissolved oxygen in the high pressure-treated food product. In most cases, as stated earlier, the HPP is always known as an expensive method as compared to conventional processing technologies (Yaldagard et al., 2008).

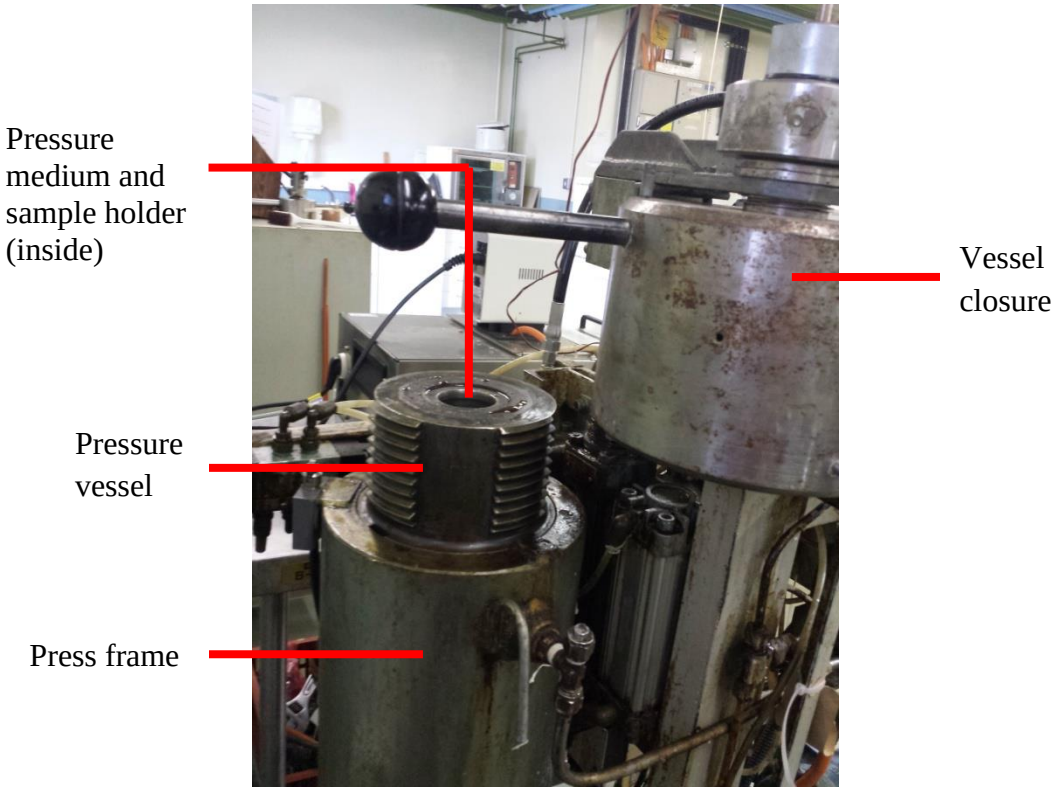
2.4.1 High pressure processing equipment and principles

A high pressure vessel is the main element of a HPP system which in many cases, is a forged monolithic, cylindrical form. It is constructed of low alloy steel with high tensile strength. In the case of monobloc vessel, the maximum working pressure is normally limited to 400-600 MPa, depends on the vessel's wall thickness and internal diameter. The use of higher pressure levels require pre-stressed vessel designs such as multilayer vessels or wire-wound vessels (Mertens, 1995). A HPP system also consists of the high pressure vessel's closure, a pressure generation system, and a temperature controlling device (Fig. 2.3 (a) and (b)).

The food samples are normally packed and sealed in a suitable packaging material (e.g. in Fig. 2.3(c)) which is sufficiently flexible and are able to withstand the high pressure compression. One of the criterion evaluated in a food preservation method is the cost per amount of treated product (Ting and Farkas, 1995) which is very much dependent on the operating parameters and the scale of operation (Rastogi et al., 2007). The large capital investment and higher operating cost is overcome by operating the HPP plants at full capacity. Therefore, in the case of processes involving seasonal commodities, there is a need to identify a product mix in order to maximally utilize the equipment (Torres and Velazquez, 2005). Table 2.7 summarizes the HPP systems, requirements, costs, and changes taking place during pressurization and depressurization of food samples.


Top closure with air purge
Pressure vessel
Fluid under compression
Prepackaged foods
Pump
Top closure
Press frame
Pressure vessel
Pressure meduim
End closure
Intensifier
Pressure medium tank

(a)


Pressure medium and sample holder (inside)
Pressure vessel
Press frame
Vessel closure

(b)

(c)

Figure 2.3 (a) Indirect compression in a high pressure processing system (Adapted from Mertens, 1995 and Yaldagard et al., 2008), (b) high pressure processing machine (Stansted Fluid Power Ltd., Essex, England) displaying the press frame, pressure vessel, vessel closure, and the location of pressure medium and sample holder, and (c) example of water/kernel (1:1 w/w) in the packaging medium after vacuum-sealed prior to insertion into the sample holder.

Table 2.7 Overview of high pressure processing systems with reference to Mertens (1995), Rastogi et al. (2007), Yaldagard et al. (2008), and USFDA (2014)

High pressure processing		
Main type	Batch / Conventional	• Processing of pre-packed liquid and solid products • The overall cycle time take into account each of single step: filling and closing the pressure vessel, pressure increment, pressure holding, pressure decrement or release, opening the vessel's closure, and taking out the treated product
	Semi-continuous	• Processing of liquid products • The use of a pressure vessel containing a free piston to compress the liquid foods • The treated liquid food can be filled aseptically into pre-sterilized containers
	Continuous	• As of December 2014, no commercial continuous HPP systems are operating (USFDA) • The process involves compression of liquid food, provide a plug flow hold tube or hold vessel to achieve a specified process time • The decompressed, treated liquid can be sent to a sterile hold tank for eventual aseptic filling
Method of HP generation	Direct compression	• Piston-type compression • Direct pressurization of the pressure medium by a piston in the HP vessel, driven by a low pressure pump at its larger diameter end
	Indirect compression	• The pressure medium is pumped by a HP intensifier from the reservoir into the HP vessel which is closed and de-aerated, until the desired pressure level is achieved • commonly been applied in the food industry, regardless of the required temperature - cold, warm, or hot isostatic system
Pressure-transmitting fluids	• Water • Solutions of food-grade glycol-water • Ethanol solution • Sodium benzoate solution • Silicone oil • Castor oil • Inert gases	

Table 2.7 (Continue)

High pressure processing	
Packaging materials	• ethylene vinyl alcohol copolymer (EVOH) • polyvinyl alcohol (PVOH)
Cost	Cost per amount of treated product (Thakur and Nelson, 1998; Balasubramaniam, 2003): • HPP: 5-50 cents per liter or kilogram • Thermal: ~5 cents per liter or kilogram Cost per amount of treated product (Hendrickx et al., 2005): • HPP: 10-20 cents per liter • Thermal: 2-4 cents per liter or kilogram Cost of HP vessel of commercial scale (Hendrickx et al., 2005): • USD 500,000 to USD 2.5M (depends on the equipment capacity and degree of automation)
During pressurization	• In the HP vessel, the pressure-transmitting fluids transmit pressure uniformly and instantaneously to the food samples • The product is iso-pressed - the product's internal pressure is equal to the external one. • The liquids and solids (product) remain according to their compressibility • The gaseous part of the product nearly disappears • 400-800 MPa: The pressure medium and the packed food sample are compressed to about 80-90% of their original volumes • Rasanayagam et al. (2003): Increment in temperature of the food's liquid component by approximately 3°C per 100 MPa, to as high as 8-9 °C per 100 MPa in the case of food containing a significant amount of fat such as butter or cream
During de-pressurization	• The treated product returns to its original volume and temperature; the latter occurs if isothermal condition is achieved (i.e. no heat transfer across the walls of the pressure vessel) during the holding stage Rovere (2001): • Liquids and solids undergo low volume contraction, thus particulate food products are not mechanically damaged • The elasticity in most food types allow them to recover their original shape and structure

2.4.2 Effect of high pressure processing on food protein

High pressure treatment alters large molecules including proteins, enzymes, polysaccharides, and nucleic acid, while small molecules such as amino acids, vitamins, and flavor compounds remain unaffected (Balci and Wilbey, 1999). Primary structure of protein consists of linear sequence of varying types of amino acids which structurally organize into three or four conformation levels (Yaldagard et al., 2008). Protein structure with considerable amount of amino acids with hydrophobic groups is less soluble in an aqueous phase than those containing greater hydrophilic groups (Anglemier and Montgomery, 1976). Interactions within the protein chains are determined by the amino acid sequence and its interaction with the surrounding solvent. Changes in external factors including the composition, temperature, and pressure of the surrounding solvent may induce changes in the protein-protein and/or protein-solvent interactions which further causes (complete) unfolding / denaturation of the polypeptide chain (Yaldagard et al., 2008).

The effect of high pressure in inducing protein denaturation is summarized in Table 2.8, which is very much dependent on the nature and concentration of protein, the processing conditions, the pressure applied, and the characteristics of the surrounding environments such as pH or ionic strength (Yaldagard et al., 2008; Rastogi et al., 2007; Rovere, 2001). According to Masson (1992), the term 'denaturation of protein' refers to rupture and unfolding of protein of higher structure into a number of denatured products due to changes in surrounding conditions, while the primary structure or covalent bonds are unaffected. At 400 MPa, 19.2 kJ energy is needed to compress 1 liter of water, which is lower than 20.9 kJ of energy used to heat the same amount of water from 20-25 °C.

Therefore, the covalent bonds in food constituents are less affected by high pressure as compared to other interactions including hydrogen, ionic, and hydrophobic bonds (Yaldagard et al., 2008; Rastogi et al., 2007). Additionally, the covalent bonds are almost unaffected by high pressure at 0-40 °C (Hendrickx et al., 1998).

Upon denaturation, the proteins may dissolve, precipitate, and form gels, or aggregates, which are generally reversible at 100-300 MPa and irreversible at pressures greater than 300 MPa (Rastogi et al., 2007). The non-covalent interactions in the protein tertiary structure are de-stabilized upon high pressure, yet much of their secondary structure are retained with small degree of unfolding (Pittia et al., 1996; Pittia et al., 1996a; Tedford et al., 1999). The protein unfolding exposes its hydrophobic regions, followed by formation of hydrophobic and disulphide bonded protein aggregates after pressure release (Mozhaey et al., 1996; Funtenberger et al., 1997; Tedford et al., 1999). Following denaturation, there is a possibility for reformation of an intra and inter molecular bonds within or between the protein molecules (Rastogi et al., 2007).

Table 2.8 Effect of high pressure processing on food protein structure

Pressure applied	Finding	Reference
>200 MPa	Observable changes in protein tertiary structure	Masson (1992), Hendrickx et al. (1998)
>700 MPa	Irreversible denaturation in the protein secondary structure	Balny and Masson (1993)
400-800 MPa	Reversible unfolding of small proteins (e.g. ribonuclease A)	Rastogi et al. (2007)
At low pressures	Enhancement of cleavage of hydrogen bonds in secondary structure	Knorr (1999)

Denaturation of protein as affected by high pressure was first observed by Bridgman (1914). According to this study, the pressure induced protein coagulum in egg albumin, yet the appearance was quite different from that induced by thermal treatment. Heat denaturation occurs due to violent movement of protein molecules which cause breakage of the non-covalent bonds. Ghosh et al. (2001) reported relatively compact and retained secondary structure of denatured protein upon high pressure, while the denatured protein upon thermal treatment exhibited extended and nearly random coil configurations. Additionally, Bridgman (1914) suggested that the use of low temperatures increased the ease of pressure in inducing the coagulation.

Following this observation were numerous studies in relation to the effect of high pressure on the protein component in different types of food products including beef and pork meat (Bouton et al., 1977; Beilken et al., 1990; Ananth et al., 1995; Carlez et al., 1995; Fernandez-Martin et al., 1997; Galazka and Ledward, 1998; Chapleau and de Lamballerie, 2003; Ma and Ledward, 2004), carp muscles (Shoji and Saeki, 1989), fish (Ohshima et al., 1993; Angsupanich and Ledward, 1998; Lanier, 1998; Angsupanich et al., 1999; Etienne et al., 2001), surimi (Okamoto et al., 1990; Yoshioka and Yamada, 2002), surimi gels from marine species including pollack, sardine, skipjack, tuna, and squid (Farr, 1990; Thakur and Nelson, 1998; Venugopal et al., 2001), milk (Dumay et al., 1994; Galazka et al., 1996a; Dickinson et al., 1997; Olsen et al., 1999), and cheese (Messens et al., 2000; Johnson and Darcy, 2000). As stated earlier, the findings varied based on types of foods and conditions applied during the high pressure treatment.

In reference to Anglemier and Montgomery (1976), upon denaturation, the peptide bonds of the protein become more available or more susceptible to hydrolysis by

proteolytic enzymes, besides lowering the protein solubility. Similarly, beyond 300 MPa, there is a tendency for oligomeric proteins to dissociate into subunits which are vulnerable to proteolysis in contrast with monomeric proteins (Thakur and Nelson, 1998). Omi et al. (1996) reported that the solubility of soybeans' protein in aqueous phase increased with pressure from 100MPa to 400MPa, and decreased thereafter. Kato et al. (2000) also reported considerable release of proteins from polished rice grains soaked in a mixture of enzyme and distilled water and treated with HPP at 100-400 MPa. In this case, it was suggested that the high pressure induced rupture of grains' cotyledon cells, further enhanced the permeability of the surrounding solution into rice grains, followed by solubilization and release of part of the protein component into the surrounding solution.

2.4.3 Effect of high pressure processing on food microstructure

The potential of HPP in disruption of food microstructure has not widely been explored. In the case of oil seeds, earlier studies on AEE processes have critically emphasized the importance of cells rupture in order to release the oil content. Most studies reported lower oil yields from MO kernels as compared to solvent-extracted oil due to insufficient cells rupture during the AEE process (Table 2.6). Similar findings of lower enzymatically extracted oil yield than that of solvent-extracted oil were reported in the case of ground canola seeds (Latif et al., 2008), ground Kalahari melon seeds (Nyam et al., 2009), and yellow horn seed kernels (Li et al., 2013), among others. Therefore, a number of studies have tested different types of pre-treatments (Table 2.4) for greater extent of cells rupture which may facilitate in the following enzymatic hydrolysis for higher oil yield. The use of HPP as a pre-treatment however was rarely been conducted.

Jung and Mahfuz (2009) reported increment of 3.20% and 1.20% in the soybean oil yield at 200 MPa and 500 MPa, respectively, yet the increment was not statistically significant as compared to the use of AEE alone. The study suggested that the HPP did not promote rupture of cotyledon cells of soybean seeds, yet induced formation of protein aggregates which were further hydrolyzed by the added enzymes in the following AEE process. There was also no significant difference in tiger nuts oil yield from an AEE alone and from AEE with HPP pre-treatment at 50-700 MPa (Ezeh et al., 2016). It was reported that the parenchyma cells of the tubers exhibit cross linking of diferulic acid with arabinoxylans which contribute to its tough texture, even tougher than potatoes (Parker et al., 2000), thus the tuber's cells were not affected by the HPP pre-treatment.

Despite these findings, there were studies reported on detrimental effect of HPP treatment on food microstructure (Table 2.9), especially those of fragile foods containing entrapped air such as strawberries or lettuce (Rastogi et al., 2007). In overall, the effect of HPP treatment varies with types of foods which exhibit different microstructures. Therefore, the potential of HPP on other oil-bearing materials prior to an AEE method for higher oil release should further be explored.

Table 2.9 Effect of high pressure processing on food microstructure

Sample	Pressure applied	Finding	Reference
Cherry tomatoes	200-400 MPa	• Cell rupture at increasing pressure • Decrease in the product's firmness	Tangwongchai et al. (2000)
	500-600 MPa	Less observable damage	
Onion	200 MPa	Destabilization of the cell membrane	Gonzalez et al. (2010)

2.4.4 Effect of high pressure processing on emulsification properties of food protein

Generally, as explained earlier, the high pressure treatment promotes rupture of non-covalent bonds within protein molecules. These changes further induce formation of new intra and intermolecular bonds, causing formation of new complexes between proteins, promote protein aggregation, modify protein surface properties, and therefore imply new emulsifying properties to the protein structure (Chapleau and de Lamballerie-Anton, 2003, 2003a; Masson, 1992). Unfolding of protein molecule imparts a significant effect in development of an important specific surface area and formation of droplets. High pressure treatment on protein compound increases the electrostatic repulsion between the droplets, and absence of an emulsifying agent to completely saturate the droplets' surface highlights the importance of hydrophobic interactions in emulsions.

Improvement of emulsifying capacity upon high pressure treatment resulted in an emulsion with better textural properties, thus indicate its potential in development or improvement of emulsion-based food products. However, the effect of high pressure treatment in modifying the emulsifying properties of food protein has boosted controversial results (Chapleau and de Lamballerie-Anton, 2003a). Improved emulsifying capacity upon high pressure was reported in the case of soy protein isolate (Denda and Hayashi, 1992) and soymilk (Kajiyama et al., 1995). Zhang et al. (2005) proved formation of greater hydrophobic regions in soy protein upon high pressure treatment, which further breaks into subunits. In some cases, the subunits formed insoluble aggregates. In the case of lupin proteins, high pressure treatment at 400 MPa promotes aggregation and denaturation of 11S and 7S globulin, respectively, thus increased the ability of lupin proteins to be adsorbed at an oil/water interface (Chapleau and de Lamballerie-Anton, 2003a). Similar

observation was also reported in soybean protein isolates at pH 3 and pH 8, where the high pressure (200, 400, and 600 MPa at 10 °C for 10 min) decreased bridging flocculation and increased the amount of adsorbed proteins, regardless of the pH used (Puppo et al., 2005). Improvement of protein emulsifying capacity however is a disadvantage in an AEE method as described in section 2.2, due to formation of emulsified oil which needs further de-emulsification method for higher free oil release. This disadvantage was reported in the case of *Isatis indigocita* seeds (Gai et al., 2013), bayberry kernels (Zhang et al., 2012), and soybean seeds (Lamsal and Johnson, 2007).

Despite these findings, contrast outcomes were observed in milk proteins treated in similar conditions as in the study done by Dumay et al. (1994) and Galazka et al. (1996). Poor emulsifying properties of pressurised *Vicia faba* 11S protein was also reported by Galazka et al. (2000) due to protection of globulin against pressure-induced aggregation. Additionally, the emulsification property decreased in the case of soy protein at same pH and concentration (Molina et al., 2001), and in the soy 11S globulin, its emulsification property decreased at pressure higher than 400 MPa due to its aggregation.

To conclude, the effects of HPP vary with the types of foods and processing conditions applied. The high pressure treatment may affect the seed's microstructure and emulsification properties of the seed's proteins. To date, no studies have been done on the use of HPP as a pre-treatment prior to AEE on MO seed kernels.

2.5 Oxidative properties of *Moringa oleifera* oil

2.5.1 Introduction to oxidation of fats and oils

Fats and oils exhibit unsaturated bonds which represent active centers for reaction with oxygen. This reaction induces formation of primary, secondary, and tertiary oxidation products contributing to off flavors and quality deterioration in the fat or fat-containing foods, thus make them unsuitable for consumption (deMan, 1999). The rate of oxidative deterioration, normally referred as rancidity, is affected by the following factors as summarized by Bendini et al. (2010), deMan (1999), and Meyer (1960):

a) degree of unsaturation of the lipids
b) type of packaging material
c) amount of oxygen present
d) storage temperature
e) light exposure, particularly that in the ultraviolet or near ultraviolet
f) presence of water content
g) presence of antioxidants
h) presence of prooxidants (particularly copper and some organic compounds, e.g. heme-containing molecules and lipoxidase)

Presence of light induces photooxidation, while in the absence of light, these factors induce autoxidation reaction in fats and oils which follows three main stages - initiation, propagation, and termination. Free radicals are formed in the initiation stage, due to abstraction of hydrogen at the carbon atom next to the double bond in an oleifinic compound. This reaction highlights the significant effect of an oil's degree of unsaturation

on the autoxidation. The free radical formed further combines with oxygen to form a peroxy-free radical. This radical can abstract hydrogen from another unsaturated molecule, yielding a peroxide and a new free radical. These phenomena starts the propagation reaction which may repeat up to several thousand times and exhibits nature of a chain reaction. If the free radicals react with themselves, the propagation can be followed by termination with formation of non-active products (deMan, 1999).

Farmer (1946) first proposed the hydroperoxide mechanism of autoxidation. During the propagation step, the peroxides and hydroperoxides formed are the primary oxidation products which do not impart significant effect on the flavor deterioration. These products slowly increase in its amount in a period known as induction period. The progress of oxidation within this period is determined by measuring the oil's peroxide value (PV). A sudden increase in peroxide content occurs at the end of the induction period. These primary oxidation products reflects the oil's oxidative level and its tendency to become rancid. Additionally, the products are generally unstable and further decompose into various secondary oxidation products. Therefore, absence or low PV does not necessarily indicate that an oil is not oxidized. A product with PV of 1-5 meq/kg is categorized as exhibiting low oxidation rate, followed by PV of 5-10 meq/kg as moderate oxidation rate, while a product with PV of higher than 10 meq/kg is considered as having high oxidation rate (deMan, 1999; Moigradean Diana et al., 2012).

Keeney (1962) described the decomposition of hydroperoxides into various volatile and non-volatile secondary oxidation products; the most important are carbonyl compounds such as aldehydes and ketones, besides esters, alcohols, and hydrocarbons. These secondary products are responsible for changes in fats organoleptic properties and oxidized

flavor, particularly the volatile aldehydes, and can be measured by various procedures including the benzidine value. This method however was replaced with the determination of *p*-anisidine value (*p*-AV) due to the toxicity of the benzidine (IAFMM, 1981). An oil is considered as having good quality if its *p*-AV is less than 10.0 according to Rossell (1989), or less than 2.0 according to Subramaniam et al. (2000). Decomposition and oxidation of aldehydes causes formation of free fatty acids (FFA) which are considered as tertiary oxidation products (deMan, 1999). These compounds are also measured as the oil's acid value (AV) which according to Aziz (1982), an oil having AV greater than 2.0 is unsuitable for edible purposes. These acids are also responsible for the off -flavor and off-odor in fats and oils products (Noor and Augustin, 1984).

The oxidation state of an oil is better represented by measuring the combined index of primary and secondary oxidation products which is expressed as total oxidation products (i.e. TOTOX value) (deMan, 1999). Lower TOTOX value indicates better oil quality (Moigradean Diana et al., 2012). The fat composition, i.e. the type of unsaturated fatty acids present and its degree of unsaturation primarily determine the rate and course of autoxidation takes place. It is also possible for saturated fatty acids to undergo oxidation. In the case of 18-carbon-atom fatty acids, the relative oxidation rate was in the ratio of 1:100:1200:2500 for 18:0, 18:1, 18:2, and 18:3. The oxidation may change the double bond position and may also causes isomerization from *cis* to *trans* (deMan, 1999). Lundberg (1961) discovered that 90% of the peroxides formed may be in the *trans* configuration.

Oxidation reaction can be prevented by removal of oxygen from foods, yet this is difficult to be done in practice. Decrement in the oxidation rate can be achieved in the presence of antioxidants; the natural ones are mainly the tocopherols which can be found in

many foods. Vegetable oils contain greater amount of tocopherols, thus exhibiting greater stability than that of animal fats. Besides naturally present, antioxidants particularly tocopherols may also be induced by processes such as roasting or smoking, and may also be added into food products as synthetic additive. Antioxidants react with free radicals, causes formation of fatty acid free radical or peroxy free radical, thus terminating the chain. Furthermore, this reaction results in antioxidant free radical which oxidizes to quinones and finally terminate the chain (deMan, 1999).

2.5.2 Effects of storage conditions on oxidative properties of fats and oils

Oxidative deterioration is the main problem of fats and oils, especially during storage, due to production of offensive odors and off-flavor which decreases their nutritional quality and limit their use (Shao et al., 2015; Frankel et al., 1984). Some of the oxidation products are also known to cause harmful effects on human health (Ohfuji and Kaneda, 1973; Khattab et al., 1974; Alexander, 1978; June, 1981). Oxidation rate determines the shelf life of an oil, and in accord to Pristouri et al. (2010), the most important oil physicochemical properties been evaluated including AV, PV, and iodine value (IV). The study conducted by Moigradean Diana et al. (2012) indicated high stability of coconut oil towards atmospheric oxidation, which was also reported by Gopala Krishna et al. (2010). Theoretically, this is due to low degree of unsaturation in coconut oil. Additionally, tomato seed oil exhibits properties within the range shown in Codex standard, CAC (1999), thus it can be potentially used for edible purpose (Shao et al., 2015). A number of studies highlighted the effect of storage condition on the oxidative stability of seed and fruits oils (Table 2.10).

Table 2.10 Effect of storage condition on the oxidative properties of oil samples

Oil sample	Storage condition	Findings	Reference
Cotton seed oil, canola oil	Room temperature, 6 months	• Increment in AV and PV • Decrement in IV • Greater oxidative deterioration rate in oil samples without addition of antioxidants	Iskander et al. (2009)
Extra virgin olive oil	13 °C, 22 °C, and 35C, in dark, 12 months	• Increment in temperature (22 °C to 35 °C) caused greatest AV (0.83-0.85) after 12 months • At 22 °C, AV and PV remained within their limits after 12 months • At 35 °C, AV and PV exceeded their limits within the first 3 months of storage	Pristouri et al. (2010)
Coconut oil	12 months	• The PV within 12 months (0.24-0.49 meq/kg) was much lower than the maximum limits • PV increased gradually up to 9 months and decreased afterwards from 0.49 by 0.44 meq/kg, indicating appearance of secondary oxidation products • The *p*-AV ranged from 0.19 to 0.87, yet was still lower than 1.0, indicating the good oil quality	Moigradean Diana et al. (2012)
Tomato seed oil	25 °C, 210 days 35 °C, 120 days	• Changes in the oil quality attributes were more significant at 35 °C than that at 25 °C • Increment in AV and PV at both storage temperatures • Decrement in IV at both temperatures	Shao et al. (2015)

AV, acid value; IV, iodine value; PV, peroxide value; *p*-AV, p-anisidine value

Oxidation reaction leads to hydrolysis of some phosphatides and triglycerides into glycerol and free fatty acids which further cause oil degradation and affects the oil's freshness. Greater degeneration occurs in the oil with higher AV and PV (Iskander et al., 2009; Shao et al., 2015; Liauw et al., 2008). Despite this fact, in the case of olive oil, the oil with relatively high AV may possess a highly desirable aroma, while the oil with low AV may exhibit less aroma (Kiritsakis, 1998). During storage, the IV of oil samples also

decreased (Iskander et al., 2009; Shao et al., 2015), mainly due to production of hydroperoxides and intermediate compounds which resulted in saturation of the fatty acids' double bonds. This finding was also reflected in decrement in the unsaturated fatty acids (poly or mono) in the oil samples (Iskander et al., 2009).

2.5.3 Oxidative properties of *Moringa oleifera* oil

Abundant studies reported on the effect of different extraction methods on the quality characteristics of MO oil. Specifically, the oxidative properties are highlighted in Table 2.11. The MO oil contains high amounts of tocopherols and oleic acid, which contribute to the oil's oxidative stability and lower the risk of developing coronary heart disease (Rahman et al., 2009; Abdulkarim et al., 2007; Anwar et al., 2007; Lalas and Tsaknis, 2002; Tsaknis and Lalas, 2002; Tsaknis et al., 1999). These properties led to studies conducted on the use of MO oil for frying purpose. According to Tsaknis and Lalas (2002), solvent-extracted MO oil underwent greater oxidative deterioration as compared to cold-pressed oil upon frying of potatoes and cods. On the other hand, Abdulkarim et al. (2007) reported greater stability of solvent-extracted MO oil than that of conventional frying oils i.e. canola oil, soybean oil, and palm olein after frying of potato chips.

Fotouo-M. et al. (2016) demonstrated the effect of storage of MO seeds on the quantity and quality of the MO oil for its potential in biodiesel production. The seeds were stored in different packaging materials (paper and aluminium bags) at different temperatures (-19 °C, 4 °C, 20 °C, and 30 °C) for up to 24 months. The total MO oil content was extracted at the end of the storage periods with the use of combination of both screw press machine and by using solvent. Additionally, storage of MO oil (in dark glass

bottles) extracted from freshly harvested seeds was also done at ambient temperature. The findings revealed that the FFA (% oleic acid equivalent) of the MO oil remained unchanged after 6 months of storage (1.75%). However, the FFA started to increase after 12 months (2.20%), up to 24 months (2.93%). The increment of FFA was highly assumed due to combination of both hydrolysis and oxidation, since the water content of the oil was not measured prior to the storage.

Table 2.11 Physico-chemical and oxidative properties of *Moringa oleifera* oil from different extraction methods

Oil characteristics	*M. oleifera* seeds: Origin	Extraction methods						References
		Solvent treatment						
		n-hexane	Chloroform : Methanol (1:1)	Petroleum ether	Cold-pressing	Aqueous enzymatic extraction (AEE)	Supercritical fluid extraction (CO_2)	
Iodine velue (g iodine / 100 g oil)	Kenya	66.83	66.66	*	66.81	*	*	Tsaknis et al. (1999)
	India	65.58	*	*	65.73	*	*	Tsaknis and Lalas (2002), Lalas and Tsaknis (2002)
	Malaysia	*	*	65.4	*	66.1	*	Abdulkarim et al. (2005), Abdulkarim et al. (2006)
	Bangladesh	68.9	67.3	66.9	*	*	*	Rahman et al. (2009)
Free fatty acid (% as oleic acid)	India	1.12	1.39	*	1.94	*	*	Tsaknis and Lalas (2002), Lalas and Tsaknis (2002)
	Malaysia	*	*	2.48	*	1.13	*	Abdulkarim et al. (2005), Abdulkarim et al. (2006)
	Bangladesh	0.73	0.74	1.14	*	*	*	Rahman et al. (2009)
Acid value (KOH / g oil)	Kenya	0.85	0.91	*	1.01	*	*	Tsaknis et al. (1999)
Peroxide value (meq O_2/kg oil)	Kenya	1.80	0.94	*	0.36	*	*	Tsaknis et al. (1999)
	India	1.83	*	*	0.11	*	*	Tsaknis and Lalas (2002), Lalas and Tsaknis (2002)
	Bangladesh	1.50	0.86	0.87	*	*	*	Rahman et al. (2009)
p-anisidine value	Pakistan	1.85	*	*	*	1.60-1.92	*	Latif et al. (2011)

Table 2.11 (Continue)

Oil characteristics	*M. oleifera* seeds: Origin	Extraction methods						References
		Solvent treatment						
		n-hexane	Chloroform : Methanol (1:1)	Petroleum ether	Cold-pressing	Aqueous enzymatic extraction (AEE)	Supercritical fluid extraction (CO_2)	
Fatty acid composition (%)								
Oleic acid, C18:1 (%)	Kenya	73.6	73.91	*	75.39	*	*	Tsaknis et al. (1999)
	India	71.21	71.22	*	71.60	*	*	Tsaknis and Lalas (2002), Lalas and Tsaknis (2002)
	Malaysia	*	*	67.9	*	70.0	*	Abdulkarim et al. (2005), Abdulkarim et al. (2006)
	Bangladesh	74.4	71.9	71.3	*	*	*	Rahman et al. (2009)
	Philippines	70.23	*	*	*	*	69.7	Nguyen et al. (2011)
	Agric. Food[a]	65.87	*	*	*	*	66.69	Zhao and Zhang (2013)
Ben acid, C22:0 (%)	Kenya	6.73	6.38	*	5.83	*	*	Tsaknis et al. (1999)
	India	6.41	6.28	*	6.21	*	*	Tsaknis and Lalas (2002), Lalas and Tsaknis (2002)
	Malaysia	*	*	6.20	*	5.80	*	Abdulkarim et al. (2005), Abdulkarim et al. (2006)
	Bangladesh	6.16	6.22	6.58	*	*	*	Rahman et al. (2009)
	Philippines	7.55	*	*	*	*	6.71	Nguyen et al. (2011)
	Agric. Food[a]	6.85	*	*	*	*	6.36	Zhao and Zhang (2013)
Total polyunsaturated fatty acids (%)	Kenya	0.95	0.91	*	0.92	*	*	Tsaknis et al. (1999)
	India	0.83	0.83	*	0.97	*	*	Tsaknis and Lalas (2002), Lalas and Tsaknis (2002)
	Malaysia	*	*	1.3	*	0.9	*	Abdulkarim et al. (2005), Abdulkarim et al. (2006)
	Bangladesh	1.45	1.14	1.17	*	*	*	Rahman et al. (2009)
	Philippines	1.23	*	*	*	*	1.12	Nguyen et al. (2011)
	Agric. Food[a]	0.78	*	*	*	*	0.79	Zhao and Zhang (2013)

Table 2.11 (Continue)

Oil characteristics	*M. oleifera* seeds: Origin	Extraction methods						References
		Solvent treatment			Cold-pressing	Aqueous enzymatic extraction (AEE)	Supercritical fluid extraction (CO_2)	
Total saturated fatty acids (%)	Kenya	20.98	20.17	*	19.1	*	*	Tsaknis et al. (1999)
	India	23.79	23.45	*	23.23	*	*	Tsaknis and Lalas (2002), Lalas and Tsaknis (2002)
	Malaysia	*	*	27.0		24.8	*	Abdulkarim et al. (2005), Abdulkarim et al. (2006)
	Bangladesh	21.92	21.85	23.57	*	*	*	Rahman et al. (2009)
	Philippines	24.54	*	*	*	*	24.51	Nguyen et al. (2011)
	Agric. Food[a]	23.64	*	*	*	*	22.87	Zhao and Zhang (2013)
Tocopherol composition								
α-tocopherol (mg/kg oil)	Kenya	98.82	105.02	*	101.46	*	*	Tsaknis et al. (1999)
	India	15.38	*	*	5.06	*	*	Tsaknis and Lalas (2002), Lalas and Tsaknis (2002)
	Bangladesh	127	154	121	*	*	*	Rahman et al. (2009)
γ-tocopherol (mg/kg oil)	Kenya	27.90	33.45	*	39.54	*	*	Tsaknis et al. (1999)
	India	4.47	5.52	*	25.40	*	*	Tsaknis and Lalas (2002), Lalas and Tsaknis (2002)
	Bangladesh	62.2	77.4	64.0	*	*	*	Rahman et al. (2009)
δ-tocopherol (mg/kg oil)	Kenya	71.16	77.60	*	75.67	*	*	Tsaknis et al. (1999)
	India	15.51	12.67	*	3.55	*	*	Tsaknis and Lalas (2002), Lalas and Tsaknis (2002)
	Bangladesh	62.3	58.2	57.7	*	*	*	Rahman et al. (2009)

*not reported

CHAPTER 3

AQUEOUS ENZYMATIC EXTRACTION OF *Moringa oleifera* OIL

3.1 Introduction

A number of techniques are available for oil extraction from *Moringa oleifera* (MO) seed kernels of different origins, which include solvent extraction, cold-pressing, supercritical fluid extraction, and aqueous enzymatic extraction (AEE) methods. The advantages of AEE over solvent extraction are mainly due to its environmental-friendly nature and are explained in details in Chapter 2 (section 2.2). Despite the advantages, the oil yield - measured as the mass of oil extracted per unit mass of the seed taken - was lower than in the case of solvent extraction. Another disadvantage of AEE is that it results in the formation of oil-in-water cream emulsions which requires separation to recover oil (Latif and Anwar, 2011; Long et al., 2011; Chabrand et al., 2008).

Mat Yusoff et al. (2015) and Rosenthal et al. (1996) have reviewed AEE and explored the links between the microstructure of a seed or kernel and the choice of enzyme which can potentially be employed. The enzymes play an important role in rupturing the major components of the cell wall and facilitate oil release. Additionally, these papers summarized the factors affecting oil yield in AEE which include the particle size of the oil-bearing material, the amount of enzyme and water added, the pH of the mixture, and the incubation temperature, time, and shaking speed. These factors inevitably influence the nature and stability of the emulsions formed after extraction, and hence downstream processing. Statistical optimization of the AEE parameters resulting in the highest oil yield

possible, was reported for pine kernels (Yang Li et al., 2011), watermelon kernels (Xiaonan Sui et al., 2011; Shan Liu et al., 2011), and bayberry kernels (Zhang et al., 2012).

In the case of MO kernels, the effect of different AEE parameters on MO oil yield and recovery has been reported by Abdulkarim et al. (2006, 2005) and Latif et al. (2011). However, no systematic studies have been conducted so far on the microstructure of MO kernels and statistical optimization of its AEE parameters. According to Sineiro et al. (1998a), the main purpose in optimizing AEE parameters is to obtain the conditions that best incorporate with the enzymes added, besides leading to greater cell rupture. Although this statement is not incorrect, it is important to note that cell rupture does not only determine the amount of oil released from the kernel, but it also plays a critical role in the structure and stability of the emulsion formed. So far, no studies have been carried out on the formation of MO cream emulsion as affected by AEE parameters.

This chapter has been published as: "Mat Yusoff, M., Gordon, M. H., Ezeh, O., and Niranjan, K. (2016). Aqueous enzymatic extraction of *Moringa oleifera* oil. *Food Chemistry, 211*, 400-408". The main objectives of this chapter are to examine the microstructure of MO kernels and identify the type of enzymes which can be employed in the aqueous enzymatic extraction AEE method. In addition, this work reports on the effect of AEE parameters on the yield and recovery of MO oil. This chapter also discusses the effect of selected AEE parameters on the nature of MO cream emulsions. Finally, the resulted aqueous phase from the AEE conducted by using the optimized AEE parameters was re-used in another AEE process in order to evaluate the re-usability of the enzymes.

3.2 Materials and methods

3.2.1 Materials

Mature MO seeds (PKM1 hybrid) were purchased from Genius Nature Herbs Pvt ltd., Coimbatore, India. All solvents and enzymes used in this study were purchased from Sigma-Aldrich Company Ltd., Dorset, UK.

3.2.2 Statistical analysis

All statistical analyses in this study were done by using Minitab® 14.12.0 Statistical Software. A 2-Sample t-test was used to determine significant differences involving two samples (each sample with replicates data), while a 1-Sample t-test was performed when a sample (with replicates data) was statistically compared with another sample which has one datum only. A one-way analysis of variance (ANOVA) with Tukey's multiple comparison test was applied for the determination of significant differences involving more than two samples (each sample with replicates data).

3.2.3 Preparation of *Moringa oleifera* kernels and determination of the kernels' moisture and protein content

Figure 3.1 shows the processing steps involved in the preparation of MO kernels to be used in the oil extraction processes. The conditioned kernels (50 °C, 8 hr) were kept in darkness at 4 °C until use (Zhao and Zhang, 2013). The protein content of the kernels was determined by Kjeldahl method (AOAC official method 955.04, 2000), while the moisture content was determined by using oven drying method at 130±5 °C for 17±2 hr (Mani et al., 2007; AOAC, 1990). Determination of these protein and moisture contents was performed in five replicates.

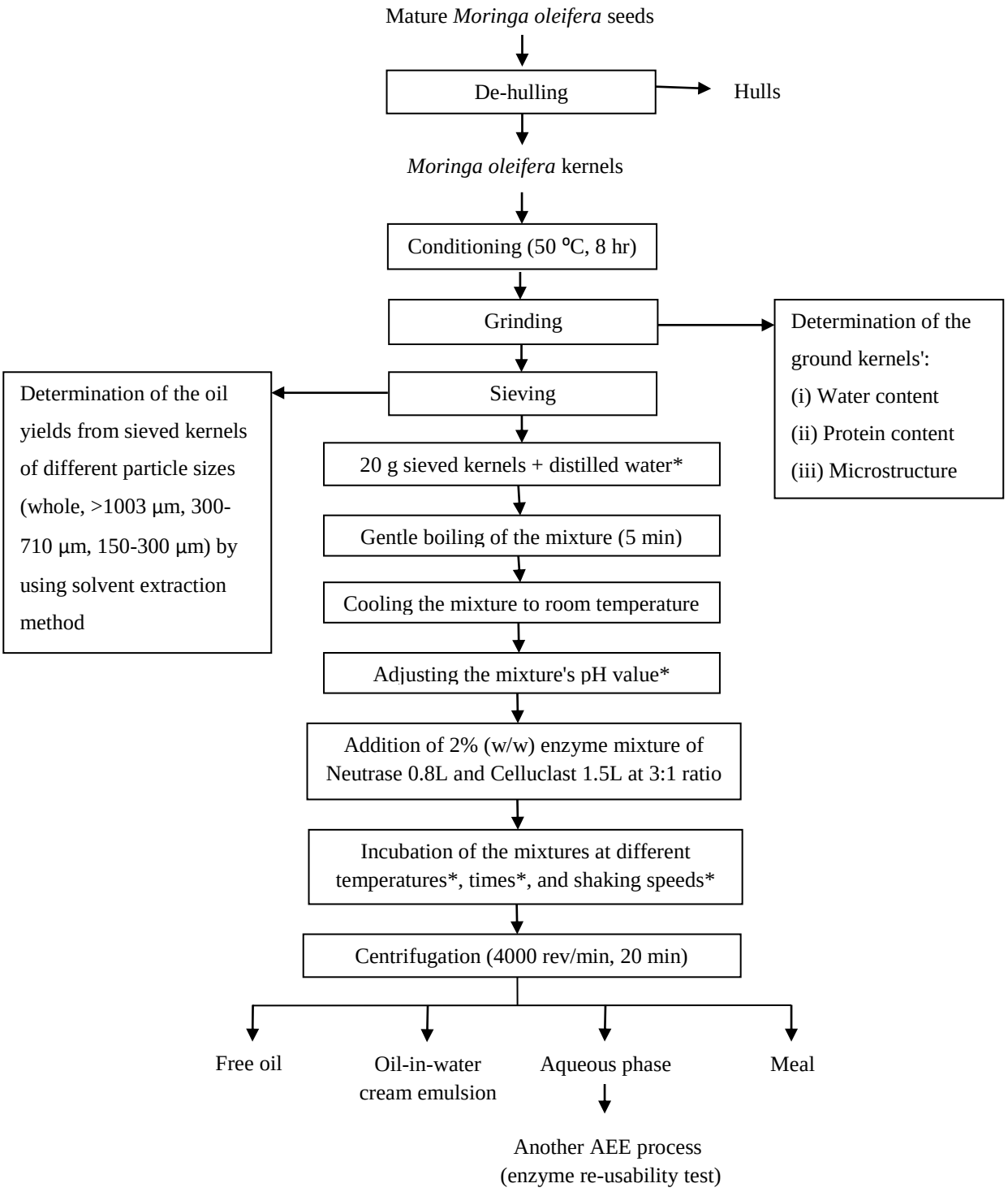


Figure 3.1 Processes involved in the preparation of *Moringa oleifera* kernels, and extraction of *Moringa oleifera* oil via aqueous enzymatic (AEE) and solvent extraction methods. The (*) represents the AEE parameters tested.

3.2.4 Aqueous enzymatic extraction and experimental design

Figure 3.1 shows the steps involved in conducting the AEE method. In order to study the effect of different AEE parameters on the oil yield and recovery, Box Behnken experimental design was applied by using Minitab® 14.12.0 Statistical Software. The range of variables involved were: the mixture's pH value (pH 4-8), temperature (40-60 °C), water content (80-160 g, which correlated to 4:1-8:1 g water / g kernel ratio), incubation time (1-24 hr), and shaking speed (40-300 stroke/min). Specifying these ranges to the software resulted in 46 experimental designs with 5 centre points (Table 3.1), while the enzyme concentration used in all AEE processes was 2% (g enzyme / g kernel).

Table 3.1 Possible combinations of aqueous enzymatic extraction parameters as obtained from Box-Behnken design (MINITAB™ Statistical Software: MINITAB Release 14.12.0, New York, USA), based on the pre-determined range of each parameter.

	pH	Temperature (°C)	Water content (g)	Time (hr)	Shaking speed (stroke/min)
Range	4-8	40-60	80-160	1-24	40-300
Run Order					
1	6	50	80	1.0	170
2	4	50	80	12.5	170
3	8	50	160	12.5	170
4	6	50	120	1.0	40
5	4	50	160	12.5	170
6	6	50	160	1.0	170
7	6	50	80	24.0	170
8	6	50	160	24.0	170
9	4	40	120	12.5	170
10	8	60	120	12.5	170
11	4	60	120	12.5	170
12	6	50	120	12.5	170

13	8	50	80	12.5	170
14	6	50	120	1.0	300
15	6	50	120	24.0	40
16	6	50	120	12.5	170
17	6	50	120	12.5	170
18	8	40	120	12.5	170
19	6	40	120	12.5	300
20	6	50	120	24.0	300
21	6	60	120	12.5	300
22	6	60	120	12.5	40
23	6	40	120	12.5	40
24	8	50	120	12.5	300
25	6	50	120	12.5	170
26	4	50	120	1.0	170
27	6	40	80	12.5	170
28	6	50	160	12.5	40
29	6	60	120	24.0	170
30	4	50	120	12.5	300
31	8	50	120	12.5	40
32	6	50	80	12.5	40
33	6	40	160	12.5	170
34	6	60	80	12.5	170
35	4	50	120	24.0	170
36	4	50	120	12.5	40
37	6	40	120	24.0	170
38	6	50	120	12.5	170
39	6	50	80	12.5	300
40	6	60	120	1.0	170
41	6	50	120	12.5	170
42	6	60	160	12.5	170
43	6	40	120	1.0	170
44	8	50	120	1.0	170
45	6	50	160	12.5	300
46	8	50	120	24.0	170

These pre-determined values were based on earlier studies on AEE which showed that the oil yield decreased or remained the same beyond the ranges stated above (Yang Li et al., 2011; Nyam et al., 2009; Zhang et al., 2007; Abdulkarim et al., 2006; Sharma et al., 2002; Hanmoungjai et al., 2001; Dominguez et al., 1996). According to Jiang et al. (2010), this trend may be due to saturation of the substrates, while Zuniga et al. (2003) assumed that the oil released was limited due to caramelization of soluble sugars. In addition, in terms of pH value, a mixture of two enzymes used may exhibit a different optimum pH, despite the optimum pH of each individual enzyme. The incubation time range chosen was also based on its acceptability in practice (Passos et al., 2009). Moreover, very long incubation time may results in lower oil quality (Jiang et al., 2010), the use of higher energy, and may result in the production of undesirable products (Abdulkarim et al., 2006). In terms of shaking speed, a preliminary study was carried out to determine the maximum shaking speed to be used which was 300 stroke/min. The enzyme concentration was based on most earlier studies on AEE which reported steady or decline oil yield with the use of concentration of beyond 2% (g enzyme / g material), for examples in the case of *Moringa oleifera* kernels (Abdulkarim et al., 2006), peanut (Jiang et al., 2010), pine kernels (Yang Li et a., 2011), and *Sylibum marianum* seeds (Li et al., 2012). According to Jiang et al. (2010), the decrease in oil yield may be due to saturation of the substrates. The use of too high enzyme concentration may also results in extraction of other undesirable components from the materials which possibly contribute to bitterness and off flavors (Jiang et al., 2010). Therefore, 2% (g enzyme / g kernel) enzyme concentration was used in this study.

3.2.5 Recovery of free oil

The centrifuged mixture (Figure 3.1) was kept frozen at -20 °C for 24h, and the solidified oil was transferred to a crucible of pre-determined weight, and heated in an oven at 60 °C for 15 min to ensure complete removal of any aqueous phase that may present in the recovered oil. The crucible containing the oil was cooled to room temperature in a desiccant containing silica gel for approximately 10 min before been weighed. The free oil yield and recovery was calculated as follows:

$$\text{Free oil yield (\%)} = \frac{[\text{Mass of crucible containing the oil (g)} - \text{Mass of crucible (g)}] \times 100}{\text{Mass of kernels initially taken (g)}}$$

$$\text{Free oil recovery (\%)} = \frac{\text{Mass of oil extracted from a given mass of kernel (g)} \times 100}{\text{Mass of oil contained in the kernels initially taken (g)}}$$

3.2.6 Re-usability of the resulted aqueous phase from an aqueous enzymatic extraction process

The aqueous phase resulted from the optimized AEE parameters was filtered by using a filter paper and used for another optimized AEE process as shown in Figure 3.1. This step was carried out in order to evaluate the re-usability of the enzymes in the aqueous phase. It was assumed that the enzymes still present and are still active, thus the gentle boiling step for 5 min prior to adjusting the mixture's pH value (Figure 3.1) was not applied to prevent denaturation of the enzymes. The optimized AEE was carried out for up to six times in order to obtain sufficient amount of aqueous phase for this re-usability test which was further conducted in triplicate.

3.2.7 Solvent extraction of oil from ground *Moringa oleifera* kernels

It is always assumed that the oil yield from solvent extraction method represents the total amount of oil present in the seeds (Soto et al., 2007). The dried MO kernels were ground in a coffee grinder (De'Longhi KG49 Electric Coffee Grinder, Hampshire, UK) and sieved using a vibratory sieve shaker (Fritsch, Analysette 3E) into four different particle size ranges: size greater than 1000 µm, 710-1000 µm, 300-710 µm, and 150-300 µm. Solvent extraction was performed on each of the particle sizes using hexane following the procedure of Zhao and Zhang (2013). Extractions conducted for 6 hr and 8 hr resulted in insignificantly different ($P > 0.05$) MO oil yield (data not shown), thus 6 hr extraction time was sufficient to determine the oil content of the kernels. The hexane was evaporated from the extracted oil in a round bottom flask of pre-determined weight by using a rotary evaporator (60 °C, 10 min), followed by heating in an oven (100 °C, 10 min). The difference between the initial (empty) and final (containing the extracted oil) weight of the round bottom flask used was measured as the oil yield in the meal by normalizing this against the weight of the kernels taken initially. This solvent extraction was conducted for up to five times at different time intervals using two different water baths in order to ensure that the oil content in the kernel samples did not undergo any significant changes upon storage.

3.2.8 Confocal laser scanning microscopy

Confocal laser scanning microscopy, CLSM (Leica TCS SP2 AOBS, Heidelberg, Germany) was used to visualize the structure of MO kernel cell. The procedure of Gallier et al. (2012, 2010) was followed with modifications, where sample dilution was not performed due to different types of samples used in these studies. A thin slice of the kernel was mounted on a slide and stained with Nile Red for 1 min, followed by immersion in distilled water. Slide staining was then repeated with Fast Green FCF, and observed under argon laser illumination. The Nile Red and Fast Green FCF specifically stained the oil and protein components of the kernel cells, respectively. The kernels for this experiment were randomly selected, and up to forty images were taken in order to obtain and confirm on the final image.

3.2.9 Scanning electron microscopy

High vacuum scanning electron microscopy, SEM (Cambridge 360 Stereoscan) was used to determine the microstructure of MO kernels under different magnifications. The sample preparation was done according to Sineiro et al. (1998a) with slight modification. The MO kernels were dried in an oven at 40 °C for 1 hr before been placed in a desiccator for 30 min in order to obtain dehydrated kernel tissues. A thin slice of MO kernel was mounted on a stub and coated with gold before been subjected to SEM analysis. The kernels for this experiment were randomly selected, and up to 25 images were taken under different magnifications in order to obtain and confirm on the final images.

3.3 Results and discussion

3.3.1 Effect of particle sizes of *Moringa oleifera* kernels on oil yield

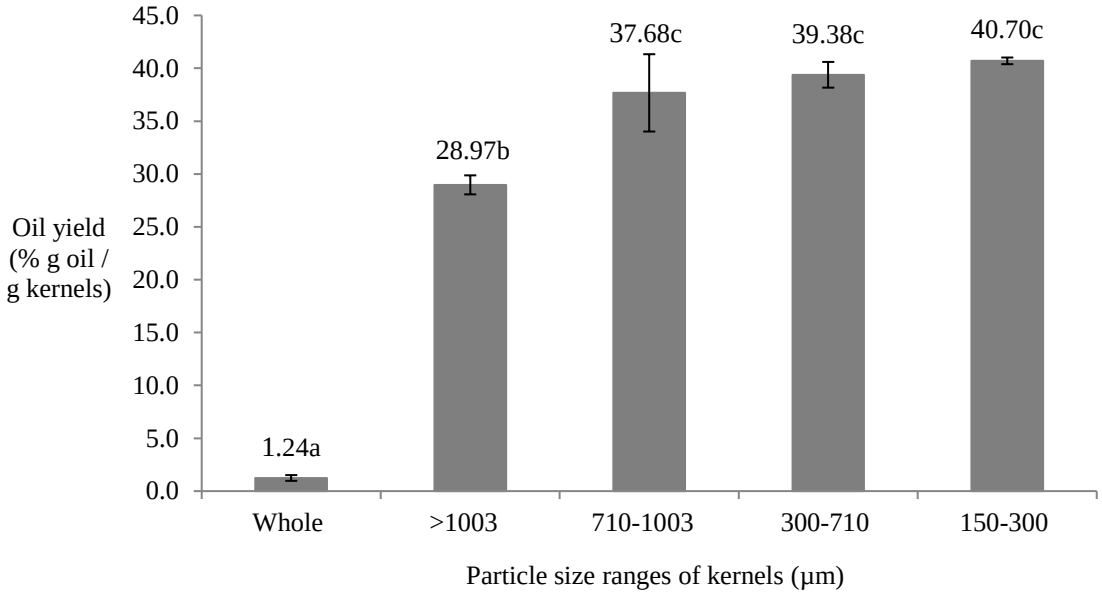


Figure 3.2 *Moringa oleifera* oil yield from ground kernels of different particle sizes as determined by solvent extraction method using hexane. Mean values followed by different letters are significantly different ($P < 0.05$).

In reference to Figure 3.2, as low as 1.24±0.27% (w/w) oil was extracted from the whole kernels, which indicated that within the 6 hr extraction time, the solvent was unable to penetrate the cells and extract the oil out. The oil yield increased with decrement in the particle size. Another solvent extraction was conducted on ground MO kernels which passed a 710 µm sieve only, and the oil yield was 41.03±1.07% (w/w) within a 6 hr extraction time. This value was concluded as the total oil content in the MO kernels used in this study, and further oil extractions were conducted on ground MO kernel fractions of less than 710 µm size.

3.3.2 Microstructure of *Moringa oleifera* kernels

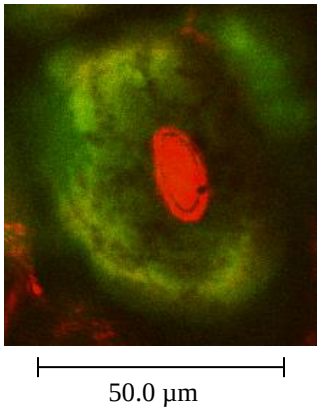
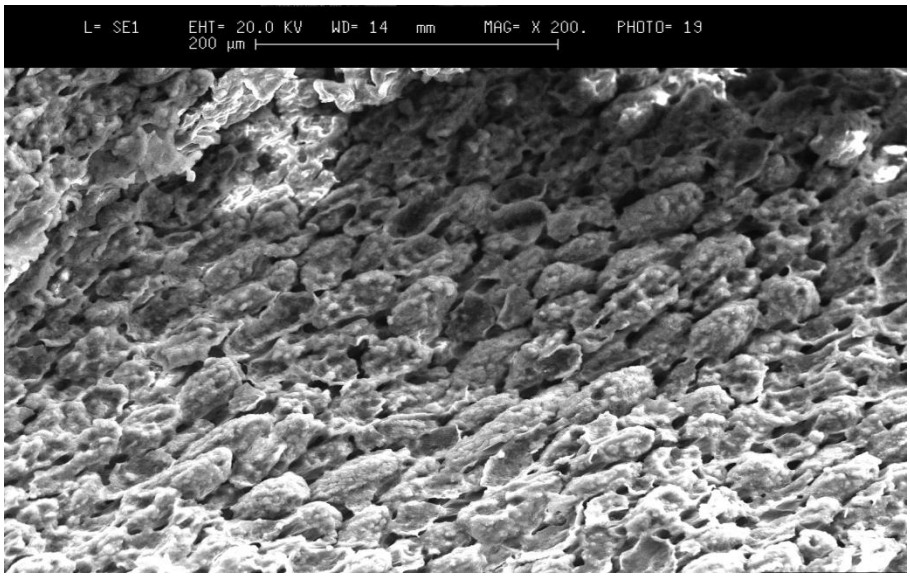
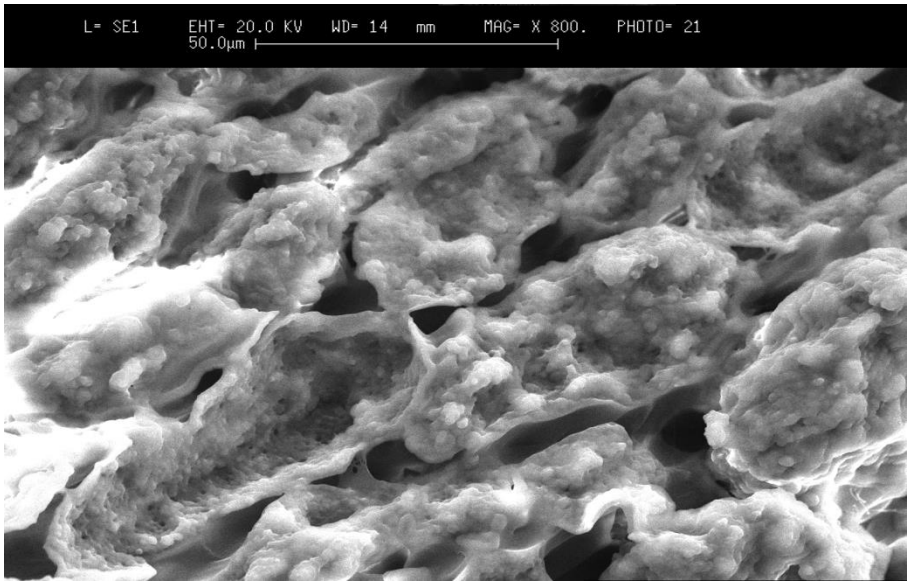


Figure 3.3 Microstructure of *Moringa oleifera* kernel cell as observed by using Confocal Laser Scanning Microscopy (CLSM). Green, protein; red, oil.



(a)



(b)

Figure 3.4 Microstructure of *Moringa oleifera* kernels obtained by high vacuum scanning electron microscopy (SEM, Cambridge 360 Stereoscan) under (a) 200x and (b) 800x magnifications.

The moisture content of MO kernels used in this study was 7.33±1.15% (g moisture / g kernel), while the protein content was 35.10±0.35% (g protein / g kernel). Figure 3.3 proved the presence of protein as the major component of MO cell wall which surrounds the oil, while Figure 3.4 displays additional microscopic observations on the MO cells under different magnifications of SEM. In the case of soybean and rapeseeds, protein and pectin were reported as the major component in their cell walls, respectively, and degradation of these components allowed easier oil release from the cells (Santos and Ferrari, 2005; Jung et al., 2009; Zhang et al., 2007). As far as the authors' concern, no studies have reported the microstructure of MO kernel cell. Based on Figure 3.3 and due to the high protein content surrounding the oil, commercial protease and cellulase enzymes were selected for the AEE process with higher amount of protease at 3:1 ratio. The protease enzyme was from *Bacillus amyloliquefaciens* (Neutrase 0.8L), while the cellulase was from *Trichoderma resei* (Celluclast 1.5L) which exhibits optimum pH of 6.8 and 4.8, respectively. Latif et al. (2011) and Abdulkarim et al. (2006, 2005) had also proved that the use of protease resulted in higher MO oil yield than the use of cellulases, pectinase, and other enzymes mixtures.

3.3.3 Optimization of the aqueous enzymatic extraction parameters for highest *Moringa oleifera* oil recovery

Table 3.2 Possible combinations of aqueous enzymatic extraction parameters as obtained from Box-Behnken design (MINITAB™ Statistical Software: MINITAB Release 14.12.0, New York, USA) based on the pre-determined range of each parameter, and the *Moringa oleifera* oil recovery from each run order.

	pH	Temperature (°C)	Water content (g)	Time (hr)	Shaking speed (stroke/min)	Oil recovery (% g oil / g hexane-extracted oil)
Range	4-8	40-60	80-160	1-24	40-300	-
Run Order						
1	6	50	80	1.0	170	46.46
2	4	50	80	12.5	170	35.89
3	8	50	160	12.5	170	21.26
4	6	50	120	1.0	40	51.38
5	4	50	160	12.5	170	50.84
6	6	50	160	1.0	170	54.34
7	6	50	80	24.0	170	63.32
8	6	50	160	24.0	170	63.14
9	4	40	120	12.5	170	45.85
10	8	60	120	12.5	170	48.69
11	4	60	120	12.5	170	48.77
12	6	50	120	12.5	170	44.53
13	8	50	80	12.5	170	36.87
14	6	50	120	1.0	300	65.13
15	6	50	120	24.0	40	50.74
16	6	50	120	12.5	170	44.25
17	6	50	120	12.5	170	43.87
18	8	40	120	12.5	170	42.68
19	6	40	120	12.5	300	72.75
20	6	50	120	24.0	300	68.87

21	6	60	120	12.5	300	66.87
22	6	60	120	12.5	40	53.40
23	6	40	120	12.5	40	46.40
24	8	50	120	12.5	300	59.03
25	6	50	120	12.5	170	41.89
26	4	50	120	1.0	170	25.92
27	6	40	80	12.5	170	36.29
28	6	50	160	12.5	40	46.79
29	6	60	120	24.0	170	54.65
30	4	50	120	12.5	300	63.15
31	8	50	120	12.5	40	52.97
32	6	50	80	12.5	40	43.54
33	6	40	160	12.5	170	50.38
34	6	60	80	12.5	170	57.74
35	4	50	120	24.0	170	57.64
36	4	50	120	12.5	40	43.76
37	6	40	120	24.0	170	52.01
38	6	50	120	12.5	170	45.48
39	6	50	80	12.5	300	64.25
40	6	60	120	1.0	170	41.82
41	6	50	120	12.5	170	41.89
42	6	60	160	12.5	170	42.14
43	6	40	120	1.0	170	50.79
44	8	50	120	1.0	170	19.50
45	6	50	160	12.5	300	68.93
46	8	50	120	24.0	170	49.05

Table 3.2 displays the 46 design points i.e. run orders resulted from the pre-determined AEE parameters, and the MO oil recovered from each run order. Highest oil recovery of 72.75% (w/w) was obtained in Run 19 (pH 6, 40 °C, 6:1 water/kernel ratio, 12.5 hr incubation time, 300 stroke/min shaking speed). Statistical optimization was carried out, and the combination of parameters which was predicted to result in highest oil

recovery was the use of a mixture at pH 4.5 and 8:1 water/kernel ratio, incubated at 300 stroke/min shaking speed. Both the incubation temperature and time did not have significant effect ($P > 0.05$) on the oil recovered within the ranges studied, and the predicted oil recovery was 72.49% (w/w). The regression model equation representing the oil recovery (response, Y) from AEE as significantly ($P < 0.05$) affected by pH value (X_1), water content (X_2), and shaking speed (X_3) is as follow:

$$Y = 30.9859\ X_1 + 0.5942\ X_2 - 0.1553\ X_3 - 1.7356\ X_1^2 + 0.0007\ X_3^2 - 0.0955\ X_1X_2$$

Statistically, the R^2 value represents the changes in Y that can be determined by the changes in the X values in the particular model, if the experiment and estimation is done accordingly. The equation with high R^2 value (maximum = 1.0000) indicates better 'explanatory power', and vice versa, while the p-value further indicates the validity of the R^2 value. If the p-value is less than 0.05, its R^2 value is not equal to zero or not meaningless which thus confirms the model's validity (Hair et al., 1998). In this study, the model did not exhibit a great 'explanatory power' since the R^2 value was 0.5920 (Personal communication), yet the p-value was less than 0.05. Therefore, the model was valid to be used statistically for estimating the changes in MO oil recovery based on the changes in pH value, water/kernel ratio, and shaking speed.

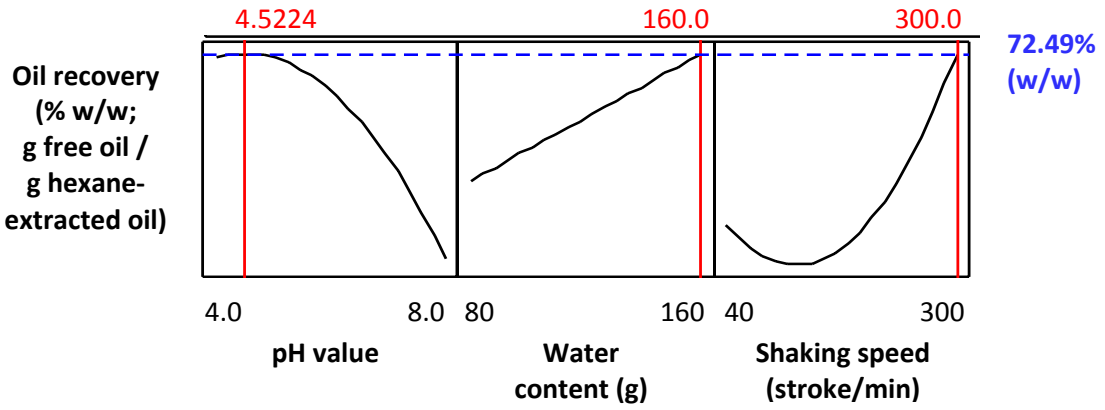


Figure 3.5 The optimized aqueous enzymatic extraction (AEE) parameters (in red) which is predicted to yield in approximately 72.49% (w/w) *Moringa oleifera* oil recovery (in blue; g free oil / g hexane-extracted oil), and the effect of each parameter of pH value, water content, and shaking speed (in range) on the oil recovery; as generated by MINITAB™ Statistical Software: MINITAB Release 14.12.0, New York, USA, with modification.

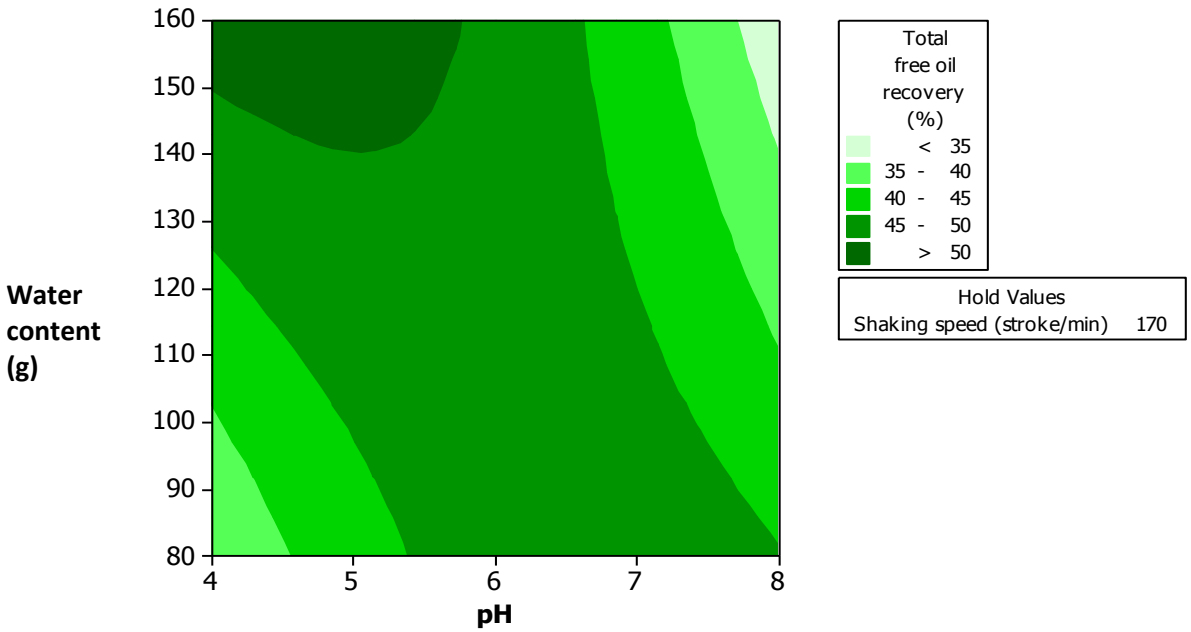


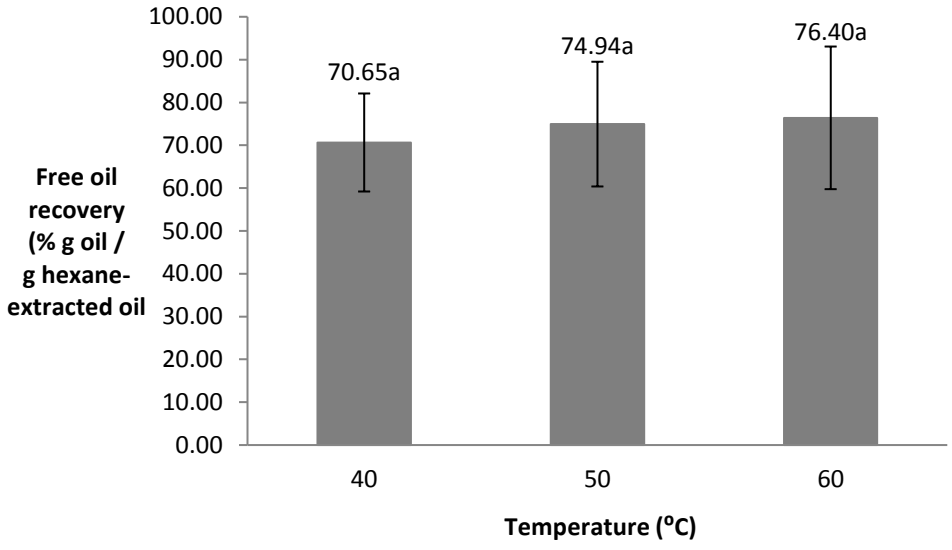
Figure 3.6 Contour plot of the interaction effect between the pH value and water content (g) of a mixture undergoing an AEE process at 170 stroke/min incubation shaking speed on the free *Moringa oleifera* oil recovery (% g oil / g hexane-extracted oil) as generated by MINITAB™ Statistical Software: MINITAB Release 14.12.0, New York, USA with modification.

According to Figure 3.5, the free oil recovery increased with the use of lower pH value, higher water content, and higher shaking speed. Additionally, the statistical model above showed that there was a significant interaction effect ($P < 0.05$) between the pH value (X_1) and the water content (X_2) of the mixture which underwent the AEE process. The interaction between these two factors at 170 stroke/min was generated by the statictical software and is presented in Figure 3.6. Free MO oil recovery of greater than 50 % (w/w) can be achieved if the mixture's pH value is less than 6.0 with water content of higher than 130 g. Lowest free oil recovery of less than 35% (w/w) is predicted with the use of higher

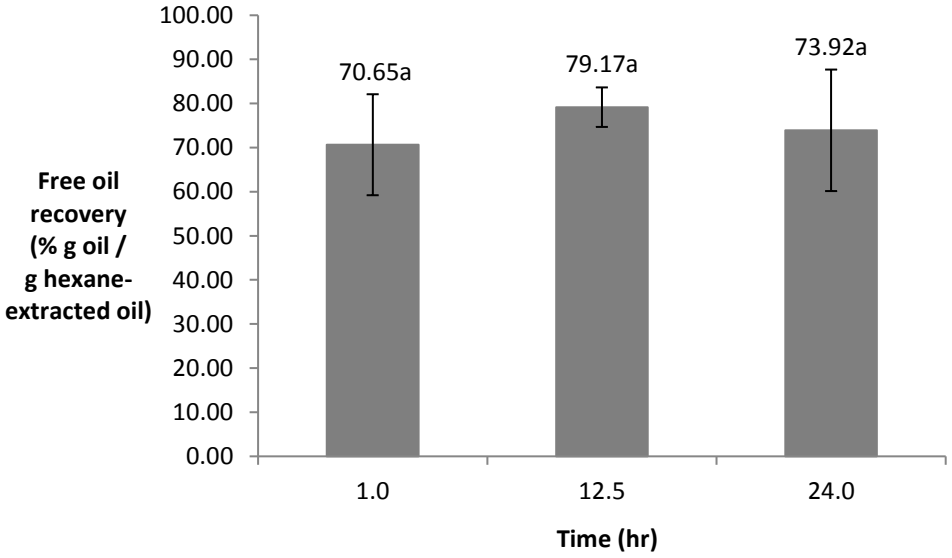
pH, up to 8, even though the highest amount of water was used in the mixture. This interaction effect explains how the different AEE parameters actually dependent on each other and significantly affect the oil recovery.

3.3.4 Data validation

Upon statistical optimization, the software predicted highest oil recovery of 72.49% (w/w) with the use of the optimal conditions - pH 4.5, 8:1 water/kernel ratio, and 300 stroke/min incubation shaking speed. The temperature and time however did not impart any significant difference ($P > 0.05$) on the oil recoveries within the ranges tested. This predicted value was validated by experimentally testing (in triplicates) the optimal conditions at different temperatures (40 °C, 50 °C, and 60 °C) and times (1 hr, 12.5 hr, 24 hr) of incubation. The optimized pH value, water/kernel ratio, and incubation shaking speed of 4.5, 8:1 (w/w), and 300 stroke/min were used, respectively. Figure 3.7(a) displays the MO oil recoveries at different incubation temperatures in 1 hr incubation time, while Figure 3.7(b) shows the oil recoveries at different incubation times at 40 °C incubation temperature. There was no significant difference ($P < 0.05$) in the MO oil recovery between different temperatures and between different time been studied, thus proved the insignificant effect imparted by these factors within the ranges tested on the oil recovery with the use of pH 4.5, 8:1 water/kernel (w/w), and 300 stroke/min shaking speed. There was also no significant difference ($P > 0.05$) between these values with that of predicted by the software (72.49% w/w), thus the experimental design is validated.



(a)



(b)

Figure 3.7 *Moringa oleifera* oil recoveries from the optimized aqueous enzymatic extraction method at different incubation (a) temperatures and (b) times. Mean values followed by different letters are significantly different ($P < 0.05$).

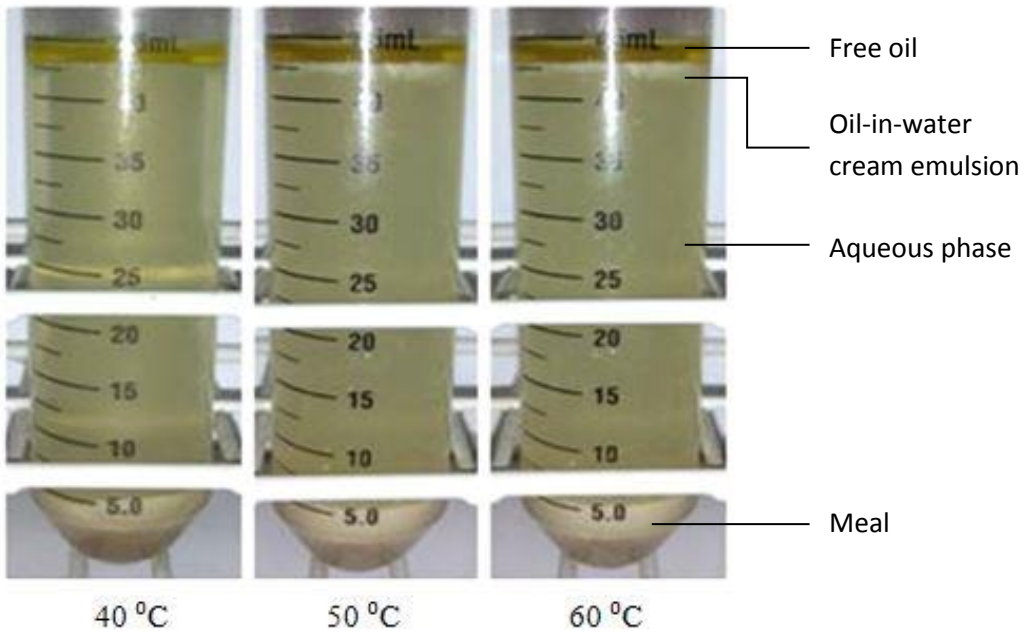
3.3.5 Effect of aqueous enzymatic extraction parameters on *Moringa oleifera* oil recovery and formation of its cream emulsions

Earlier experiments in this study have concluded that in the AEE process, the MO free oil recovery was significantly ($P < 0.05$) affected by the pH value and water content of the mixture, and the incubation shaking speed. The effects of these parameters on the free MO oil recovery in this study are summarized in Figure 3.5. On the other hand, with the use of the optimized pH value (pH 4.5), water/kernel ratio (8:1), and shaking speed (300 stroke/min), both the incubation time and temperature did not have significant effect ($P > 0.05$) on the oil recovery within the ranges been studied. Without enzymes, an aqueous extraction using the optimized parameters at 40 °C for 1 hr incubation resulted in minute amount of oil which was unable to be recovered. This observation highlighted the significance of adding the enzymes for greater oil release. Furthermore, the AEE performed on selected experimental runs from Table 3.2 which resulted in 64-80% free oil recovery (w/w) were repeated in triplicates for statistical comparison purpose. The values are displayed in Table 3.3 with the optimum values determined. Figures 3.8(a-c) display the visual outcomes of the AEE processes for these runs.

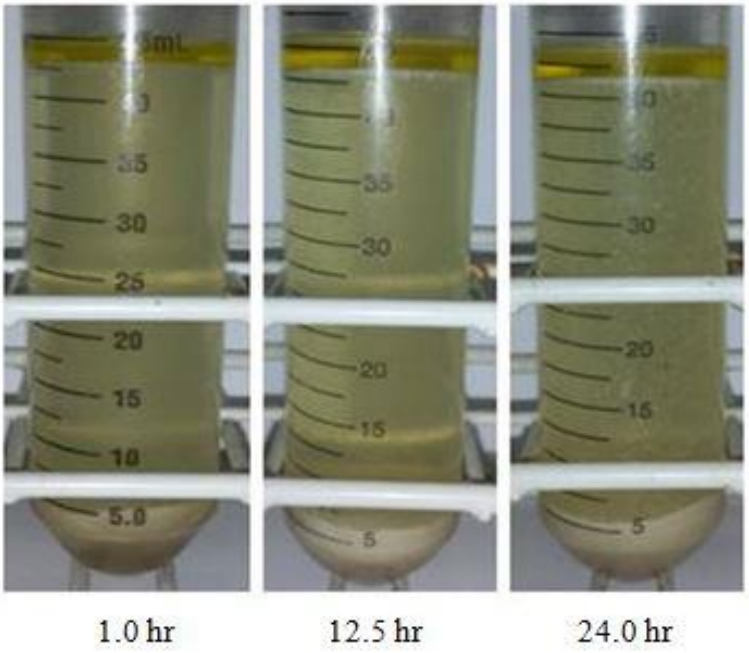
Table 3.3 *Moringa oleifera* hexane-extracted oil yield, and oil yield and recovery from different aqueous enzymatic extraction processes: the optimum parameters, selected run orders of 64-73% (w/w) free oil recoveries, and from previous studies

Hexane-extracted oil (% g oil / g kernel)	Aqueous enzymatic extraction (AEE)								
	Run / Reference	Type of enzyme	pH	Water / kernel (w/w)	Time (hr)	Temperature (°C)	Shaking speed (stroke/min)	Oil yield (% g oil / g kernel)	Free oil recovery (% g oil / g hexane-extracted oil)
Current study									
41.03 ± 1.07	Optimum	Neutrase 0.8L / Celluclast 1.5L (3:1)	4.5	8:1	1.0	40.0	300	28.99 ± 4.70[a] (Predicted = 29.74)	70.65 ± 11.45[b] (Predicted = 72.49)
	14		6.0	6:1	1.0	50.0	300	26.61 ± 0.67[a]	64.86 ± 1.63[b]
	19		6.0	6:1	12.5	40.0	300	26.98 ± 2.00[a]	65.75 ± 4.87[b]
	20		6.0	6:1	24.0	50.0	300	29.33 ± 2.37[a]	71.48 ± 5.78[b]
	21		6.0	6:1	12.5	60.0	300	27.25 ± 0.34[a]	65.26 ± 1.76[b]
	39		6.0	4:1	12.5	50.0	300	27.15 ± 1.66[a]	66.16 ± 4.04[b]
	45		6.0	8:1	12.5	50.0	300	27.77 ± 2.18[a]	67.66 ± 5.32[b]
Previous studies									
33.40	Abdulkarim et al. (2006)	Neutrase 0.8L	6.8	6:1 (v/w)	24.0	45.0	120 rpm	24.02	74.00
32.40	Latif et al. (2011)	Protex 7L	*	8:1 (v/w)	2.0	45.0	200 rpm	22.50	69.40

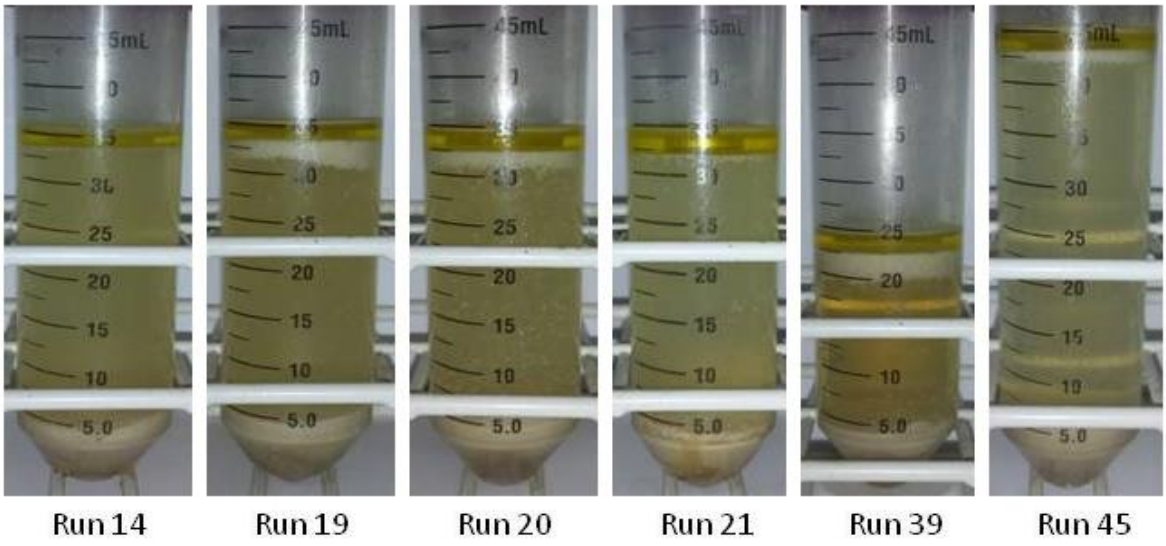
Neutrase 0.8L and Protex 7L, protease. Celluclast 1.5L, cellulase. All studies employed the enzymes at 2% (g enzyme / g kernel) concentration. The pH value (*) was the optimum pH of Protex 7L which was not stated in the study. Mean values followed by different letters in the same column are significantly different ($P < 0.05$).


Free oil
Oil-in-water cream emulsion
Aqueous phase
Meal
40 °C
50 °C
60 °C

(a)


1.0 hr
12.5 hr
24.0 hr

(b)



(c)

Figure 3.8 The effect of incubation at different (a) temperatures for 1 hr incubation time and (b) times at 40 °C incubation temperature in aqueous enzymatic extraction processes on the formation of *Moringa oleifera* oil-in-water cream emulsions. The pH value, water/kernel ratio, and shaking speed used were the optimum values of pH 4.5, 8:1 (w/w), and 300 stroke/min, respectively. Refer Table 3.2 or Table 3.3 for the parameters employed for selected run orders in Figure 3.8(c).

In terms of the effect of pH, the highest free MO oil was obtained in the range pH 4.0-4.5 (Figure 3.5). It can be concluded that this pH range is condusive for maximum activity of protease/cellulase enzymes mixture at 3:1 ratio. This pH range is also away from the isoelectric pH of the MO protein which is pH 10 and above (Gassenschmidt et al., 1995). Therefore, it is assumed that the MO protein is soluble in the solution and accesible to be hydrolyzed by the added protease, releasing the oil. The oil release was also facilitated by addition of the cellulase enzyme which disrupted other polysaccharide consitutents normally present in cell wall. Additionally, it is assumed that the hydrolyzed protein was unable to act as an emulsifier, thus resulted in thin layer of cream emulsions (Figure 3.8(a-b)).

In the review done by Mat Yusoff et al. (2015), proper selection of water content is important in an AEE process. It was difficult for the added enzyme to penetrate into the cell wall of the samples if the water content was too low, due to formation of thick suspension (Zhang et al., 2007). In contrast, addition of too much water reduced the enzyme and sample concentration, thus reducing the extraction efficiency (Yang Li et al., 2011; Zhang et al., 2007; Dominguez et al., 1996). In this study, higher water content resulted in higher MO oil recovery (Figure 3.5). This was in agreement with a study conducted by Soto et al. (2007) in the case of borage oil; the authors stated that higher extraction efficiency can be obtained from materials with higher water activitiy. Moreover, the use of high water content simultaneously resulted in a solution with lower proportion of protein content, thus may led to thinner cream emulsion layer visually observed (Figure 3.8(a-b)). When the optimum parameters are not employed, Run 39 and Run 45 with different water content yielded similar oil recovery of 66.16±4.04% (w/w) and

67.66±5.32% (w/w), respectively, yet Run 45 with higher water content exhibited thinner emulsion layer; similar to the optimum AEE. As had been discussed earlier, there is a significant interaction effect ($P < 0.05$) between the water content and the pH value, thus both of these parameters may also depend on each other in contributing any effect on the oil yield and recovery and formation of the emulsion.

In the case of shaking speed, most earlier studies as summarized by Mat Yusoff et al. (2015) reported similar observations: higher shaking speed or shaking rate assists in mixing and cell wall disruption for greater oil release (Rosenthal et al., 1998; Sineiro et al., 1998a). In this study, highest shaking speed of 300 stroke/min were used in all run orders displayed in Table 3.3 which resulted in 64-80% oil (w/w).

In terms of incubation time, most earlier studies reported that longer incubation time allows greater degradation of cell wall components which led to higher oil release (Jiang et al., 2010; Passos et al., 2009; Abdulkarim et al., 2006). In this study, there was no significant difference ($P > 0.05$) between the free oil recoveries after 1 hr up to 24 hr incubation time with the use of the optimized AEE parameters. This may be due to condusive effect of other incubation parameters applied which led to maximum enzymes' activity and therefore maximum release of oil within the short incubation time. The substrate may also have been totally depleted within the 1 hr incubation time, thus further oil release was not observed after that, up to 24 hr. In rereference to Table 3.3, when the optimized parameters were not employed, the effect of different incubation times can be seen in Run 14 (1 hr) and Run 20 (24 hr). Run 20 resulted in insignificant ($P > 0.05$) yet higher free oil recovery (71.48±5.78% w/w) and thicker emulsion layer visually observed (Figure 3.8(c)) as compared to Run 14 (64.86±1.63% w/w). It was assumed that higher oil

was actually released after the 24 hr incubation time in Run 20 which was in agreement with most earlier studies, yet the oil was emulsified and thus led to formation of thicker emulsion layer as compared to Run 14.

Besides the incubation time, there was also no significant difference ($P > 0.05$) between the incubation temperatures used when the optimum parameters were employed. Similar with the pH value, the temperatures of 40 °C, 50 °C, and 60 °C may be condusive for the enzymatic hydrolysis to take place in combination with the optimum AEE parameters, which therefore resulted in insignificantly different ($P > 0.05$) free MO oil recovery. As summarized by Mat Yusoff et al. (2015), lower processing temperature is highly favorable for most oil-bearing materials in order to preserve the oil quality. Therefore, insignificant difference ($P > 0.05$) in the oil recovery between incubation at 40 °C with 50 °C and 60 °C is of much beneficial to the MO oil quality. In accord to Zuniga et al. (2003), enzyme inactivation started at 45 °C, and further increase in temperature leads to decrease in enzymatic hydrolysis. The optimum enzymatic activity may also lie in the range of 40 °C to 55 °C, as reported by Rui et al. (2009). According to Table 3.3, Run 21 (60 °C) resulted in similar oil recovery of 65.26±1.76% (w/w) yet thinner emulsion layer than Run 19 (40 °C) with oil recovery of 65.75±4.87% (w/w), when other parameters are fixed. It is assumed that at 60 °C, protein denaturation occured which restricts its role as an emulsifier and therefore resulted in thinner emulsion layer than that of 40 °C.

In summary, all AEE parameters played significant role in affecting the oil yield and nature of the emulsion formed, and this study indicated significant interaction effect between the water/kernel ratio and its pH value within the ranges tested. It is not recommended to highlight the effect of one parameter only, if the value of other parameters

are not fixed. Moreover, different optimum AEE parameters may be discovered if the type of enzyme and/or ratio of them is changed. There are also abundant of commercial enzymes available nowadays which are manufactured from different sources. Therefore, the enzymes' activity may vary and resulted in different optimum parameters as well.

Comparison of the oil yields and recoveries were also highlighted between the present study and earlier studies as indicated in Table 3.3. Abdulkarim et al. (2006) and Latif et al. (2011) reported total solvent-extracted MO oil of 33.4% (w/w) and 32.4% (w/w), respectively, while higher total oil of 41.0% (w/w) was extracted from MO kernels used in this study. These outcomes proved that MO kernels of different origins vary in their microstructure, composition, and therefore their oil contents. Additionally, the difference may also be due to the type of solvent used and the method conducted for the solvent extraction process. In overall, despite the difference of the seeds' origins, oil recoveries in these present and earlier studies were in a range of up to 69-80% (w/w), indicating the efficiency of the AEE method conducted in each particular study as compared to the solvent extraction method.

3.3.6 How does the *Moringa oleifera* oil-in-water cream emulsion affect its total oil yield?

The optimum parameters have great advantages in terms of the ability to use lowest temperature (40 °C) and shortest incubation time (1 hr) to result in highest free MO oil recovery. A tiny amount of emulsion was also observed as compared to other run orders (Figures 3.8(a-c)). In contrast, thick-intact emulsions were observed in Run 19 and Run 39 which indicated greater emulsion stability as compared to thin emulsions in granulated

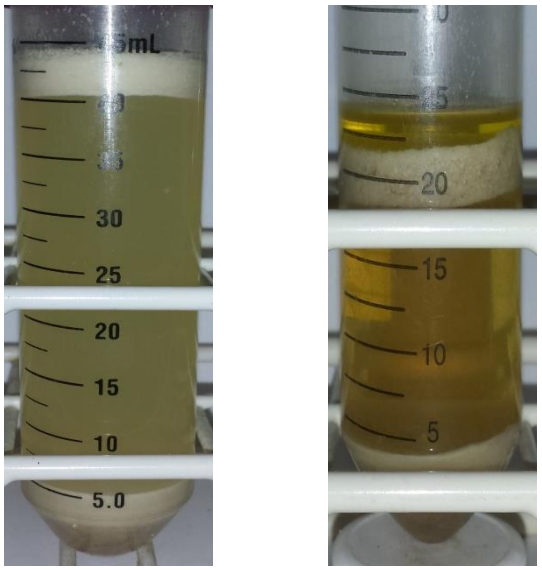
form observed in other run orders. Despite these outcomes, it is assumed that higher amount of oil was actually released from the kernels in Run 19, 20, 39, and 45, where part of the released oil was emulsified. The total oil recovered can be higher if the oil is extracted or released as free oil and does not get emulsified with the water used.

It was believed that no water content presents in the free oil after the water removal (section 3.2.5). However, minute amount of emulsion was observed which was inseparable from the free oil. In order to recover an emulsion-free MO oil, the oil was allowed to undergo gravitational separation - the emulsion settled as the bottom layer, and the oil was separated afterward by using a dropper. Despite the recoverability of the emulsion-free oil, this minute amount of emulsion may affect the total oil recovered.

Therefore, as highlighted in earlier studies on AEE method, de-emulsification was always needed in order to maximize the total oil yield. None of the AEE run orders conducted in this study resulted in zero emulsion. A number of studies have used enzymes for de-emulsification, which were thoroughly discussed in an earlier paper by Mat Yusoff et al. (2015). The enzymes added acted by hydrolyzing the interfacial proteins which are mainly responsible for stabilizing the cream emulsion. The hydrolysis resulted in a decrease in protein molecular size and oil droplet interfacial rigidity. Demulsification by using enzymes has been reported in the case of alkaline pre-treated ground peanuts (Jiang et al., 2010), coconut milk emulsion (Raghavendra and Raghavarao, 2010), extruded soybean flakes (Lamsal and Johnson, 2007; de Moura et al., 2008; Jung et al., 2009; Wu et al., 2009), ground *Perilla frutescens* seeds (Zhang et al., 2013), soybean flour (Chabrand and Glatz, 2009), and yellow mustard flour (Tabtabaei and Diosady, 2013). Chabrand and Glatz (2009) and Jung et al. (2009) also recycled the enzymes in order to improve process

economics and lower the environmental impact of the process. In the case of extruded soybean flakes, the extrusion pre-treatment has been claimed to facilitate demulsification (Jung et al., 2009). Latif et al. (2011) demulsified MO cream by freezing the cream layer (-20 °C) for 24 hr, followed by thawing in a water bath at 35 °C for 4 hr prior to centrifugation to separate oil. These studies demonstrate the critical importance of de-emulsifying the cream layer after an AEE process, for higher total oil recovery.

3.3.7 The re-usability of the aqueous phase from an aqueous enzymatic extraction of oil from *Moringa oleifera* kernels



(a) (b)

Figure 3.9 The visual outcome after an aqueous enzymatic extraction (AEE) which was carried out by re-using the aqueous phase from (a) the optimized AEE and (b) Run 39. The parameters used for the re-usability tests were similar with that of the initial AEE process.

After the AEE conducted at its optimized parameters, it was assumed that the added enzymes may still present in its active state in the resulted aqueous phase, thus it can further be re-utilized for another AEE process. Despite these assumptions, zero oil was recovered, and thick emulsion layer was observed (Figure 3.9(a)) when the following AEE was carried out by using the same optimized AEE parameters. In reference to section 3.2.6, the gentle boiling step for 5 min prior to the AEE was not applied in order to preserve the enzymes' activity that may still present in the aqueous phase. It was assumed that protein denaturation did not occur due to exclusion of this step. In addition, the following AEE process was applied at low temperature of 40 °C which is not adequate to allow protein denaturation to take place. Therefore, the protein component present in the aqueous phase and the MO sample used in the following AEE process retained their functional properties as emulsifiers which led to emulsification of the released oil.

In order to further discover the re-usability of enzymes, the aqueous phase from Run 39 was also re-utilized according to section 3.2.6, and the following AEE process was in accord to the parameters in Run 39. Similarly, a thick emulsion layer was also observed (Figure 3.9(b)), yet 59.70±3.66% (w/w) free oil was recovered. This finding confirmed the re-usability of the enzymes in the used aqueous phase, but in overall, it is highly dependent on the parameters used in the initial and the following AEE carried out. The potential of re-using the aqueous phase containing the utilized enzymes may reduces the total cost of the enzymes, besides maximizing waste utilization which further highlights the advantages of an AEE process as an environmental-friendly oil extraction method.

3.4 Conclusions

The efficiency of an AEE method depends on the type and source of oil-bearing material, the type and concentration of enzymes added, and the process parameters used. In the case of MO, ground kernels of 710-150 μm particle sizes resulted in similar solvent-extracted oil yields within 6 hr. For the AEE process, addition of protease was necessary besides the cellulase, considering the presence of protein in the kernel's cell wall. The statistical optimization revealed that within the pre-determined ranges, the MO free oil recovery increased with decreasing pH value, increasing water/kernel ratio, and increasing shaking speed, while both the incubation time and temperature did not impart any significant difference on the oil recoveries upon application of these optimum parameters. The optimum parameters resulted in highest free oil recovery of 70-80% (w/w) which was approximately similar to that of Run 14, 19, 20, 21, 39, and 45 (64-77% w/w). It was assumed that some of the released oil was emulsified, and minute emulsion was present in the recovered free oil. Furthermore, there is a potential for the resulted aqueous phase containing the utilized enzymes from an AEE process to be re-used in another AEE process, yet it is very dependent on the AEE parameters applied. In overall, it is highly suggested for further studies on AEE to focus on de-emulsification methods or pre-treatment prior to the AEE in order to restrict the formation of emulsion and therefore maximize the total oil recovered.

CHAPTER 4

HIGH PRESSURE PRE-TREATMENT OF *Moringa oleifera* SEED KERNELS PRIOR TO AQUEOUS ENZYMATIC OIL EXTRACTION

4.1 Introduction

With reference to Chapter 3, *Moringa oleifera* (MO) seed kernels contain approximately 35% (w/w) protein and up to 40% (w/w) total oil content extracted using hexane. The confocal laser scanning microscopy (CLSM) revealed that protein is the one of the major components in the cell walls surrounding the oil globules (Figure 3.3), and degradation of the protein may allow easier oil release from the cells. Therefore, in the aqueous enzymatic extraction (AEE) of MO oil conducted, the types of enzymes selected were protease and cellulase at 3:1 (w/w) ratio. The AEE parameters studied (Table 3.1, Table 3.2) were the ratio of water/kernel (4-8:1w/w), the mixture's pH value (pH 4-8), and the parameters used in incubating the mixture i.e. the temperature (40-60 °C), time (1-24 hr), and shaking speed (40-300 stroke/min). These parameters were pre-determined according to earlier studies and the available experimental set up in the laboratory. The AEE process was followed by centrifugation (4000 rev/min, 20 min) of the incubated mixture which resulted in four distinct layers of free oil at the top, followed by cream emulsion, aqueous phase, and meal at the bottom (Figure 3.1). The amount of oil recovered from an AEE was measured based on the amount of free oil at the top in relation to the total oil extracted using hexane (i.e. g oil / g hexane-extracted oil).

Statistical optimization has proved that the use of a mixture of 8:1 water/kernel (w/w) at pH 4.5 incubated at 300 stroke/min resulted in highest oil recovery of

approximately 70% (w/w), with both the incubation temperature and time insignificantly affected the oil recovery within the ranges studied (Figure 3.5-3.7). These AEE parameters also resulted in thin cream emulsion layer (Figure 3.8) which is advantageous in lowering the load on the de-emulsification step. However, when the aqueous phase obtained after the centrifugation step (assumed to contain the used protease and cellulase enzymes) was re-used for another AEE process with the same parameters, virtually no free oil recovery with thick emulsion layer were observed (Figure 3.9). This finding highlighted that the protein component present in the aqueous phase and the MO kernel sample used in the following AEE process retained their functional properties as emulsifiers which led to the complete emulsification of the released oil. On the other hand, AEE (protease:cellulase, 3:1 w/w) of a mixture of 4:1 water/kernel (w/w) at pH 6.0 incubated at 50 °C for 12.5 hr and 300 stroke/min (Table 3.3, Run 39) resulted in approximately 66% (w/w) oil recovery, and 60% (w/w) oil was recovered upon re-using the aqueous phase. This outcome confirmed the re-usability of the enzymes in the used aqueous phase which is highly dependent on the parameters used in the initial and the following AEE carried out.

Despite the ability of the aqueous phase to be re-used, Run 39 resulted in thick emulsion layer (Figure 3.9) which needs to be de-emulsified for higher total oil recovery. Formation of this cream emulsion is another challenge in an application of an AEE process, besides its lower oil recovery as compared to the solvent extraction method. The de-emulsification was particularly difficult because of the added emulsion stability imparted by the proteins. Earlier studies suggest that oil recoveries can be enhanced by decreasing the emulsifying capacity of the proteins, possibly by altering their structures. The alteration of protein structure may be induced by both hydrolysis and denaturation, which can be

promoted by subjecting the kernels to treatments such as high pressure processing (HPP). The effect of HPP on food protein and microstructure are already discussed in Chapter 2 (section 2.4.2-2.4.3).

The role of protein as an emulsifier varies greatly as reported by Denda and Hayashi (1992), Kajiyama et al. (1995), Galazka et al. (2000), Molina et al. (2001), and Chapleau and de Lamballerie-Anton (2003a). Additionally, the factors affecting protein denaturation and hydrolysis may or may not affect the role of the protein as an emulsifier. Based on these hypotheses and outcomes of earlier studies, it appears that a combination of protein denaturation caused by HPP, followed by protein hydrolysis employing AEE may significantly influence MO oil recovery. This chapter aims to investigate this hypothesis as a novel application of HPP. To the best of our knowledge, there are, no studies reporting on the use of HPP pre-treatment to limit the stability of the cream emulsion formed after AEE.

The main objective of this study is to explore the effect of different HPP parameters on the stability of cream emulsion formed after AEE on MO kernels and on the total recoveries of oil. The effect of different particle sizes of the kernels for the HPP pre-treatment (i.e. the whole and ground-sieved kernels) on the oil recovery after the AEE process was also determined. Additionally, optimization of the HPP parameters has been undertaken to determine the most suitable conditions which result in highest MO oil recovery. With reference to Figure 3.1, a gentle boiling step was used on the mixture of MO kernels and water prior to pH adjustment in order to inactivate the natural hydrolytic enzymes in the seed kernels (Zhang et al., 2007). It was hypothesized that replacing this boiling step with the HPP could potentially affect the oil recovery and the cream emulsion formed; this has been established towards the end of this chapter. The boiling step

mentioned above is termed as boiling pre-treatment in this study. Further, the effects of both boiling and HPP pre-treatments prior to applying an AEE method on the MO oil quality attributes are discussed in Chapter 5.

4.2 Materials and methods

4.2.1 Materials

Mature MO seeds (PKM1 hybrid) were purchased from Genius Nature Herbs Pvt ltd., Coimbatore, India. All solvents and enzymes used in this project were obtained from Sigma-Aldrich Company Ltd., Dorset, UK.

4.2.2 Statistical analysis

Minitab® 14.12.0 Statistical Software: MINITAB Release 14.12.0, New York, USA was used for all statistical analyses and determination of significant differences between the data obtained in this study. A 1-Sample t-test was applied when one datum is compared with a sample with replicates data. When two replicates were compared, a 2-Sample t-test was used, while analysis of variance (ANOVA) with Tukey's multiple comparison test was applied when more than two samples (each sample with replicates data) were involved. The confidence level used in this study was 95.0.

4.2.3 High pressure processing and experimental design

The HPP parameters involved in this study were the pressure (50-450 MPa), temperature (20-60 °C), and processing time (10-60 min). The pressure range was determined based on earlier studies, where the amount of protein released from soybean seeds (Omi et al., 1996) and rice grains (Kato et al., 2000) increased up to 400 MPa only, besides decrease in the amount of soybean oil extracted at 500 MPa pre-treatment prior to an AEE method (Jung and Mahfuz, 2009). In terms of the temperature range, most types of protein are fully denatured at 60 °C (Scopes, 1994) and above if held for sufficient time. In the case of the processing time, due to adiabatic temperature increase during the pressure treatment, the temperature was unstable in the first 2-3 min of pressurization period based on a preliminary study conducted. Therefore, a minimum of 10 min was used in order to allow enough time for the temperature to stabilize. The statistical software (section 4.2.2) was used to generate the Box Behnken experimental design used in this study, where the pre-determined HPP ranges resulted in 15 design points (i.e. 15 run orders) with 3 centre points (Table 4.1).

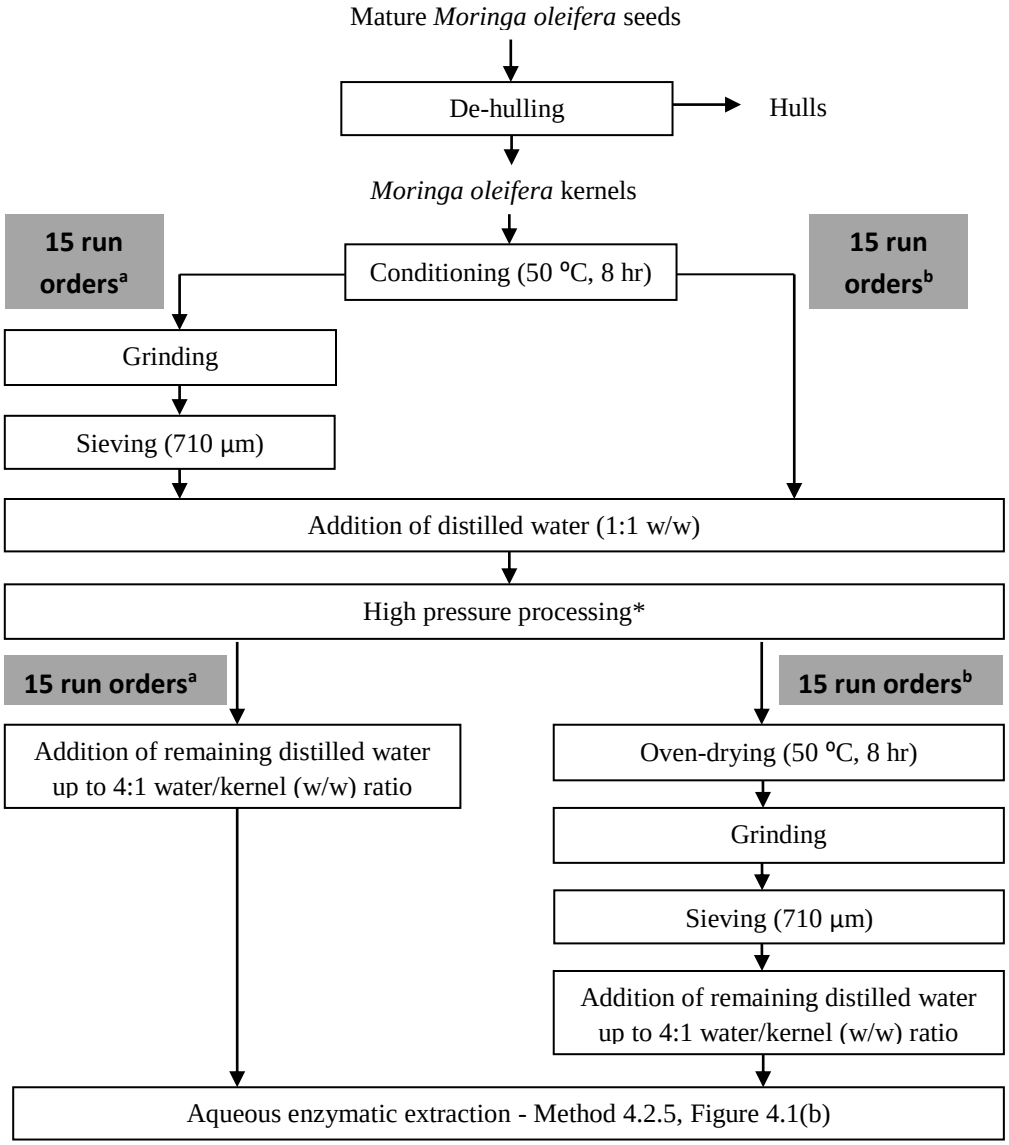
Table 4.1 High pressure processing (HPP) parameters as obtained from Box-Behnken design (MINITAB^{TM} Statistical Software: MINITAB Release 14.12.0, New York, USA), based on the pre-determined range of each parameter

Run order	Pressure (50-450 MPa)	Temperature (20-60 °C)	Time (10-60 min)
1	250	20	60
2	250	40	35
3	250	60	10
4	450	20	35
5	450	40	10
6	450	60	35
7	250	40	35
8	50	40	60
9	50	40	10
10	50	20	35
11	250	40	35
12	250	60	60
13	450	40	60
14	250	20	10
15	50	60	35

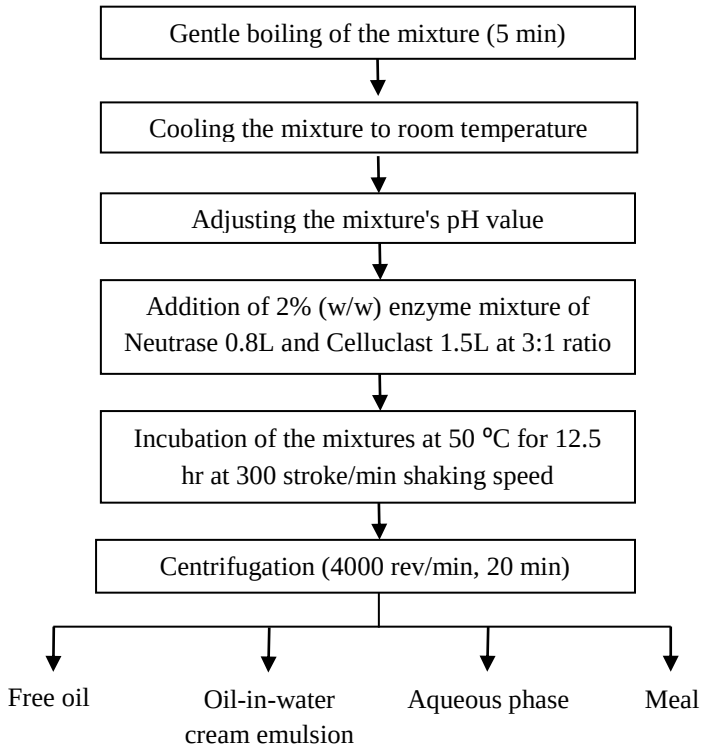
4.2.4 Preparation of *Moringa oleifera* kernels for oil extraction methods

The process flow for MO sample preparation, HPP, and the AEE methods are illustrated in Figure 4.1. With reference to Chapter 3, ground MO kernels of less than 710 μm particle size resulted in highest hexane-extracted oil yield of 41.03% (g oil / g kernel), thus samples having this particle size range were used as the starting material in this study. For comparison purpose, HPP was also conducted on the MO kernels which were not ground, termed as whole kernels.

A mixture of MO sample and distilled water (1:1 ratio) was transferred into a polyester bag and vacuum-sealed in order to minimize the headspace. The prepared sample was further treated according to the parameters displayed in Table 4.1 by using a Food-Lab 900 High-Pressure Food Processor (Stansted Fluid Powder Ltd., Stansted, UK) with a mixture of 1,2-propanediol and water (70:30 v/v) as the pressure transmitting fluid.


Mature *Moringa oleifera* seeds
De-hulling
Hulls
Moringa oleifera kernels
15 run orders[a]
Conditioning (50 °C, 8 hr)
15 run orders[b]
Grinding
Sieving (710 μm)
Addition of distilled water (1:1 w/w)
High pressure processing*
15 run orders[a]
15 run orders[b]
Addition of remaining distilled water up to 4:1 water/kernel (w/w) ratio
Oven-drying (50 °C, 8 hr)
Grinding
Sieving (710 μm)
Addition of remaining distilled water up to 4:1 water/kernel (w/w) ratio
Aqueous enzymatic extraction - Method 4.2.5, Figure 4.1(b)

(a)



(b)

Figure 4.1 The process flow of preparation of *Moringa oleifera* kernels for (a) high pressure processing (HPP) method followed by (b) aqueous enzymatic extraction (AEE). *High pressure processing, the 15 different parameters (Table 4.1) conducted on ground-sieved[a] and whole[b] kernels.

4.2.5 Aqueous enzymatic extraction and determination of free oil yield and recovery

The AEE method carried out after the HPP pre-treatment is displayed in Figure 4.1(b), and the parameters used were in reference to Run 39 (Chapter 3). In the AEE process, centrifugation of the incubated mixture resulted in four distinct layers of free oil at the top, followed by a cream emulsion layer, an aqueous phase, and meal at the bottom. After storage for 24 hr at -20 °C, the solidified free oil was separated into a crucible of pre-determined weight. The oil was heated in a drying oven for 15 min at 60 °C before been transferred into a desiccant containing silica gel for approximately 10 min. These steps were conducted to ensure complete removal of aqueous phase that may still present in the separated free oil. The amount of extracted oil was measured as follows:

$$\text{Free oil yield (\%)} = \frac{[\text{Mass of crucible containing the oil (g)} - \text{Mass of crucible (g)}] \times 100}{\text{Mass of kernels initially taken (g)}}$$

$$\text{Free oil recovery (\%)} = \frac{\text{Mass of oil extracted from a given mass of kernel (g)} \times 100}{\text{Mass of oil contained in the kernels initially taken (g)}}$$

4.3 Results and discussion

4.3.1 Effect of high pressure pre-treatment prior to aqueous enzymatic extraction of *Moringa oleifera* oil from ground-sieved and whole kernels

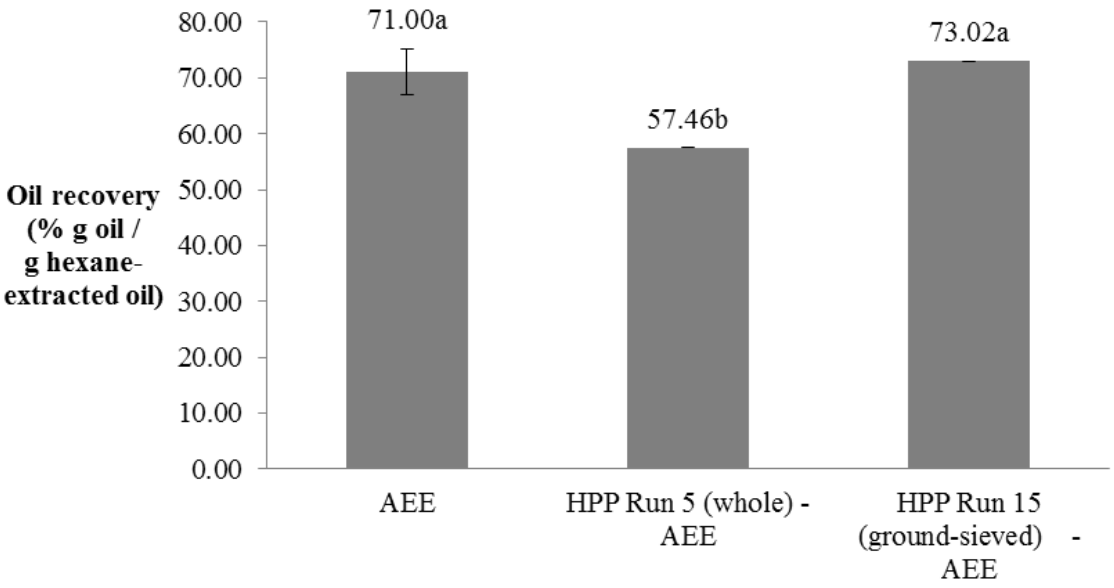
Table 4.2 High pressure processing (HPP) parameters as obtained from Box-Behnken design (MINITAB^{TM} Statistical Software: MINITAB Release 14.12.0, New York, USA), based on the pre-determined range of each parameter. Each HPP run order is carried out on whole and ground-sieved *Moringa oleifera* kernels, followed by an aqueous enzymatic extraction method (4:1 water/kernel (w/w) ratio at pH 6.0, incubated at 50 °C for 12.5 hr at 300 stroke/min shaking speed)

Run order	Pressure (50-450 MPa)	Temperature (20-60 °C)	Time (10-60 min)	Oil recovery (% g oil / g hexane-extracted oil)	
				Whole kernels	Ground-sieved kernels
1	250	20	60	41.47	54.09
2	250	40	35	45.62	57.33
3	250	60	10	55.68	64.35
4	450	20	35	51.53	47.23
5	450	40	10	57.46	58.43
6	450	60	35	46.19	66.15
7	250	40	35	47.60	57.33
8	50	40	60	52.59	68.34
9	50	40	10	51.11	58.68
10	50	20	35	47.17	50.04
11	250	40	35	49.91	57.49
12	250	60	60	52.51	64.95
13	450	40	60	46.03	70.01
14	250	20	10	50.29	50.17
15	50	60	35	54.12	73.02

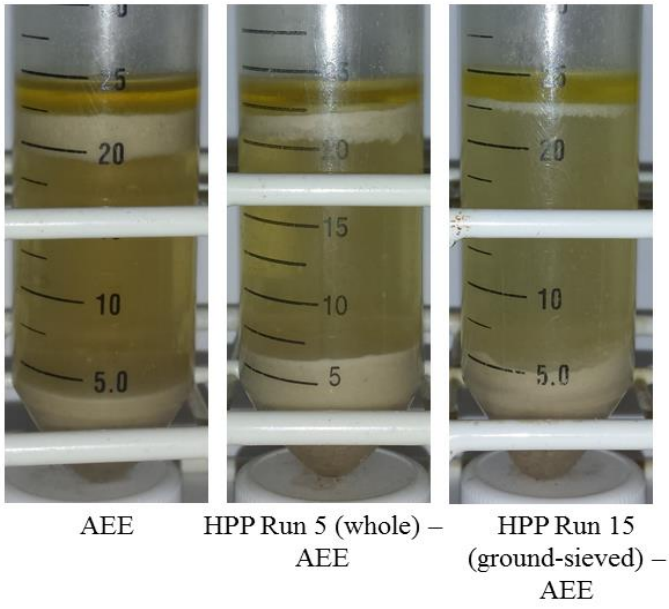
With reference to Figure 4.2 (a-b), without the HPP pre-treatment, the AEE alone resulted in 71.00±4.04% (w/w) oil recovery with thick emulsion layer. Table 4.2 displays the MO oil recoveries from AEE where the ground-sieved and whole kernels are pre-treated with high pressures. The highest oil recoveries were observed in the cases of Run 15 (73.02% w/w) and Run 5 (57.46% w/w) for ground-sieved and whole kernels, respectively. In the case of AEE process, theoretically, the size reduction of starting materials should result in greater cell wall disruption which leads to lower diffusion path for enzymes and the cellular components, therefore resulting in higher oil yields (Mat Yusoff et al., 2015). This theory is shown to be applicable in the case of HPP processed kernels as well, where a greater amount of oil was recovered from ground-sieved kernels as compared to the whole kernels for all run orders, except runs 4 and 14. It is possible that the grinding effect of the kernels may have disrupted the protein components and increased its susceptibility to the actions of pressure, heat, and enzymes during the process. Moreover, all AEE with HPP pre-treatments on ground-sieved kernels resulted in thinner cream emulsion layers as compared to the use of whole kernels and the use of AEE alone. These observations indicated the potential of HPP within the ranges of parameters been used in altering the MO protein structure into a form of less emulsifying functional properties, thus limiting the released oil from getting emulsified.

With reference to Table 4.2, the centre points generated in the experimental design were Run 2, 7, and 11 at pressurization pressure, temperature, and time of 250 MPa, 40 °C, and 35 min, respectively. Conducting these run orders resulted in smaller variation in the oil recoveries from ground-sieved kernels (57.33-57.49 w/w) as compared to the whole kernels (45.62-49.91 w/w). This finding indicated that the ground-sieved kernels exhibited

smaller variation in their physicochemical characteristics as compared to the whole kernels, thus assisted in minimizing the variation in their oil recoveries and ensure its repeatability.



(a)



(b)

Figure 4.2 The *Moringa oleifera* (a) oil recovery and (b) nature of cream emulsion formed from aqueous enzymatic extraction (AEE) method, and from AEE with high pressure processing (HPP) pre-treatment on both whole and ground-sieved kernels. Oil recoveries followed by the different letters are significantly different ($P < 0.05$).

4.3.2 Optimization of high pressure processing parameters for highest *Moringa oleifera* oil recovery

Optimization was carried out for both types of starting materials to obtain the combination of HPP parameters which may result in highest MO oil recovery. In the case of ground-sieved kernels, both, time and temperature had significant effects ($P < 0.05$) on the oil recovery, but not the pressure applied ($P > 0.05$). The linear relationship between the MO oil recovery (Y) and the significant HPP parameters ($P < 0.05$) - time (X_1) and temperature (X_2) - is given by: $Y = 0.1287X_1 + 0.4183X_2$ with R^2 =0.77. The validity of the R^2 value was confirmed from the regression p-value obtained which was less than 0.05. This p-value indicated that the R^2 value is not meaningless or not equal to zero. Therefore, statistically, the equation can be used to estimate the changes in MO oil recovery as a function of high pressure application time and temperature (Hair et al., 1998). The linear relationship clearly indicates that the extracted oil increased with increase in both the time and temperature in the range of parameters studied. This relationship is also graphically shown in Figure 4.3, and the use of HPP for 60 min at 60 °C is predicted to result in oil recovery of 71.43% (w/w), regardless of the pressure applied. Additionally, there was no interaction effect ($P > 0.05$) between the HPP parameters used. In contrast to ground-sieved kernels, none of the HPP parameters significantly influenced oil recovery ($P > 0.05$) from whole kernels. Therefore, further experiments in this study focused on the ground-sieved kernels as the starting material.

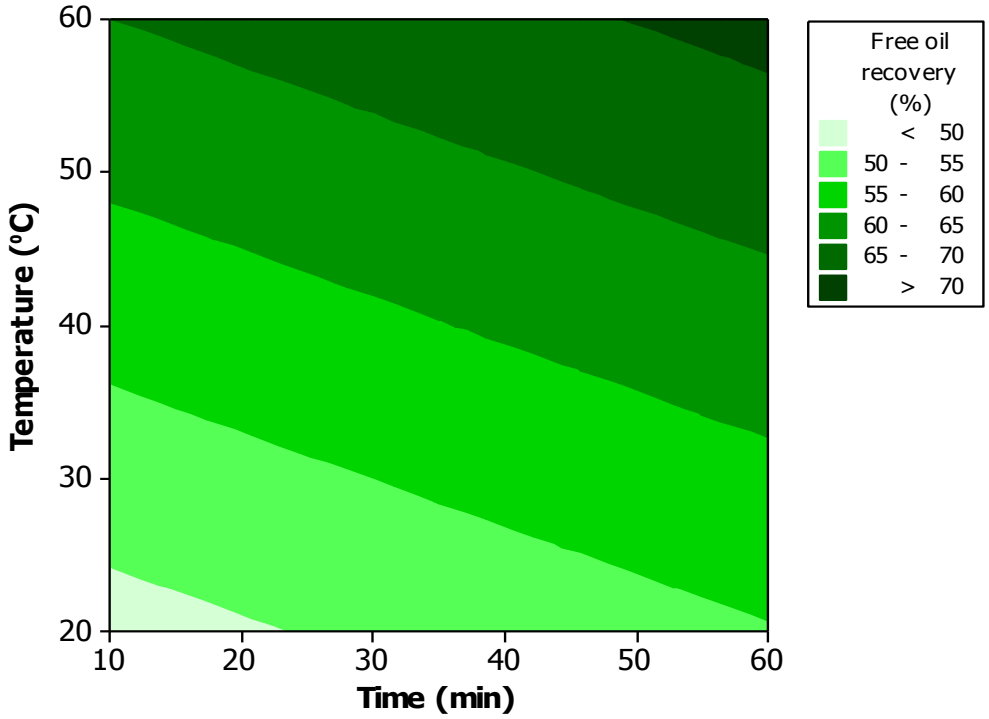


Figure 4.3 The linear relationship between the high pressure processing (HPP) temperature and time as a pre-treatment on ground-sieved *Moringa oleifera* kernels prior to aqueous enzymatic extraction on the oil recoveries (% g oil / g hexane-extracted oil), generated by Minitab® 14.12.0 Statistical Software: MINITAB Release 14.12.0, New York, USA.

Based on the experiments conducted with ground-sieved kernels (Table 4.2), Run 15 at 50 MPa (60 °C, 35 min) resulted in higher oil recovery of 73.02% (w/w) as compared to Run 6 at higher pressure of 450 MPa (60 °C, 35 min) which yielded 66.15% (w/w) oil. Run 15 also yielded highest oil recovery than that of other run orders. Therefore, an additional experiment was carried out at similar pressure of 50 MPa in combination with the optimized parameters (60 min, 60 °C), and this resulted in significantly lower ($P < 0.05$) oil recovery of 65.91±1.76% (w/w) as compared to Run 15, despite the longer pressurization time. This oil recovery was also significantly lower ($P < 0.05$) than the value of 71.43% (w/w) predicted in the experimental design. According to Anglemier and Montgomery (1976), denaturation of native protein occurs under heat or pressure, but there is also a possibility for the denaturation to be reversible when the proteins are smaller and/or the denaturing conditions are milder. On the other hand, there is also a possibility for the protein to aggregate after applying high pressures for a given time, which can re-emulsify the oil released and therefore decrease the free oil recovery. Therefore, due to significantly higher ($P < 0.05$) oil recovery at shorter pressurization time of 35 min, Run 15 was more acceptable as compared to the optimized HPP parameters and was further been used in this study.

4.3.3 Effect of boiling and high pressure pre-treatments prior to aqueous enzymatic extraction of *Moringa oleifera* oil

In section 4.3.1-4.3.2, it was revealed that the HPP pre-treatment on ground-sieved MO kernels prior to the AEE did not significantly enhance the MO oil recovery, yet thinner cream emulsion layer was formed as compared to the AEE alone. This finding indicates the potential of HPP in altering the MO protein structure into a form which exhibits less emulsifying functional properties, thus minimizing the amount of cream emulsion formed and further reducing the load of de-emulsification step. Despite this advantage, with reference to Figure 4.1(b), the AEE itself was preceded with a gentle boiling step on the water/kernel mixture for 5 min prior to adjusting the mixture's pH. Adding an HPP pre-treatment prior to the gentle boiling step may not be of great advantage particularly in terms of the overall processing cost. Therefore, additional experiments were carried out to observe the effect of replacing the gentle boiling step with the HPP pre-treatment. For this purpose, the boiling step was termed as boiling pre-treatment, and the AEE itself was carried out without the gentle boiling step and termed as AEE* to avoid confusion. These new experimental set ups are summarized in Figure 4.4 for better understanding. In the following experiments, the AEE* with boiling pre-treatment is termed as B-AEE*, while the AEE* with HPP pre-treatment is termed as HPP-AEE*. All experiments were performed in triplicate.

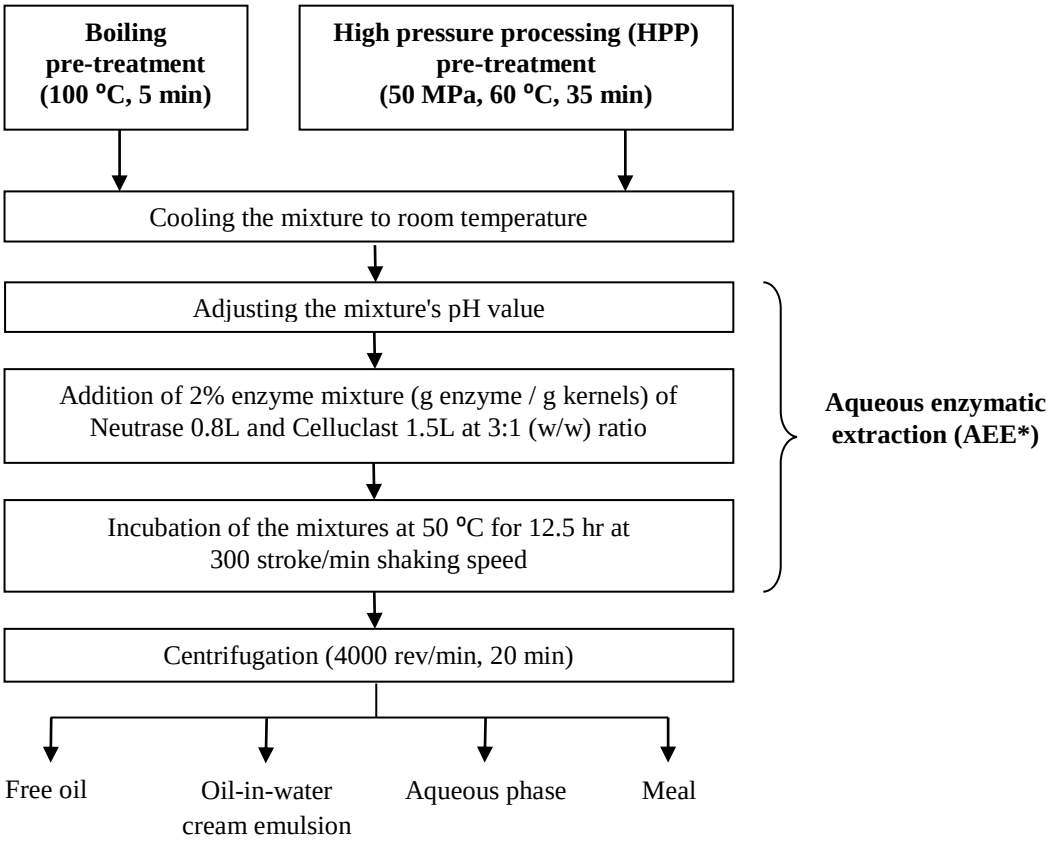
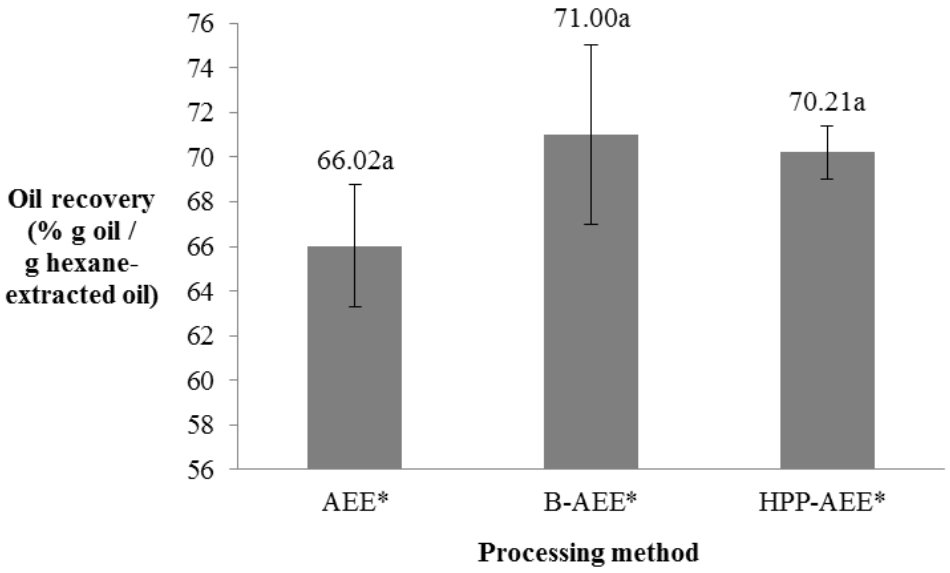


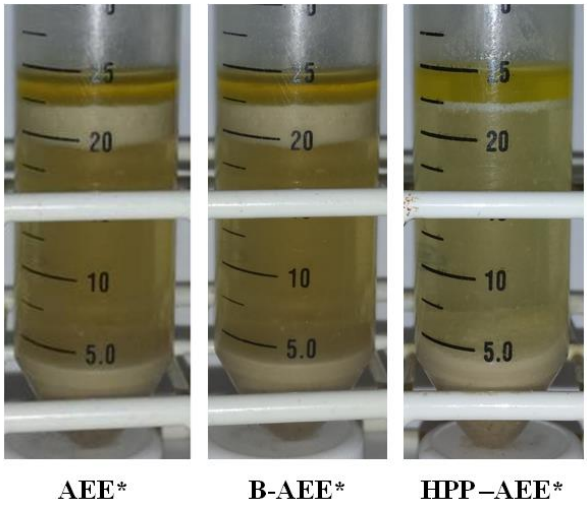
Figure 4.4 The new experimental procedure to determine the effect of boiling and high pressure processing pre-treatments prior to the aqueous enzymatic extraction (AEE*) method. The AEE* with boiling pre-treatment is termed as B-AEE*, while the AEE* with high pressure processing pre-treatment is termed as HPP-AEE*.

With reference to Figure 4.5, there was no significant difference ($P > 0.05$) between the oil recoveries from AEE*, B-AEE*, and HPP-AEE* methods, yet the HPP-AEE* resulted in thinner cream emulsion layer as compared to the other processes. Despite the insignificant difference in the oil recoveries, the B-AEE* resulted in 4.98% higher oil recovery than the AEE* alone. It was assumed that higher amount of oil was released from the MO kernels, yet certain amount of the released oil was emulsified into thick cream emulsion layer. On the other hand, HPP-AEE* resulted in 4.19% higher oil recovery than that of AEE* alone. The higher oil recovery may be due to the effect of HPP in altering the MO protein structure into a form of less emulsifying ability, thus preventing the released oil from getting emulsified and resulted in thin cream emulsion layer. Similar finding was reported in the case of *Vicia faba*, where its 11S proteins exhibited poor emulsifying properties upon HPP treatment (Galazka et al., 2000). However, Chapleau and de Lamballerie-Anton (2003a) reported contrast result, where the emulsifying properties of lupin proteins improved upon HPP at 400 MPa.

These findings proved that combination of different running time, temperature, and pressure in the HPP resulted in different oil recoveries and cream emulsions which is very much dependent on changes in the protein structure. Similar types of protein molecules display varying structural differences or microstates upon exposure to the same denaturation treatment. Additionally, native proteins require very high activation energy for denaturation to take place (Anglemier and Montgomery, 1976). These changes in the protein structure may increase its susceptibility for enzymatic hydrolysis, or may increase its efficiency as an emulsifier. Both of these situations may also occur simultaneously, or vice versa.



(a)



(b)

Figure 4.5 The *Moringa oleifera* (a) oil recovery and (b) nature of cream emulsion formed from aqueous enzymatic extraction (AEE*) method, and from AEE* with boiling (B-AEE*) and high pressure processing (HPP-AEE*) pre-treatment, with ground-sieved kernels as starting material. Oil recoveries followed by the same letter are not significantly different ($P > 0.05$).

4.3.4 Potential of high pressure process scale-up

Experiments on process and product development always start at laboratory scale. From an industrial viewpoint, when the process is scaled up to production-scale in later stages, similarity between different scales is required for efficient process development. At the very least, it is significant to understand the fundamental differences of different scales (Håkansson, 2017).

Section 2.4 discussed on the advantages and application of HPP in food industry which led to recent studies on the process scale-up. The use of HPP as a pre-treatment prior to an AEE process is an ongoing study, thus as far as the authors' concern, no studies have reported on the HPP scale-up for this particular purpose. Sevenich et al. (2016) conducted trials of HPP scale-up for inactivation of spores (*B. amyloliquefaciens*) on tested food systems of tuna in brine, tuna in sunflower oil, sardine in olive oil, and vegetable baby food puree. The machine used in this study was capable of combining high temperature (up to 121 °C) and high pressure (600 MPa, come-up time about 3 min). It was specially designed in the year 2009 to develop high pressure thermal sterilization at the industrial level. This study revealed that 7 out of 9 calculated high pressure conditions were successful, and further suggested that it is possible to go from lab scale into a pilot scale system with economic feasible temperature in combination with pressurization time of less or equal to 10 min.

On the other hand, Håkansson (2017) reported contrast finding upon the use of high pressure homogenizers for food emulsification. It was revealed that the functionality of this equipment is greatly dependent on the scales used – different scales performed principally

different emulsification processes. Therefore, its functionality at different scales are not readily be translated.

Process scale-up is not performed in this particular study. However, with reference to the earlier studies above, it can be concluded that the success of the high pressure process scale-up depends on the application of the machine itself. This varies from inactivation of microorganisms, enzymes and spores, to food emulsification, food homogenization, and destruction or preservation of cells' microstructure, among others. In this study, the end product of the HPP pre-treatment, i.e. the mixture of sample and water, can directly be used in the following AEE process which also utilizes a mixture of sample and water as a starting material. Therefore, the process scale-up can potentially be carried out on both the HPP pre-treatment and the following AEE process simultaneously. The process scale-up may assist in cost estimation and production of by-products in comparison with conventional extraction process.

4.4 Conclusions

The effect of HPP as a pre-treatment prior to AEE was more pronounced on the starting materials with low particle size, i.e. the ground-sieved kernels, and the emulsion layers formed after the AEE were thinner as compared to the use of AEE alone. Highest oil was recovered in Run 15 (73.02% w/w) and Run 5 (57.46% w/w) for the ground-sieved kernels and whole kernels, respectively. The optimization of the HPP parameters revealed that the MO oil recovery increased significantly ($P < 0.05$) with increased in both the temperature and time, but not the pressure level. Run 15 (50 MPa, 60 °C, 35 min) resulted in significantly higher ($P < 0.05$) oil recovery (73% (w/w)) than the optimized HPP

parameters (50 MPa, 60 °C, 60 min, 65% w/w) due to possibility of reversible denaturation or aggregation of protein upon longer pressurization time. Additional experiments were conducted on the mixture of water/kernel samples treated with AEE without the pre-boiling step (termed as AEE*), with the pre-boiling step (termed as B-AEE*), and with the HPP pre-treatment (HPP-AEE*). Both the B-AEE* and HPP-AEE* resulted in 4.98% and 4.19% higher oil recoveries than that of AEE* alone, respectively, yet the differences were statistically insignificant ($P > 0.05$), and the HPP-AEE* resulted in thinner cream emulsion layer than that of other processes. It was assumed that higher oil was released in the case of B-AEE*, yet part of the released oil was emulsified. In the case of HPP-AEE*, the higher oil recovery may be due to the effect of HPP in altering the MO protein structure into a form of less emulsifying ability, thus preventing the released oil from getting emulsified. The load of de-emulsification method afterwards can therefore be minimized, or may not be necessary at all. Despite these findings, the oil recoveries were still lower than that of solvent extraction method. Further studies are recommended on the quality attributes of MO oils from solvent and aqueous enzymatic extraction methods with different pre-treatments.

CHAPTER 5

OXIDATIVE PROPERTIES OF *Moringa oleifera* OIL FROM DIFFERENT EXTRACTION METHODS DURING STORAGE

5.1 Introduction

Chapter 4 discussed the effect of different high pressure processing (HPP) parameters of [pressure (50-450 MPa), temperature (20-60 °C), and time (10-60 min)] as a pre-treatment prior to aqueous enzymatic extraction (AEE) of *Moringa oleifera* (MO) oil. Within these HPP parameter ranges tested, higher oil recoveries in the clear layer and the formation of thinner cream emulsion layers were observed when ground-sieved MO kernels were used as the starting material as compared to whole (unground) MO kernels. The optimization of the HPP parameters revealed that the MO oil recovery increased significantly with increase in both the temperature and time, but not the pressure level. Run 15 (Table 4.2) at 50 MPa (60 °C, 35 min) resulted in 73% (w/w) oil recovery which was greater than 66.15% (w/w) oil from Run 6 at higher pressure of 450 MPa (60 °C, 35 min), and also greater than 65.91% (w/w) oil from the optimized HPP parameters at 50 MPa (60 °C, 60 min); the latter may be due to possibility of reversible denaturation or aggregation of protein upon longer pressurization time. Moreover, despite the insignificant difference between its oil recovery (73% w/w) with that of AEE alone (71% w/w), thinner cream emulsion layer was observed which is advantageous in reducing and simplifying downstream separation of oil. Therefore, HPP of Run 15 was selected for further experiments relating to oil quality.

As mentioned earlier in Chapter 4, AEE is normally preceded with a gentle boiling step in order to inactivate the natural hydrolytic enzymes on the seed kernels (Zhang et al., 2007), thus it was also part of the AEE method used in this study (Figure 4.1(b)). However, adding an HPP pre-treatment prior to the gentle boiling step may not be of great advantage particularly in terms of the overall processing cost. Therefore, with reference to Figure 4.4, additional AEE experiments were carried out to indicate the effect of replacing the gentle boiling step with the HPP (Run 15). To avoid confusion, the AEE without the gentle boiling step is termed as AEE*, while the process employing the boiling step is termed as boiling pre-treatment. The AEE* with the boiling pre-treatment (termed as B-AEE*) and with the HPP pre-treatment (termed as HPP-AEE*) resulted in 4.98% and 4.19% increase in oil recoveries than that of AEE* alone, respectively. It was assumed that higher oil was released in the case of B-AEE*, yet part of the released oil was emulsified into thick cream emulsion layer. In the case of HPP-AEE*, the higher oil recovery may be due to the effect of HPP in altering the MO protein structure into a form of less emulsifying ability, thus preventing the released oil from getting emulsified and resulted in thin cream emulsion layer. Despite these findings, the oil recoveries were still lower than that obtained by solvent extraction method.

The main objective of this chapter is to further explore the differences, particularly in oxidative properties of MO oil from different extraction methods - the solvent extraction (SE), aqueous enzymatic extraction (AEE*), AEE* with boiling pre-treatment (B-AEE*), and AEE* with high pressure processing pre-treatment (HPP-AEE*). The MO oils were stored for 140 days at different temperatures of 13 °C, 25 °C, and 37 °C, and changes in the oxidative properties were evaluated during the storage period in terms of their peroxide

value (PV), *p*-Anisidine value (*p*-AV), total oxidation (TOTOX) value, free fatty acids (FFA), iodine value (IV), fatty acid composition (FAC), and tocopherol content.

5.2 Materials and methods

5.2.1 Materials

Mature MO seeds (PKM1 hybrid) were purchased from Genius Nature Herbs Pvt ltd., Coimbatore, India. All solvents and enzymes used in this project were obtained from Sigma-Aldrich Company Ltd., Dorset, UK.

5.2.2 Statistical analysis

All statistical analyses in this study were done by using Minitab® 14.12.0 Statistical Software. A one-way analysis of variance (ANOVA) with Tukey's multiple comparison test (confidence level 95.0%) was applied for the determination of significant differences between more than two samples (each sample with replicates data). A 2-Sample t-test was used to determine significant differences between two samples (replicates data for each sample), while a 1-Sample t-test was used when a sample (with replicates data) was statistically compared with another sample which has one datum only.

5.2.3 Preparation of *Moringa oleifera* kernels for oil extraction

The MO kernels were randomly collected and conditioned at 50 °C for 8 hr followed by grinding and sieving to ≤710 μm particle size. All oil extraction methods were based on Chapter 3 and Chapter 4 and were performed on the ground-sieved MO kernels.

5.2.4 Solvent extraction method (SE)

Soxhlet method was used to extract the MO oil with the use of hexane for 6 hr extraction time. The hexane was evaporated from the extracted oil in a round bottom flask of pre-determined weight by using a rotary evaporator (60 °C, 10 min), followed by heating in an oven (100 °C, 15 min). The difference between the initial (empty) and final (containing the extracted oil) weight of the round bottom flask used was measured as the oil yield in the meal by normalizing this against the weight of the kernels taken initially.

5.2.5 Aqueous enzymatic extraction method (AEE*)

A mixture of ground-sieved MO kernels and distilled water at 1:4 (w/w) ratio was prepared and adjusted to pH 6.0. A mixture of 2% (g enzyme / g kernel) of protease (Neutrase 0.8L, optimum pH 6.8) and cellulase (Celluclast 1.5L, optimum pH 4.8) enzymes at 3:1 (w/w) ratio was added into the mixture, followed by incubation at 50 °C for 12.5 hr at 300 stroke/min shaking speed. The incubated mixture was centrifuged at 4000 rev/min for 20 min which induced separation into four distinct layers of free oil at the top, followed by the cream emulsion layer, the aqueous phase, and the meal at the bottom. Recovery of the free oil is explained in section 5.2.8.

5.2.6 Aqueous enzymatic extraction with boiling pre-treatment (B-AEE*)

Similar AEE* as in section 5.2.5 was conducted, with addition of boiling pre-treatment (100 °C, 5 min), followed by cooling to room temperature prior to adjusting the mixture's pH. Recovery of the free oil is explained in section 5.2.8.

5.2.7 Aqueous enzymatic extraction with high pressure processing pre-treatment (HPP-AEE*)

The ground-sieved MO kernels were mixed with distilled water at 1:1 (w/w) ratio and vacuum sealed in polyethylene bags. The mixtures were treated with high pressure (Stansted Fluid Powder Ltd., Stansted, UK) at 50 MPa and 60 °C for 35 min, followed by addition of distilled water up to 4:1 water/kernel (w/w) ratio for the subsequent AEE* as in section 5.2.5. Recovery of the free oil is explained in section 5.2.8.

5.2.8 Recovery of free oil

The centrifuged mixtures obtained in sections 5.2.5-5.2.7 were kept at -20 °C for 24 h. The solidified oil was transferred to a crucible of pre-determined weight, and heated in an oven at 100 °C for 15 min to ensure complete removal of any aqueous phase that may present in the recovered oil. The crucible containing the oil was cooled to room temperature in a desiccant containing silica gel for approximately 10 min before been weighed. The free oil yield and recovery were calculated as follows:

$$\text{Oil yield (\%)} = \frac{[\text{Mass of crucible containing the oil (g)} - \text{Mass of crucible (g)}] \times 100}{\text{Mass of kernels initially taken (g)}}$$

$$\text{Free oil recovery (\%)} = \frac{\text{Mass of oil extracted from a given mass of kernel (g)} \times 100}{\text{Mass of oil contained in the kernels initially taken (g)}}$$

5.2.9 Storage of *Moringa oleifera* oil

MO oil samples from sections 5.2.4-5.2.7 were filled in transparent glass bottles with screw-caps, up to the bottle's neck in order to minimize the headspace. The bottles were wrapped in aluminium foil and stored in dark to avoid light exposure. In dark condition, Pristouri et al. (2010) reported greater oxidative stability of extra virgin olive oil samples stored in glass containers as compared to other packaging materials including polyethylene terephthalate (PET) and polypropylene (PP). The storage temperatures used in this study were also in reference to Pristouri et al. (2010). At 13 °C, the MO oil was stored to simulate the temperature of the cellar commercially used for storing olive oil. Storage at 25 °C and 37 °C were selected for simulating room temperature and elevated ambient temperature normally occurred during the summer, respectively. All oil samples from different extraction methods were stored in these temperatures for 140 days, and the analysis of their PV, *p*-AV, TOTOX, FFA, and tocopherol content were performed on day 0, day 60, day 120, and day 140. Additionally, differences in the IV and FAC between the extracted oils before (i.e. day 0) and after the whole storage period (i.e. day 140) were also examined. Determination of these oxidative properties was performed on each oil sample in triplicate.

5.2.10 Determination of peroxide value (PV)

Peroxide value (PV) of the oil samples was determined in reference to AOCS Official Method Cd 8-53 (2000) and MPOB Test Method p2.3 (2004) with modification. A mixture of 5.0 g oil sample and 20 ml glacial acetic acid/chloroform (1.5:1 v/v) was prepared and swirled until completely dissolved. Excess of saturated potassium iodide (KI) solution was added to the mixture, followed by swirling for 1 min. The mixture was combined with 30 ml distilled water and few drops of starch indicator, before being titrated with 0.01 N sodium thiosulphate until the blue-gray color disappeared. The above steps were repeated without adding the oil sample for blank purpose. The following formula was used to calculate the PV of the oil sample:

$$\text{Peroxide value } \left(\text{mEq}\frac{O_2}{\text{kg}}\right) = \frac{(\text{Vb} - \text{Vs}) \text{ x } 0.01 \text{ x } 1000}{\text{W}}$$

Vb = Titre for blank (ml)

Vs = Titre for sample (ml)

W = Weight of sample (g)

0.01 = Normality of titrant (N)

1000 = Unit conversion (g/kg)

5.2.11 Determination of *p*-Anisidine value (*p*-AV)

p-Anisidine (*p*-AV) value of oil samples was determined according to Diana Moigradean et al. (2012) which was in reference to AOCS Official Method Cd18-90 (2000). An oil sample of 0.5 g was weighed into a 25 ml volumetric flask and topped up with isooctane. The absorbance of the oil-isooctane solution (A_1) was determined at 350 nm against isooctane (blank 1) (Cecil CE 1021 UV/Visible Spectrophotometer 1000 series). 5 ml of the oil-isooctane solution was transferred into a 10 ml glass bottle (with screw cap), added with 1 ml of anisidine reagent (0.25% w/v anisidine reagent in glacial acetic acid), shook vigorously, and kept in dark for 10 min. Similarly, 5 ml of isooctane in a glass bottle was also added with 1 ml anisidine reagent, shook vigorously, and kept in dark for 10 min (blank 2). The absorbance of the oil-isooctane containing anidisine reagent (A_2) was determined at 350 nm against blank 2. The *p*-AV was calculated as follow:

$$p - \text{Anisidine value} = \frac{25 \text{ x } (1.2A_2 - A_1)}{W}$$

A_1 = Absorbance of the oil-isooctane solution

A_2 = Absorbance of the oil-isooctane containing anidisine reagent

25 = Volume of which the oil sample is dissolved with isooctane (ml)

1.2 = The correction factor for the dilution of the test solution with 1 ml of the anisidine reagent or glacial acetic acid

W = Weight of sample taken (g)

5.2.12 Determination of total oxidation (TOTOX) value

Total oxidation (TOTOX) value of the oil samples was determined according to Diana Moigradean et al. (2012) which was based on AOCS Official Method Cg 3-91 (2000). This value takes into account both the PV and *p*-AV of the oil sample and calculated according to the following formula: TOTOX value = 2PV + *p*-AV

5.2.13 Determination of free fatty acids (FFA)

Free fatty acids (FFA) of the oil samples was calculated from its acid value (AV) which was determined in accord to Ogbunugafor et al. (2011) with reference to AOCS Official Method Cd 3d-63 (2000). An oil sample of 0.5 g was added to 50 ml of a mixture of diethyl ether and ethanol (95% v/v) in a 250 ml conical flask. Phenolphthalein was added as an indicator, followed by titration on the whole mixture with 0.1 N potassium hydroxide (KOH) solution. The whole steps were repeated without adding the oil sample for blank purpose. The AV of the oil was calculated as follows:

$$\text{Acid value (KOH g}^{-1}) = \frac{(\text{Vb} - \text{Vs}) \text{ x } 5.61}{\text{W}}$$

Vb = Titre for blank (ml)

Vs = Titre for sample (ml)

W = Weight of sample (g)

5.61 = Mass (mg) of KOH in 1 ml of 0.1 N solution

Free fatty acids, FFA (%) = Acid value/1.99; where 1.99 is the conversion factor for oleic acid

5.2.14 Determination of iodine value (IV)

Iodine value (IV) of the oil samples was determined according to MPOB Test Method p3:2 (2004) and AOCS Official Method Cd 1d-92 (2000) with slight modification. Earlier studies revealed IV of MO oil which ranged from 60-70 I_2 / 100 g (Latif et al., 2011; Rahman et al., 2009; Tsaknis et al., 1999). Thus an oil sample of 0.2 g was used. The oil was weighed into a conical flask and added with 20 ml chloroform and 25 ml Wijs reagent. A stopper was placed followed by vigorous shaking, and the mixture was kept in dark for 30 min. Following this step was addition of 20 ml KI solution (15% w/v KI in distilled water) and 100 ml distilled water. The mixture was titrated under vigorous shaking with 0.1 M sodium thiosulphate until the yellow colour due to iodine has almost disappeared. Few drops of starch was added afterwards, and the titration was continued until the blue colour just disappeared after very vigorous shaking. The whole steps were repeated without adding the oil sample for blank purpose. The IV was calculated based on the following formula:

$$\text{Iodine value } \left(\text{g } \frac{I_2}{100 \text{ g}}\right) = \frac{0.1269 \text{ x } 0.1 \text{ x } (Vb - Vs) \text{x } 100}{W}$$

Vb = Titre for blank (ml)

Vs = Titre for sample (ml)

W = Weight of sample (g)

0.1 = Normality of titrant (N)

0.1269 = Mass of iodine in 1 ml of 1 M solution

5.2.15 Determination of fatty acid composition (FAC)

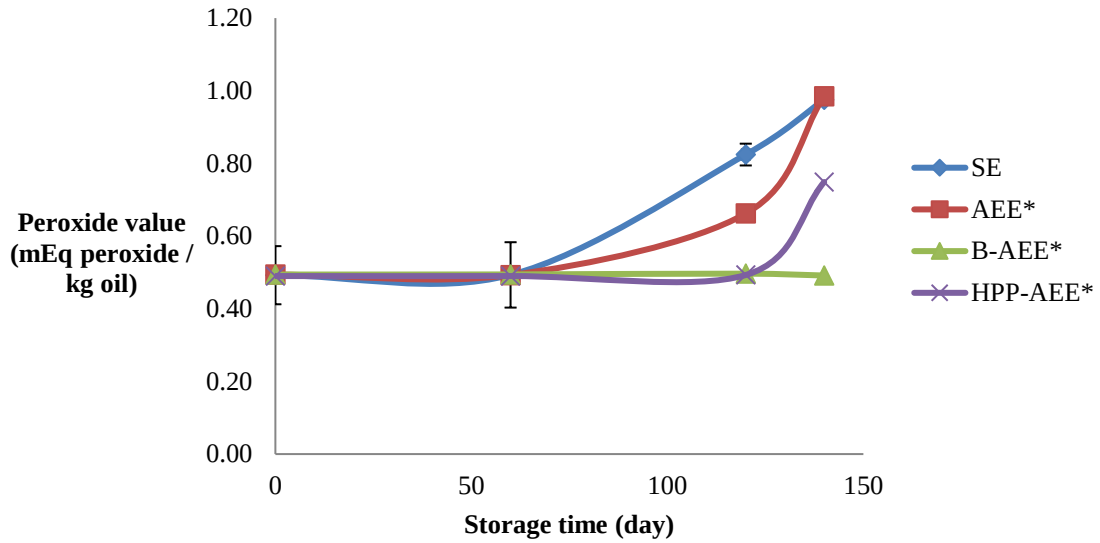
Fatty acid composition (FAC) of MO oil was determined according to Ezeh et al. (2016), Agilent Technologies, and *Trace*CERT® (Supelco®) with slight modification. Gas Chromatography (GC, Agilent HP 6890) fitted with flame ionization detector (FID) was used for the analysis, with fused silica capillary column Varian CP-Sil 88 (60 m x 0.25 mm x 0.20 μm) and helium as a carrier gas (flowrate 1.0 ml/min). The oil sample was first converted into fatty acids methyl esters (FAME) by dissolving 100 mg oil into 10 ml hexane and added with 100 μl of 2N KOH in methanol (i.e. 11.2 g KOH in 100 ml methanol). The mixture was vortexed for 30 s, centrifuged, and the clear supernatant at the upper layer was transferred into an autosampler vial. The injector and detection temperatures were 250 °C and 260 °C, respectively, while the oven temperature was 230 °C (hold 30 min). The volume of sample injected was 1 μl with split ratio of 100:1. The standard reference used was the Supelco 37 Component FAME Mix (1x1ml at varied concentrations in dichloromethane). Identification of the fatty acids was done by comparing retention times with those of standards.

5.2.16 Determination of tocopherol

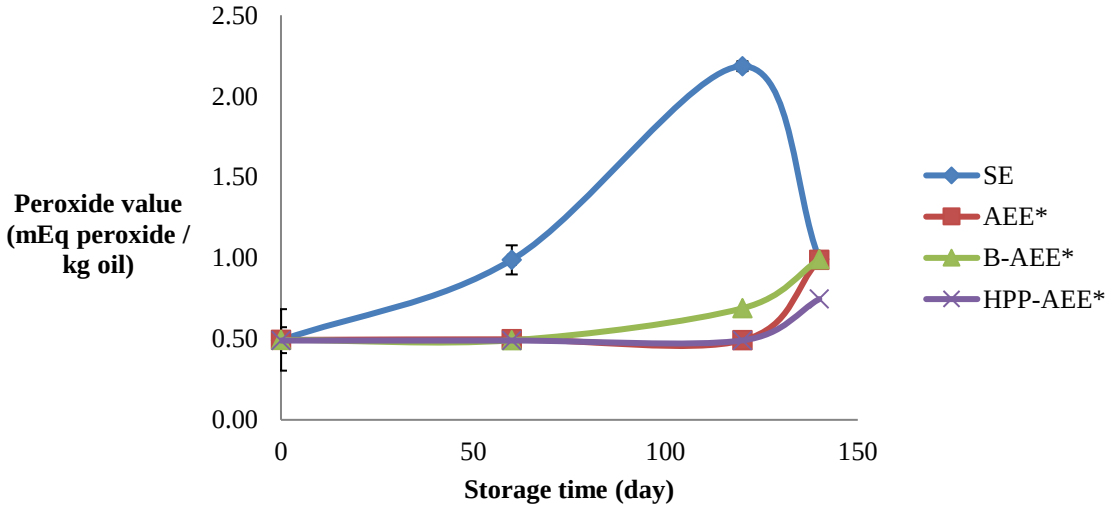
Following the method used by Ezeh et al. (2016) and Costa et al. (2010) with slight modification, the total tocopherols of the oil samples in this study was determined by HPLC-UV system (Agilent 1200, Manchester, UK). The column used was a Nucleosil C-18-100 reverse phase column (25 cm x 4.6 mm i.d.) with a particle size of 5 μm (Macherey-Nagel, Duren, Germany), while the mobile phase was a mixture of methanol:tetrahydrofuran:water (67:27:6 v/v/v) at flowrate of 0.8 ml min^{-1}. An oil sample of 0.1 ml was diluted with 1 ml of a mixture of isopropanol:chloroform (75:25 v/v). The mixture was homogenized and 10 μl was injected into the HPLC system at 25 °C and detection wavelength of 292 nm. The types of tocopherols reported are the α- and γ-tocopherols, and their standard solutions were prepared by dissolving in methanol at 0.02-1.0% (v/v) concentrations. Standard calibration curve was obtained for each type of tocopherol, and identification of the tocopherols in the oil samples was done by comparing their retention times with that of the standard solutions.

5.3 Results and discussion

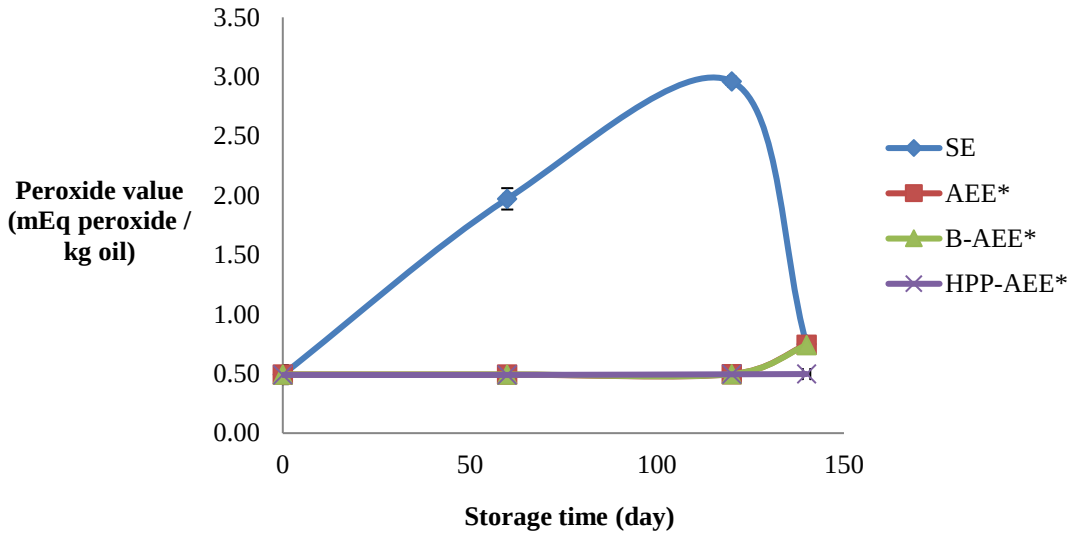
5.3.1 Effect of storage temperature on peroxide value of *Moringa oleifera* oil from different extraction methods



(a) storage temperature = 13 °C



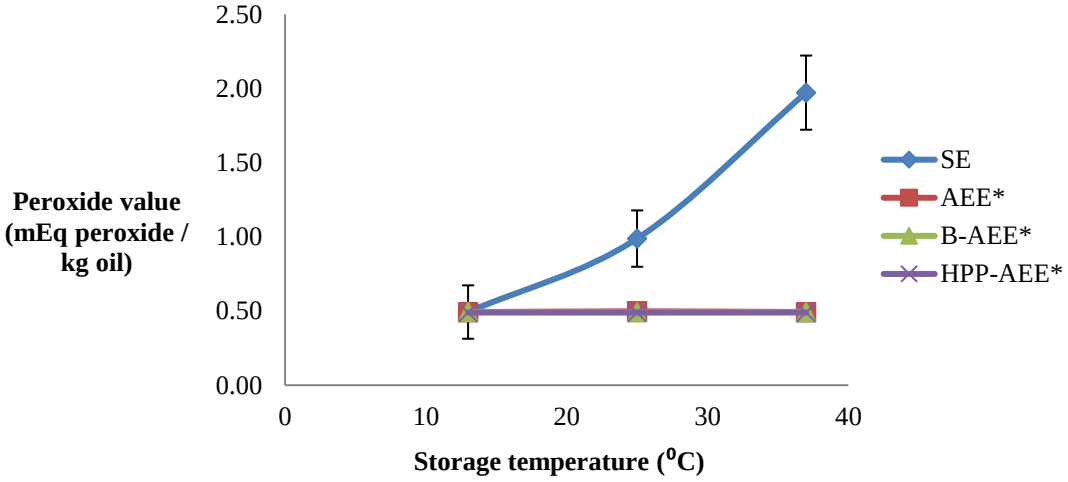
(b) storage temperature = 25 °C



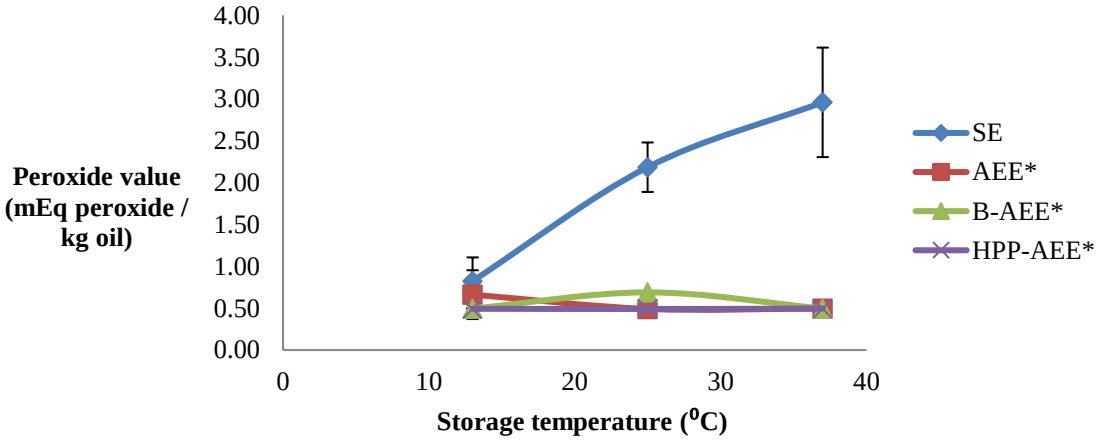
(c) storage temperature = 37 °C

Figure 5.1 Peroxide value of *Moringa oleifera* oil from different extraction methods stored at different temperatures of (a) 13 °C, (b) 25 °C, and (c) 37 °C, for different storage time of 0 day to 120 days. SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

5.3.2 Effect of storage time on peroxide value of *Moringa oleifera* oil from different extraction methods



(a) Day 60



(b) Day 120

Figure 5.2 Peroxide value of *Moringa oleifera* oil from different extraction methods stored for different storage time of (a) 60 days and (b) 120 days at different temperatures of 13 °C, 25 °C, and 37 °C. SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

5.3.3 Discussion on the effect of storage temperature and time on peroxide value of *Moringa oleifera* oil from different extraction methods

The PV indicates presence of primary oxidation products including peroxides and hydroperoxides which do not have a significant effect on the oil's flavor deterioration (deMan, 1999; Diana Moigradean et al., 2012). Higher increment rate of PV was observed in the oil from SE than that of enzymatic extraction methods. At 13 °C, Figure 5.1(a) shows increase of PV in oil samples from SE and AEE* which started after day 60. In the oil sample from HPP-AEE*, the PV started to increase after day 120, while the PV in the oil from B-AEE* remained unchanged up to day 140. Greater increase of PV in the oil from SE method as compared to other enzymatic extraction methods was observed at higher storage temperatures of 25 °C (Figure 5.1(b)) and 37 °C (Figure 5.1(c)) up to day 120, which decreased afterwards. The decrease in the PV indicated that the primary oxidation products have decomposed into secondary oxidation products.

Increase in PV was observed in oils from other enzymatic extraction methods especially on day 140, yet the values were still lower (0.49-0.99 mEq O_2/kg) than the oil from SE method. Earlier studies also reported higher PV of MO oil extracted using solvents (0.94-1.83 mEq O_2/kg) as compared to cold-pressed oil (0.11-0.36 mEq O_2/kg) (Tsaknis et al., 1999; Tsaknis and Lalas, 2002). According to O'Brien (2009) and Brink and van Duijn (2003), the quality of oil from SE method may be lower than that of pressing method due to simultaneous extraction of non-triglycerides and other undesirable minor components in the former case. Therefore in this study, the oil from SE method exhibited higher PV as compared to other extraction methods. The non-triglycerides and other minor components include fatty acids, phosphatides, sterols, tocopherols, hydrocarbons,

colorants, pigments, vitamins, sterol glucosides, protein fragments, glycolipids, traces of pesticides, trace metals, resinous, and mucilagenous materials (O'Brien, 2009; Weidermann 1981).

A product with PV of 1-5 mEq O_2/kg is categorized as exhibiting low oxidation rate, followed by PV of 5-10 mEq O_2/kg as moderate oxidation rate, while a product with PV of higher than 10 mEq O_2/kg is considered as having high oxidation rate (deMan, 1999; Diana Moigradean et al., 2012). Moreover, according to Codex (1999), the maximum PV for refined oil is up to 10 mEq O_2/kg, while for cold pressed and virgin oils, the maximum PV is up to 15 mEq O_2/kg. Despite the high PV in oil from SE method, all values were less than 3 mEq O_2/kg. Thus in terms of PV, the MO oil samples from SE and enzymatic extraction methods are categorized as oil samples with low oxidation rate within the storage conditions used.

5.3.4 Effect of storage temperature and time on *p*-Anisidine value of *Moringa oleifera* oil from different extraction methods

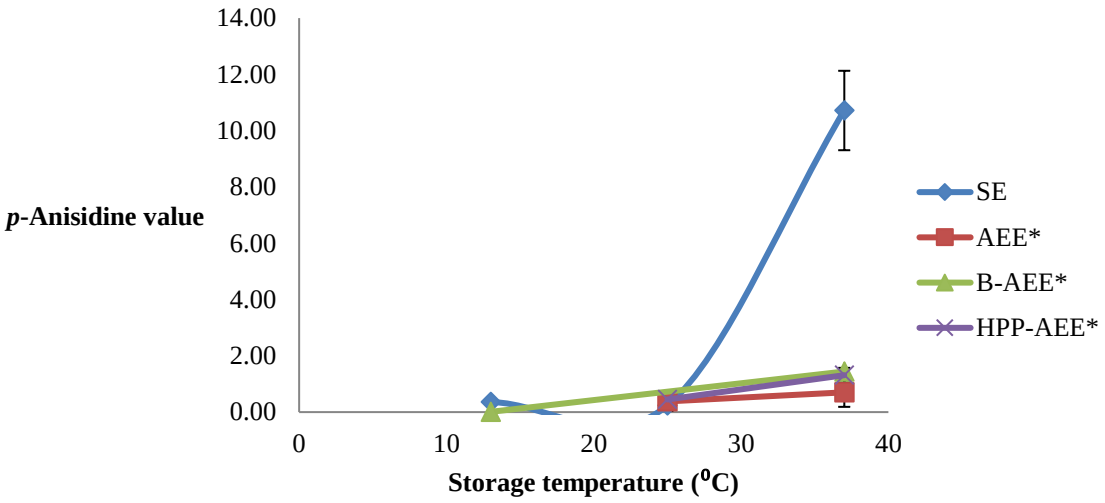


Figure 5.3 *p*-Anisidine value of *Moringa oleifera* oil from different extraction methods after 120 days of storage at different temperatures. SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

The *p*-AV represents formation of secondary oxidation products, specifically the α and β unsaturated aldehydes (O'Brien, 2009). Volatile aldehydes are responsible for changes in fats and oils organoleptic properties and oxidized flavor, besides other secondary products including ketones, esters, alcohols, and hydrocarbons (Keeney, 1962). After 120 days, decrease of PV in oil from SE method was observed at 25 °C and 37 °C (Figure 5.1(b-c)), and at the same time, the *p*-AV value increased up to 10.72±1.41 at 37 °C (Figure 5.3). Simultaneous changes in these PV and *p*-AV indicate decomposition of

primary oxidation products (causing decrease in PV) into secondary oxidation products of α and β unsaturated aldehydes (causing increase in *p*-AV) in the oil from SE method. Figure 5.3 also reveals greater oxidative deterioration in the oil from SE method as compared to other enzymatic extraction methods (*p*-AV, 0.70-1.44) after 120 days at 37 °C. Latif et al. (2011) reported approximately similar *p*-AV of MO oil extracted using solvent and enzymes which range from 1.60-1.92. An oil is considered as having good quality if its *p*-AV is less than 10.0 according to Rossell (1989), or less than 2.0 according to Subramaniam et al. (2000). Therefore, to conclude, MO oil extracted using SE method is considered as unacceptable after 120 days of storage at 37 °C due to the high *p*-AV value, but is still in good quality at lower storage temperatures of 13 °C and 25 °C. In addition to these findings, some oil samples especially those extracted using enzymes exhibited negative *p*-AV values (data not reported). According to Osawa et al. (2008), a negative *p*-AV value indicates presence of water content in any of the reagents used or in oil samples been tested. In this study, it is highly assumed that minute amount of water remained in the oil samples after the enzymatic extraction methods which involved addition of water content. The water interfered in the test conducted which led to lower *p*-AV values (i.e. negative values in this case), thus these values are not reported.

5.3.5 Effect of storage temperature and time on total oxidation value of *Moringa oleifera* oil from different extraction methods

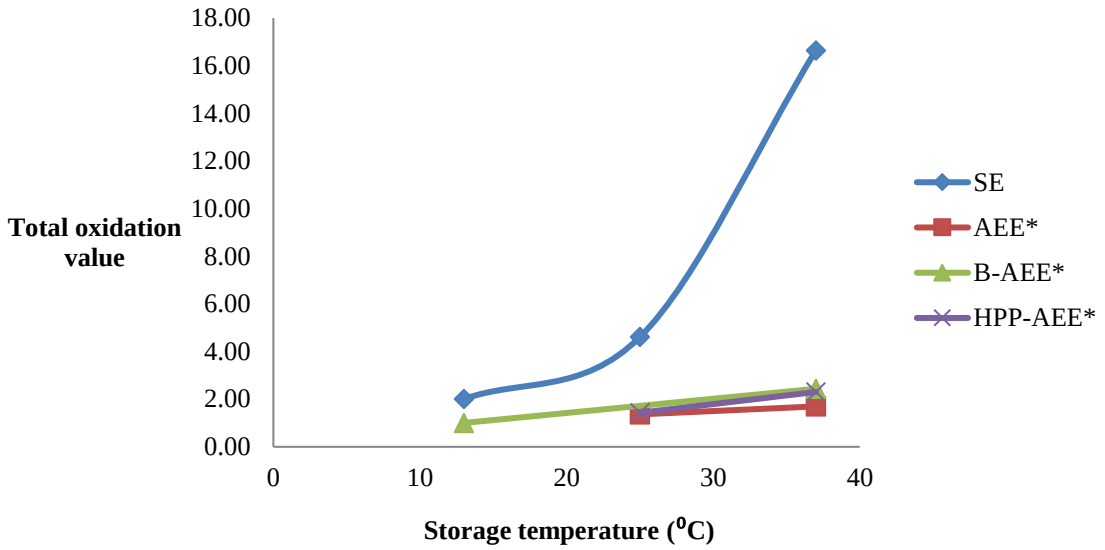
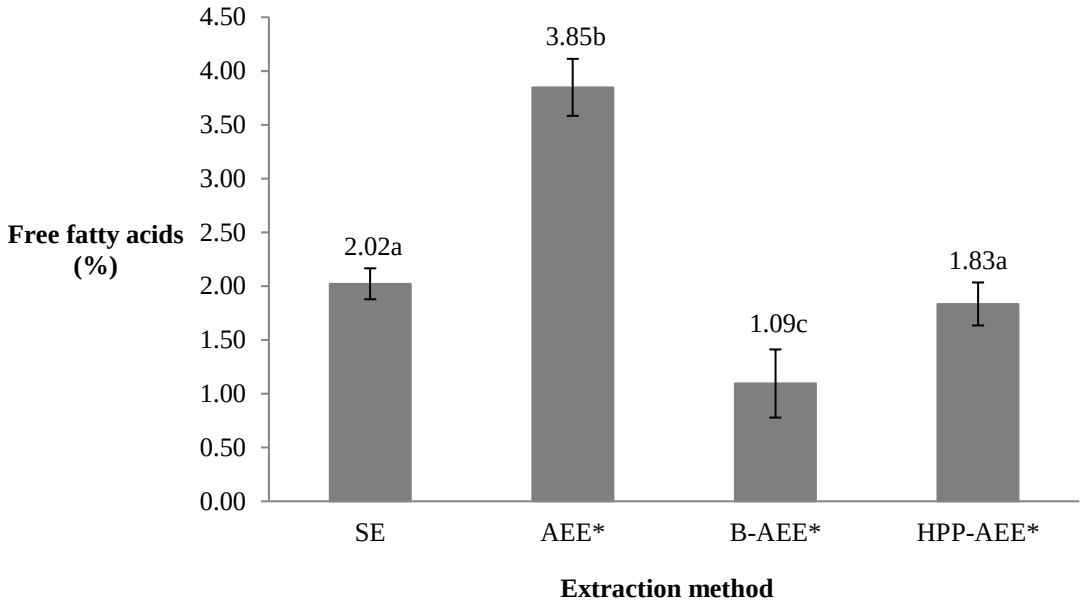


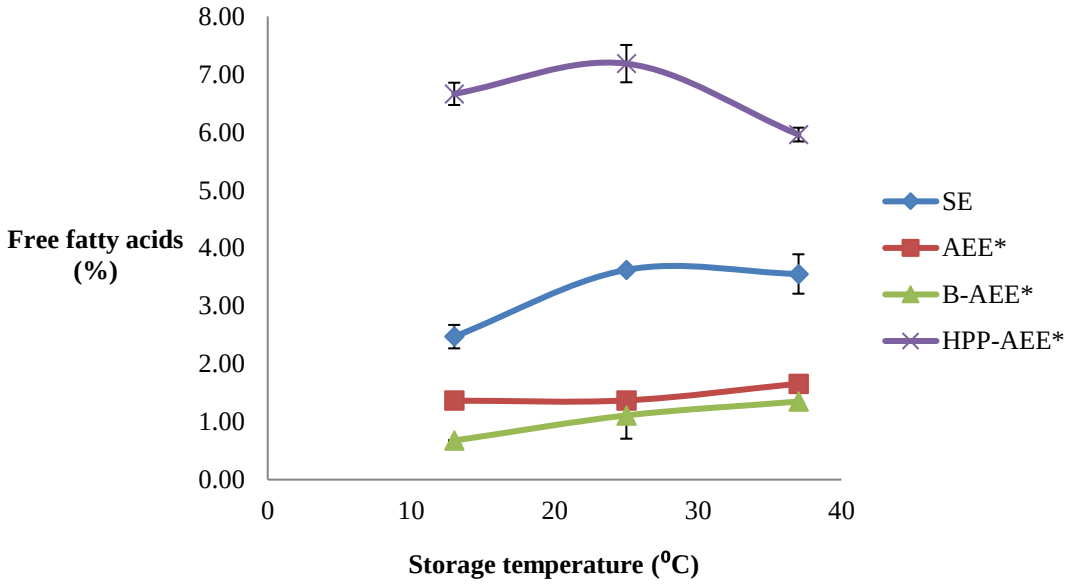
Figure 5.4 Total oxidation (TOTOX) value of *Moringa oleifera* oil from different extraction methods after 120 days of storage at different temperatures. SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

The oxidation state of an oil is better represented by measuring the combined index of primary and secondary oxidation products which is expressed as total oxidation products (i.e. TOTOX value) (deMan, 1999). After 120 days at 37 °C, the MO oil from SE method exhibited highest PV and *p*-AV as compared to other extraction methods, thus resulted in highest TOTOX value (Figure 5.4).

5.3.6 Effect of storage time on free fatty acids of *Moringa oleifera* oil from different extraction methods



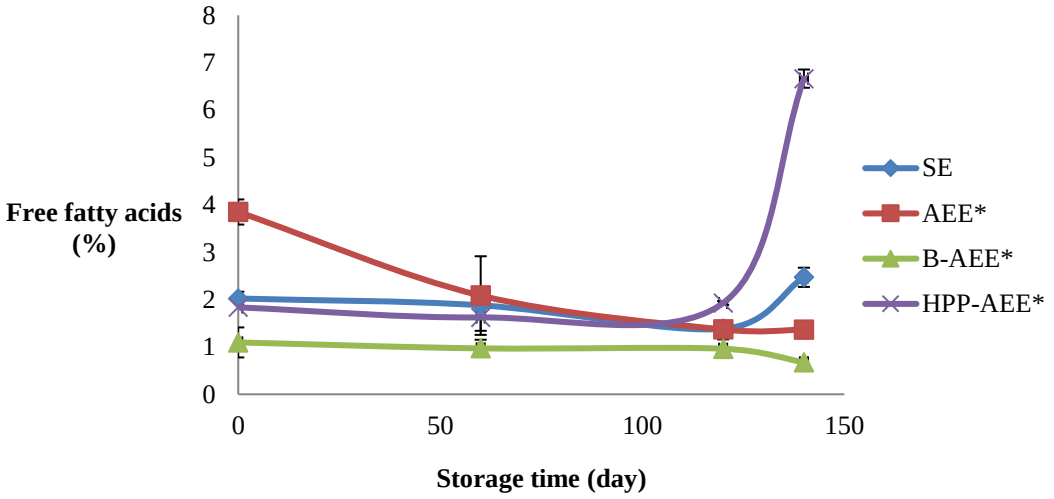
(a) Day 0



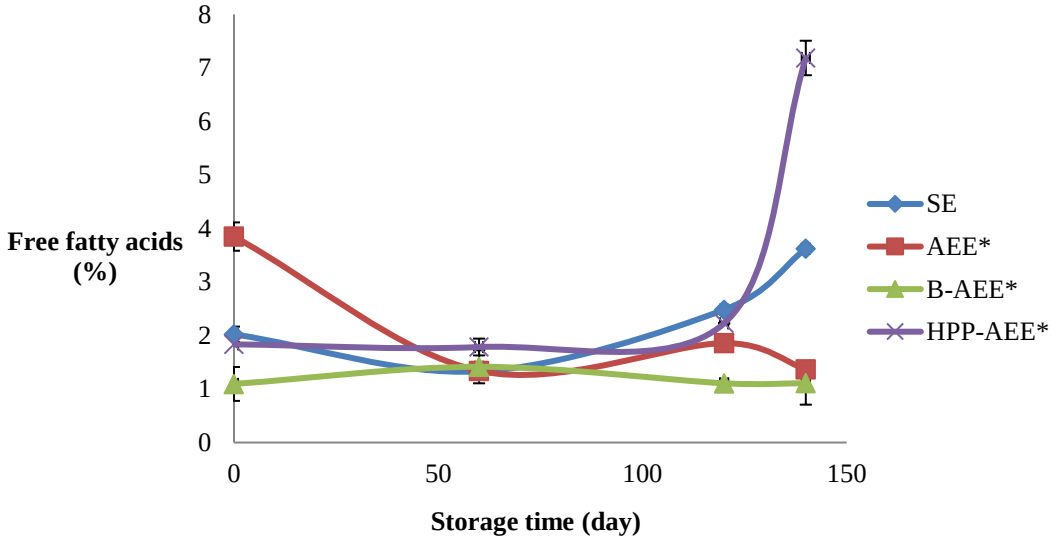
(b) Day 140

Figure 5.5 Free fatty acids (% as oleic acid) of *Moringa oleifera* oil (by weight) from different extraction methods on (a) day 0 and (b) after 140 days of storage at different temperatures of 13 °C, 25 °C, and 37 °C. On day 0 (Figure 5.5(a)), values with different letters were significantly different ($P < 0.05$). SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

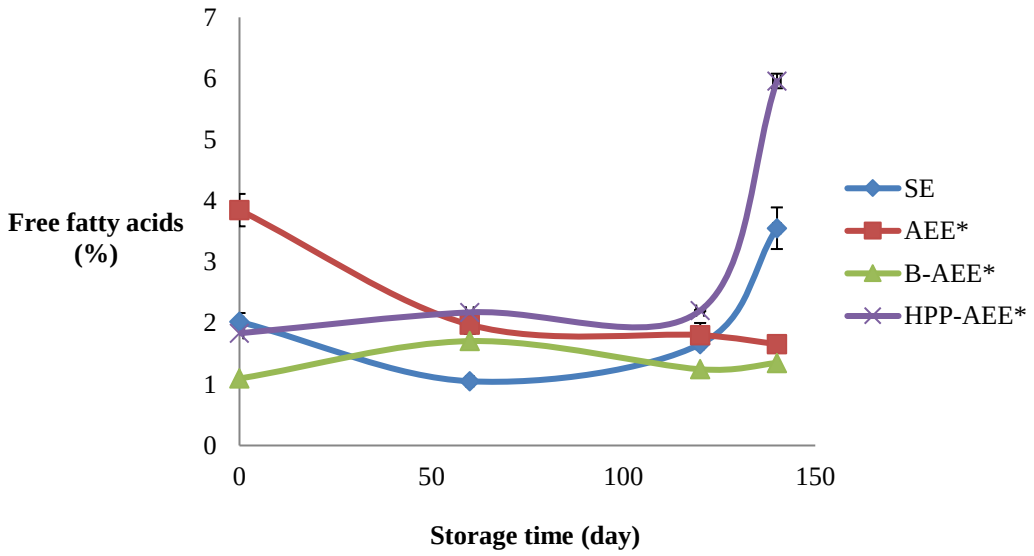
5.3.7 Effect of storage temperature on free fatty acids of *Moringa oleifera* oil from different extraction methods



(a) storage temperature = 13 °C



(b) storage temperature = 25 °C



(c) storage temperature = 37 °C

Figure 5.6 Free fatty acids (% as oleic acid) of *Moringa oleifera* oil (by weight) from different extraction methods at different temperatures of (a) 13 °C, (b) 25 °C, and (c) 37 °C. SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

5.3.8 Discussion on the effect of storage time and temperature on free fatty acids of *Moringa oleifera* oil from different extraction methods

FFA is responsible for the off -flavor and off-odor in fats and oils products (O'Brien, 2009; Noor and Augustin, 1984). Prolong storage time causes decomposition and oxidation of secondary oxidation products into FFA as tertiary oxidation product (deMan, 1999). In crude vegetable oils, improper stored or field damaged seeds contribute to abnormally high FFA level. Lipases and other enzymes in seeds and fruits are activated in the presence of water which initiates a hydrolysis reaction, causing formation of FFA (O'Brien, 2009). In this study, on day 0, the B-AEE* method resulted in MO oil with nearly 4 times lower FFA (1.09±0.32%) than the AEE* method (3.85±0.26%) (Figure 5.5(a)), and remained at low level of 0.96-1.71% throughout the whole storage conditions (Figure 5.5(b), 5.6(a-c)). Despite this finding, the FFA in oil from AEE* decreased during storage and remained between 1.34-2.08% at all storage temperatures (Figure 5.6(a-c)). These findings proved the significance of boiling pre-treatment on the ground MO kernels to inactivate the hydrolytic enzymes prior to extracting the oil. Moreover, on day 0, the oil from SE method exhibited significantly higher ($P < 0.05$) FFA than that of B-AEE* method (Figure 5.5(a)). Similar trend was reported by Abdulkarim et al. (2005) with higher FFA in oil from SE method (2.48%) as compared to an AEE* with boiling pre-treatment (1.13%). The higher FFA may be due to simultaneous extraction of other non-triglycerides and minor components by using solvent which also contributed to higher PV as explained in section 5.3.1-5.3.2.

In the case of oil from HPP-AEE* method, the FFA significantly increased ($P < 0.05$) on day 140 at all temperatures of 13 °C (6.66±0.19%), 25 °C (7.19±0.32%), and 37

°C (5.96±0.12%) (Figure 5.6(a-c)). Increase in FFA was also observed in the SE oil as compared to other extraction methods, yet still lower (2.47-3.62%) than the oil from HPP-AEE* method. As stated earlier, the boiling pre-treatment was carried out to inactivate the hydrolytic enzymes on the seed kernels prior to oil extraction. Despite this reasoning, the AEE* generally resulted in oil with approximately similar FFA with that of B-AEE* (except on day 0), and lower than that of HPP-AEE* on day 140 at all temperatures. Therefore, it is highly assumed that the sudden increase of FFA in the oil from HPP-AEE* method on day 140 is not associated with presence of hydrolytic enzymes. On the other hand, as stated in section 5.3.4, most oil samples from enzymatic extraction methods may contain minute amount of water which contributed to negative *p*-AV values. According to O'Brien (2009), presence of this water in combination with the high pressure treatment may result in acceleration of the hydrolytic reaction, therefore resulted in higher FFA on day 140 as compared to other enzymatic extraction methods.

Codex (1999) indicates maximum FFA level in oil samples in terms of its AV which is 0.6 mg KOH / g for refined oils, 4.0 mg KOH / g for cold pressed and virgin oils, and 10.0 mg KOH / g for virgin palm oils. In this study, on day 140, the AV in oil samples from SE method increased from 4.91±0.40 mg KOH / g (13 °C) to 7.21±0.04 mg KOH / g (25 °C) and 7.07±0.68 mg KOH / g (37 °C). On day 0, the AV in oil from AEE* was 7.66 mg KOH / g which decreased during storage as stated earlier. Highest FFA was observed in oil samples from HPP-AEE* method on day 140, where its AV increased up to 13.26 mg KOH / g (13 °C), 14.30±0.64 mg KOH / g (25 °C), and 11.86 mg KOH / g (37 °C). As compared to these methods, the oil extracted from B-AEE* method exhibited lowest AV of below 4.0 mg KOH / g throughout the storage conditions (1.34-3.40 mg KOH / g). This

finding further highlighted the significant effect of boiling pre-treatment in inactivating the hydrolytic enzymes, prevents enzymatic hydrolysis to take place, and thus preserving the oil's oxidative stability.

5.3.9 Effect of storage time and temperature on iodine value and fatty acid composition of *Moringa oleifera* oil

Figure 5.7 shows no significant difference ($P > 0.05$) in the IV of all oil samples from all extraction methods on day 0 (58-65 g I_2 / 100 g) and after 140 days (54-60 g I_2 / 100 g) at both 25 °C and 37 °C. Abdulkarim et al. (2005) also reported similar IV of MO oil samples extracted using solvent (65.4 g I_2 / 100 g) and AEE* with boiling pre-treatment (66.1 g I_2 / 100 g). Additionally, there was no difference in IV of MO oil samples from solvent (66.6-66.8 g I_2 / 100 g) and cold press (66.8 g I_2 / 100 g) methods (Tsaknis et al., 1999). This finding indicates that the oil did not undergo severe changes in degree of unsaturation within the storage conditions used, despite the production of oxidation products in certain oil samples as explained earlier. These outcomes are also reflected with the insignificant changes in the FAC of the oil samples (Table 5.1-5.4) at all storage temperatures. All oil samples consist of up to 76% oleic acid (C18:1) which contributes to the oil's oxidative stability and is related to reduced risk of developing coronary heart disease (Abdulkarim et al., 2007; Anwar et al., 2007). Additionally, the oil samples consist of up to 6.60% behenic fatty acid (C22:0) in all storage conditions, thus suits its other names as Ben or Behen oil as described in section 2.1.

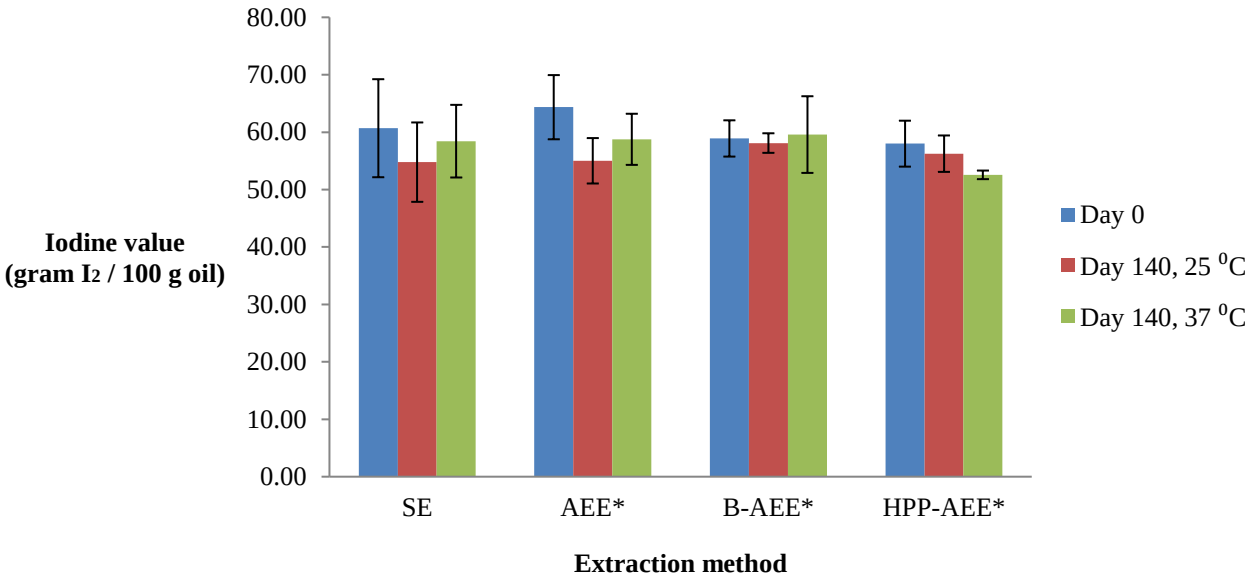


Figure 5.7 Iodine value of *Moringa oleifera* oil from different extraction methods before (day 0) and after storage (day 140) at different temperatures. There was no significant difference ($P > 0.05$) between all values. SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

Table 5.1 Fatty acid composition of *Moringa oleifera* oil from solvent (hexane) extraction method (SE) on day 0 and after 140 days of storage at different temperatures

Fatty acid (% g / 100 g)	**Day 0**	**Day 140**		
		13 °C	**25 °C**	**37 °C**
C14:0	0.02±0.00	0.03±0.00	0.03±0.00	0.02±0.00
C16:0	5.90±0.01	5.97±0.00	5.89±0.00	5.98±0.01
C16:1	1.35±0.02	1.37±0.00	1.31±0.00	1.30±0.01
C18:0	4.92±0.00	4.93±0.00	4.90±0.01	4.90±0.02
C18:1n9c	73.57±0.04	73.47±0.02	73.55±0.03	73.68±0.18
C20:0	3.72±0.00	3.70±0.00	3.72±0.01	3.71±0.01
C18:3n3	2.74±0.01	2.76±0.00	2.75±0.00	2.73±0.00
C22:0	6.32±0.02	6.32±0.01	6.36±0.00	6.26±0.07
C20:3n3	0.20±0.00	0.19±0.00	0.20±0.00	0.17±0.04
C20:4n6	0.05±0.00	0.06±0.00	0.06±0.00	0.06±0.00
C24:0	1.21±0.00	1.22±0.00	1.22±0.01	1.20±0.00
Total saturated fatty acids	22.09	22.16	22.13	22.08
Total monounsaturated fatty acids	74.92	74.84	74.86	74.98
Total polyunsaturated fatty acids	2.99	3.00	3.01	2.96

Table 5.2 Fatty acid composition of *Moringa oleifera* oil from aqueous enzymatic extraction (AEE*) method on day 0 and after 140 days of storage at different temperatures. nd, not detected

Fatty acid (% g / 100 g)	**Day 0**	**Day 140**		
		13 °C	**25 °C**	**37 °C**
C14:0	0.03±0.01	0.03±0.00	0.02±0.01	0.01±0.00
C16:0	6.09±0.04	5.86±0.01	5.90±0.02	5.87±0.01
C16:1	1.28±0.01	1.34±0.00	1.23±0.02	1.20±0.01
C18:0	5.23±0.01	4.55±0.01	4.83±0.04	4.76±0.02
C18:1n9c	72.73±0.12	76.45±0.06	73.94±0.14	74.10±0.13
C20:0	3.88±0.03	3.26±0.02	3.62±0.04	3.61±0.02
C18:3n3	2.65±0.02	2.97±0.01	2.74±0.01	2.74±0.01
C22:0	6.63±0.10	4.54±0.00	6.35±0.05	6.24±0.02
C20:3n3	0.18±0.00	0.12±0.00	0.18±0.05	0.21±0.01
C20:4n6	0.05±0.00	nd	0.05±0.02	0.05±0.02
C24:0	1.24±0.02	0.88±0.00	1.18±0.03	1.21±0.00
Total saturated fatty acids	23.10	19.13	21.89	21.71
Total monounsaturated fatty acids	74.01	77.78	75.16	75.30
Total polyunsaturated fatty acids	2.89	3.09	2.96	2.99

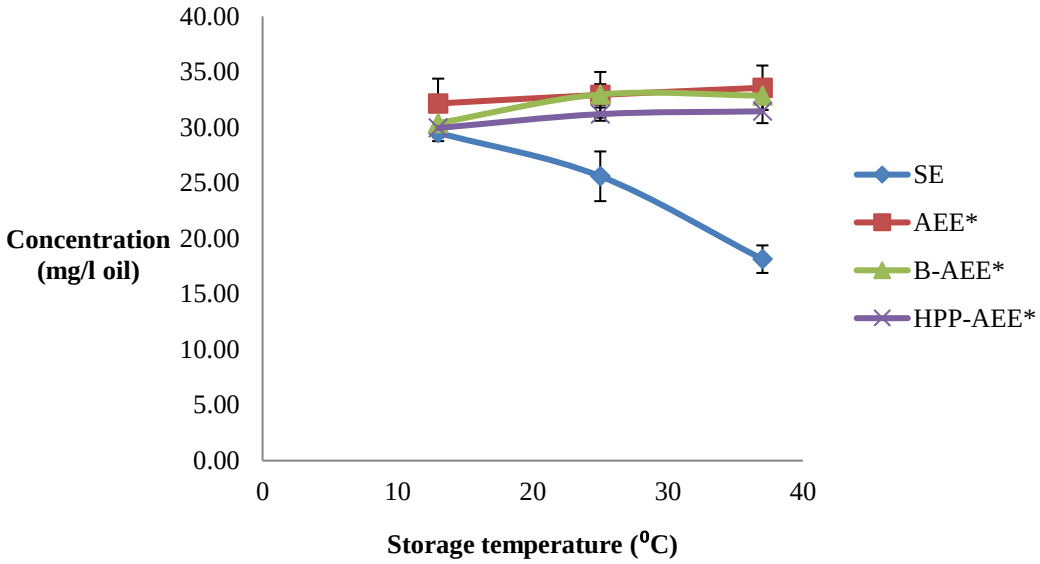
Table 5.3 Fatty acid composition of *Moringa oleifera* oil from aqueous enzymatic extraction method with boiling pre-treatment (B-AEE*) on day 0 and after 140 days of storage at different temperatures. nd, not detected

Fatty acid (% g / 100 g)	**Day 0**	**Day 140**		
		13 °C	**25 °C**	**37 °C**
C14:0	0.03±0.00	0.02±0.00	0.01±0.00	nd
C16:0	5.98±0.01	5.92±0.01	5.94±0.01	5.90±0.01
C16:1	1.26±0.01	1.25±0.01	1.21±0.01	1.21±0.01
C18:0	5.09±0.01	4.85±0.02	4.91±0.02	4.81±0.01
C18:1n9c	73.40±0.04	73.88±0.07	73.71±0.08	73.98±0.07
C20:0	3.78±0.01	3.64±0.02	3.65±0.02	3.60±0.01
C18:3n3	2.73±0.01	2.77±0.01	2.74±0.00	2.73±0.00
C22:0	6.30±0.02	6.20±0.01	6.31±0.01	6.26±0.03
C20:3n3	0.18±0.02	0.21±0.00	0.22±0.01	0.21±0.01
C20:4n6	0.04±0.02	0.06±0.00	0.06±0.00	0.05±0.02
C24:0	1.21±0.02	1.22±0.01	1.24±0.01	1.24±0.02
Total saturated fatty acids	22.39	21.84	22.06	21.82
Total monounsaturated fatty acids	74.66	75.12	74.93	75.19
Total polyunsaturated fatty acids	2.95	3.04	3.02	2.99

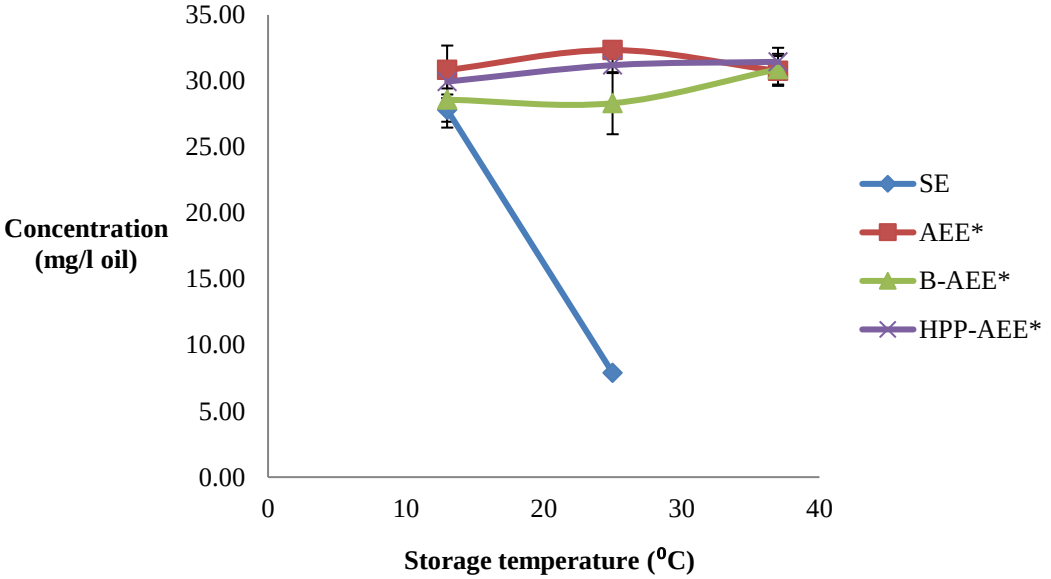
Table 5.4 Fatty acid composition of *Moringa oleifera* oil from aqueous enzymatic extraction method with high pressure processing pre-treatment (HPP-AEE*) on day 0 and after 140 days of storage at different temperatures. nd, not detected

Fatty acid (% g / 100 g)	**Day 0**	**Day 140**		
		13 °C	**25 °C**	**37 °C**
C14:0	0.02±0.00	0.01±0.00	0.02±0.00	nd
C16:0	5.85±0.02	5.79±0.01	5.86±0.03	5.79±0.01
C16:1	1.17±0.00	1.18±0.00	1.19±0.01	1.12±0.01
C18:0	4.77±0.01	4.80±0.01	4.77±0.03	4.70±0.01
C18:1n9c	74.26±0.07	74.03±0.03	74.45±0.22	74.46±0.03
C20:0	3.60±0.02	3.64±0.01	3.56±0.04	3.55±0.00
C18:3n3	2.65±0.01	2.69±0.01	2.64±0.02	2.67±0.00
C22:0	6.20±0.02	6.41±0.02	6.17±0.12	6.27±0.01
C20:3n3	0.22±0.01	0.19±0.00	0.17±0.04	0.20±0.00
C20:4n6	0.04±0.00	0.03±0.00	0.03±0.00	0.03±0.00
C24:0	1.22±0.00	1.23±0.01	1.16±0.02	1.20±0.00
Total saturated fatty acids	21.66	21.88	21.53	21.52
Total monounsaturated fatty acids	75.43	75.20	75.64	75.58
Total polyunsaturated fatty acids	2.91	2.91	2.84	2.90

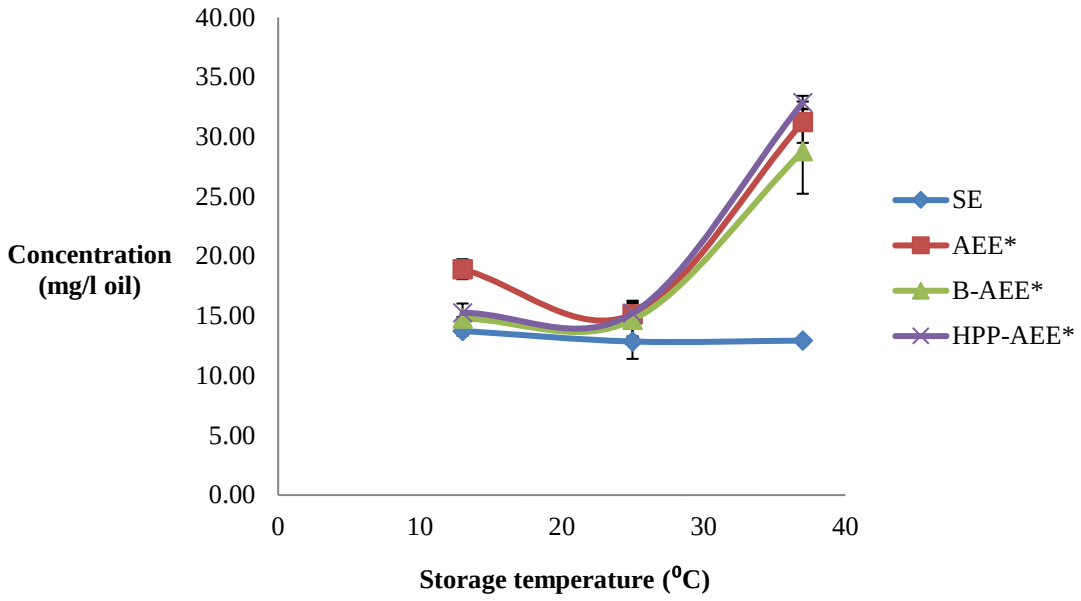
5.3.10 Effect of storage time on α-tocopherol content in *Moringa oleifera* oil from different extraction methods



(a) Day 60



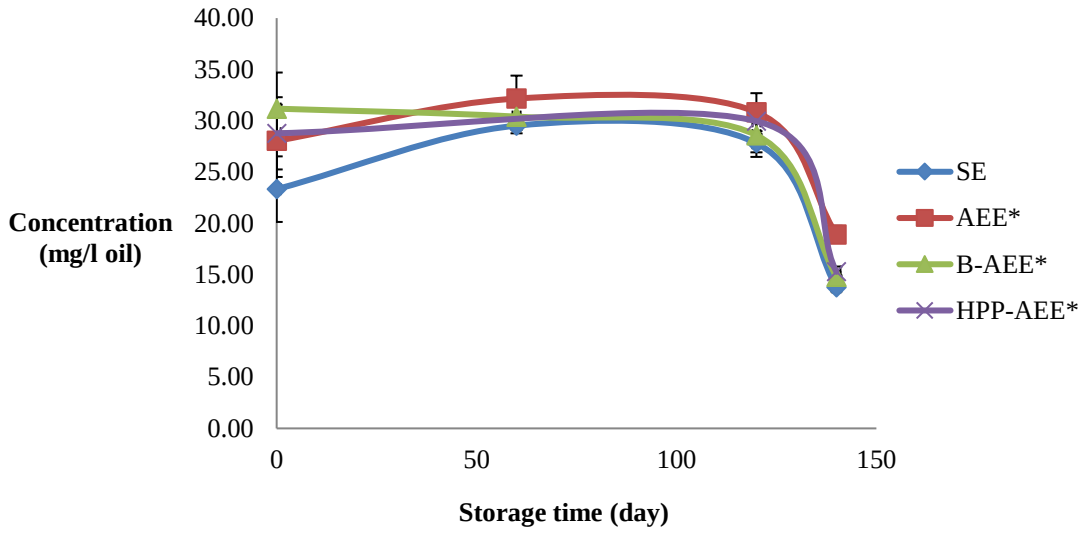
(b) Day 120



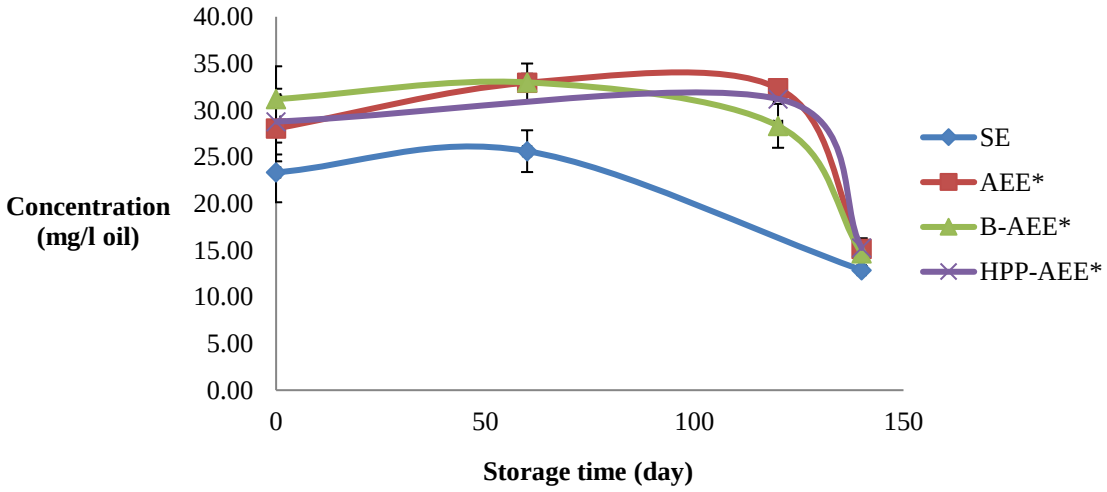
(c) Day 140

Figure 5.8 Alpha tocopherol concentration in *Moringa oleifera* oil from different extraction methods on (a) day 60, (b) day 120, and (c) day 140 at different temperatures of 13 ⁰C, 25 ⁰C, and 37 ⁰C. SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

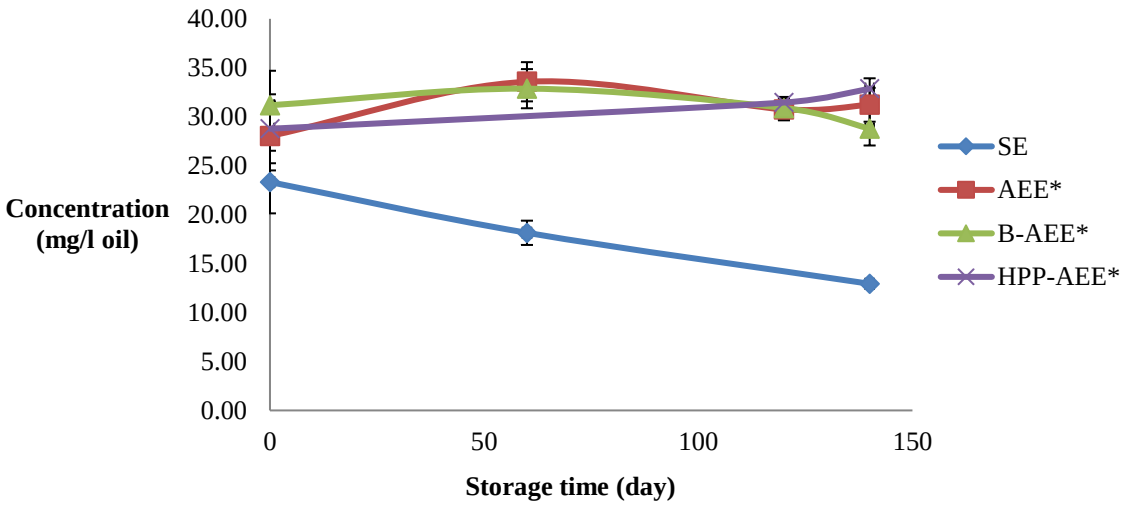
5.3.11 Effect of storage temperature on α-tocopherol content in *Moringa oleifera* oil from different extraction methods



(a) storage temperature = 13 °C



(b) storage temperature = 25 °C



(c) storage temperature = 37 °C

Figure 5.9 Alpha tocopherol concentration in *Moringa oleifera* oil from different extraction methods at different storage temperatures of (a) 13 °C, (b) 25 °C, and (c) 37 °C on different storage time of 0 day to 120 days. SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

5.3.12 Discussion on the effect of storage time and temperature on α-tocopherol content in *Moringa oleifera* oil from different extraction methods

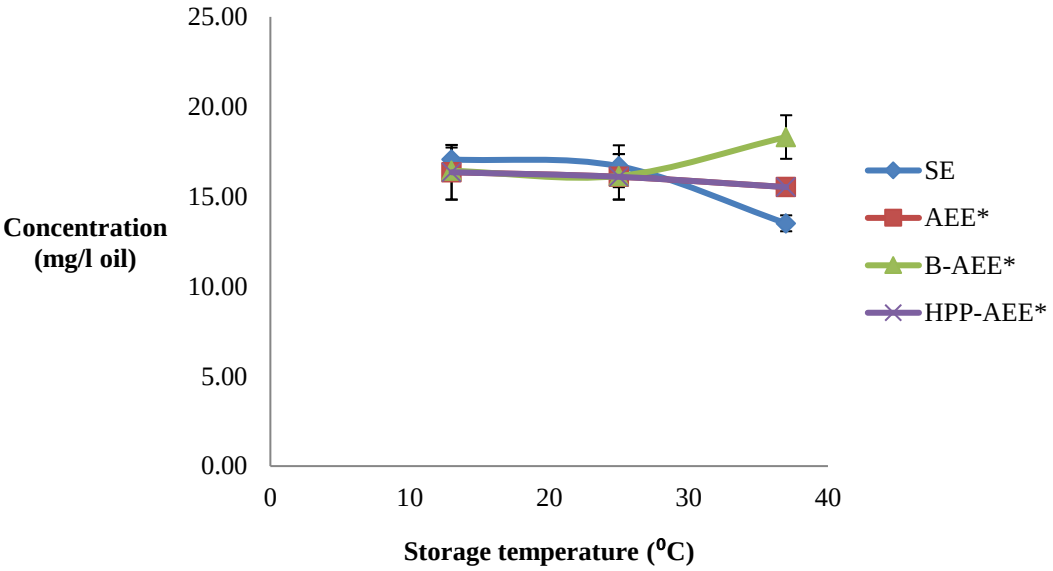
On day 0, highest α-tocopherol content was discovered in oil from B-AEE* method (31.17±3.52 mg/l) which was insignificantly different ($P > 0.05$) with the oil from AEE* (28.04±1.26 mg/l) and HPP-AEE* (28.77±1.05 mg/l) methods. As compared to these enzymatic extraction methods, significantly lower ($P < 0.05$) α-tocopherol content was observed in oil from SE method (23.33±0.99 mg/l). In a study done by Tsaknis et al. (1999) using MO seed kernels of Kenya origin, the α-tocopherol content in the oil samples were similar in the case of solvent (98-105 mg/kg) and cold press (101.46 mg/kg) methods. With the use of MO seed kernels of Bangladesh origin, Rahman et al. (2009) also revealed as high as 121-154 mg/kg α-tocopherol content in the oil extracted using different types of solvents. In another study done by Tsaknis and Lalas (2002) on seed kernels of India origin, the SE method resulted in higher α-tocopherol content (15.38 mg/kg) as compared to the cold press method (5.06 mg/kg). To summarize, regardless of the extraction methods, the α-tocopherol contents reported in this present study on day 0 (23.33-31.17 mg/l) and those reported by Tsaknis and Lalas (2002) (5.06-15.38 mg/kg) were far too low than that of reported by Tsaknis et al. (1999) (98-105 mg/kg) and Rahman et al. (2009) (121-154 mg/kg). These findings highlighted the variations in the MO seed kernels of different origins which resulted in different oil properties. Besides α-tocopherol, earlier studies reported presence of γ- and ð-tocopherols in MO oils extracted using solvents, enzymes, cold press, and supercritical fluid extraction method (Tsaknis et al., 1999; Tsaknis and Lalas, 2002; Lalas and Tsaknis, 2002; Rahman et al., 2009), yet the values

varied significantly. In this study, the tocopherols reported are the α- and γ-tocopherols only, due to low amount of ð-tocopherol detected.

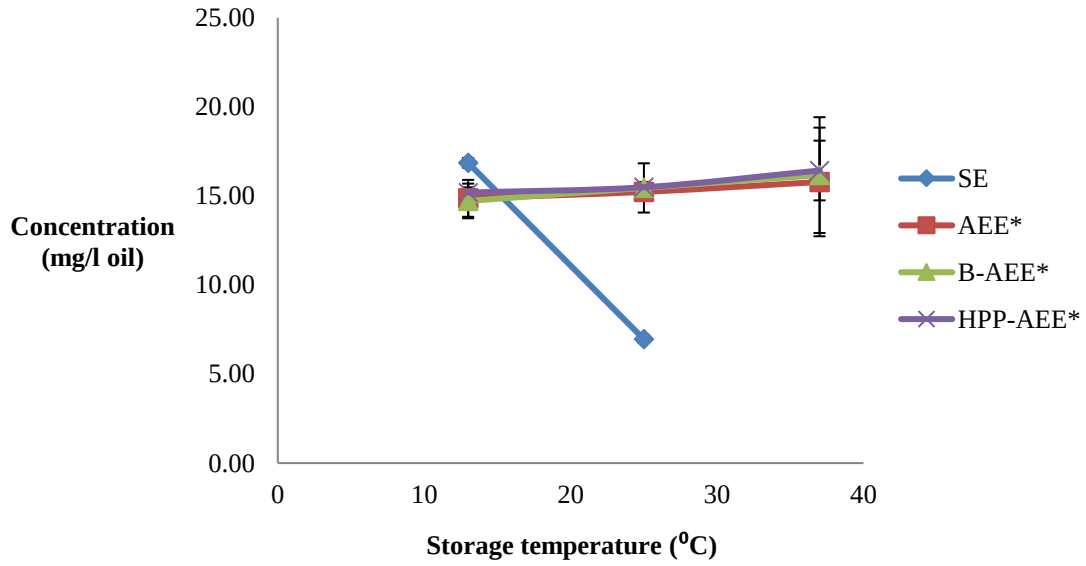
Production of greater oxidation products in oil from SE method as indicated by increase of its PV, *p*-AV, and TOTOX as compared to enzymatic extraction methods was reflected with significant decrease ($P < 0.05$) in the oil's α-tocopherol content during storage (Figure 5.8(a-b)). On day 60, the lowest α-tocopherol content was observed at 37 °C (18.14±1.24 mg/l) as compared to 13 °C (29.51±0.75 mg/l) and 25 °C (25.60±2.24 mg/l). Greatest effect of storage temperature took place on day 120 where the α-tocopherol content decreased with temperature increase from 13 °C (27.81±0.89 mg/l) to 25 °C (7.89±0.14 mg/l). On day 140 (Figure 5.8(c)), the α-tocopherol content in oil from SE method was not significantly affected ($P > 0.05$) by the storage temperatures. Yet on the same storage day, highest tocopherol content was detected at 37 °C in oil samples from AEE* (31.22±1.73 mg/l), B-AEE* (28.79±3.56 mg/l), and HPP-AEE* (32.86±0.56 mg/l) as compared to storage at lower temperatures of 13 °C (14-19 mg/l) and 25 °C (14-16 mg/l). The reason behind this finding is not yet been understood.

At 13 °C (Figure 5.9(a)), the α-tocopherol content in oil samples from all extraction methods started to decrease significantly ($P < 0.05$) after 120 days. Similar trend was observed in oil samples extracted using enzymes at 25 °C (Figure 5.9(b)). However at this temperature, the α-tocopherol content in oil from SE method decreased significantly ($P < 0.05$) after 60 days, and greater decrease was observed at 37 °C (Figure 5.9(c)).

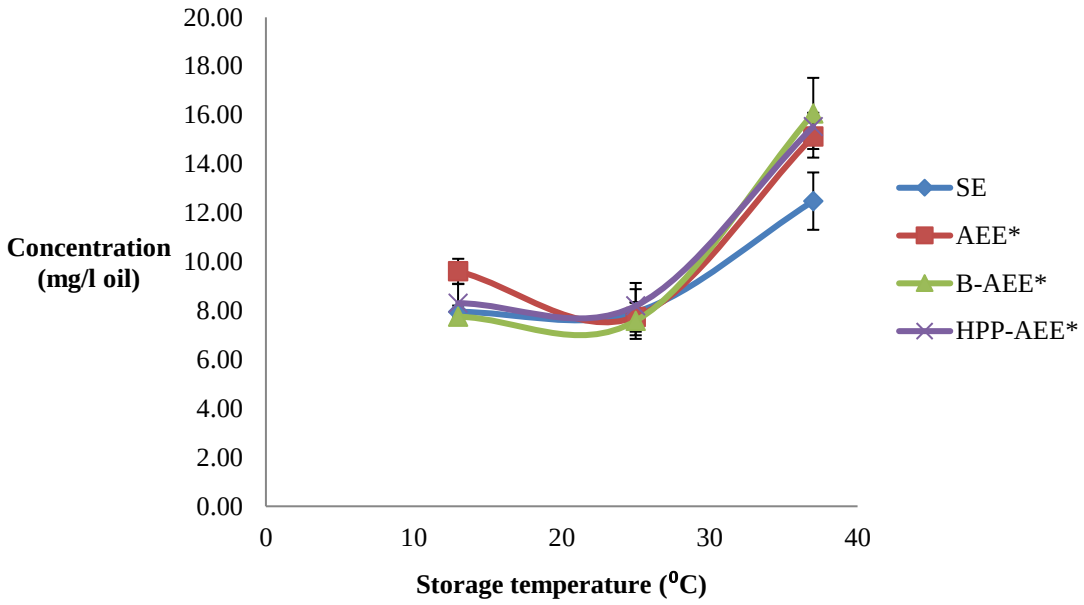
5.3.13 Effect of storage time on γ-tocopherol content in *Moringa oleifera* oil from different extraction methods



(a) Day 60



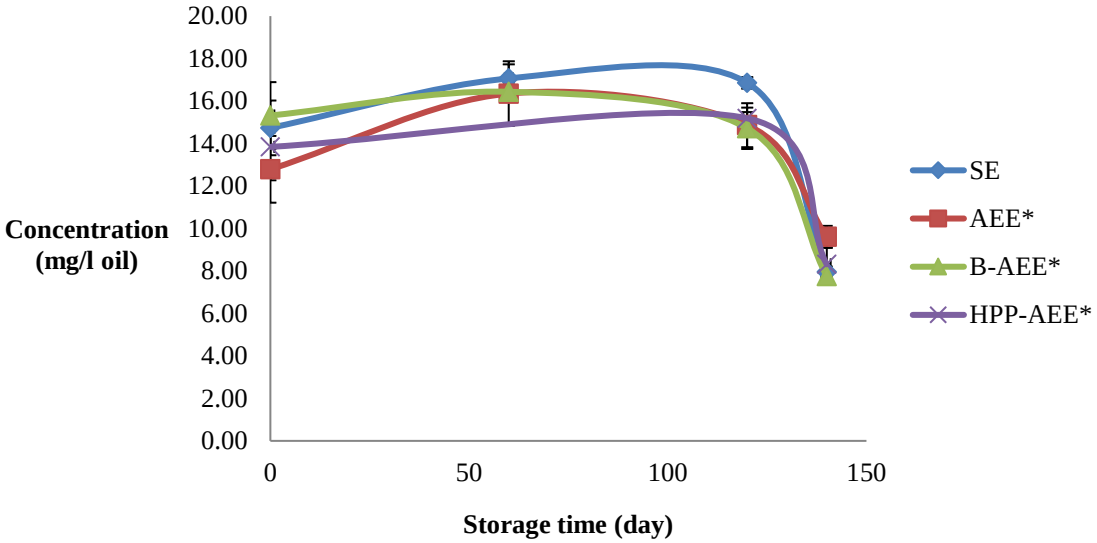
(b) Day 120



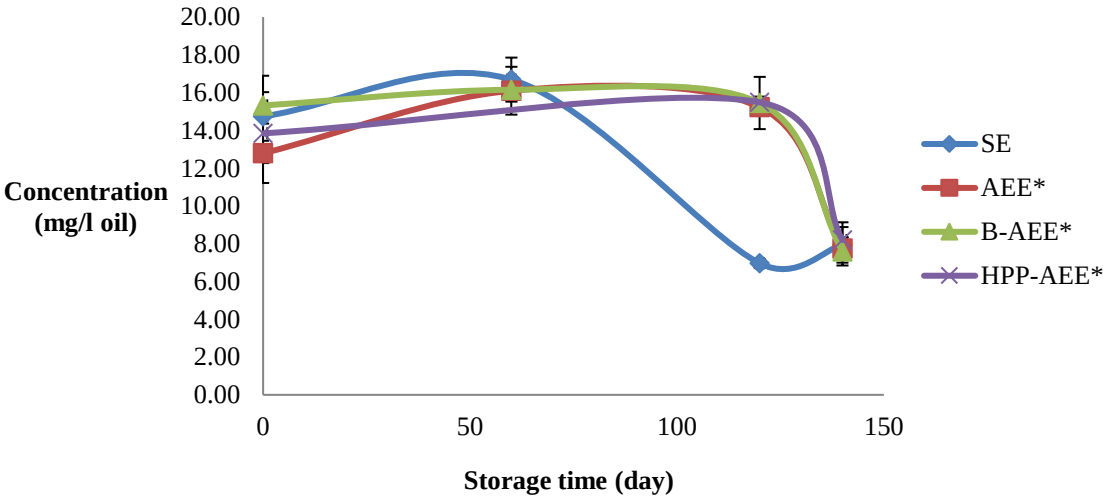
(c) Day 140

Figure 5.10 Gamma tocopherol concentration in *Moringa oleifera* oil from different extraction methods on (a) day 60, (b) day 120, and (c) day 140 at different temperatures of 13 ⁰C, 25 ⁰C, and 37 ⁰C. SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

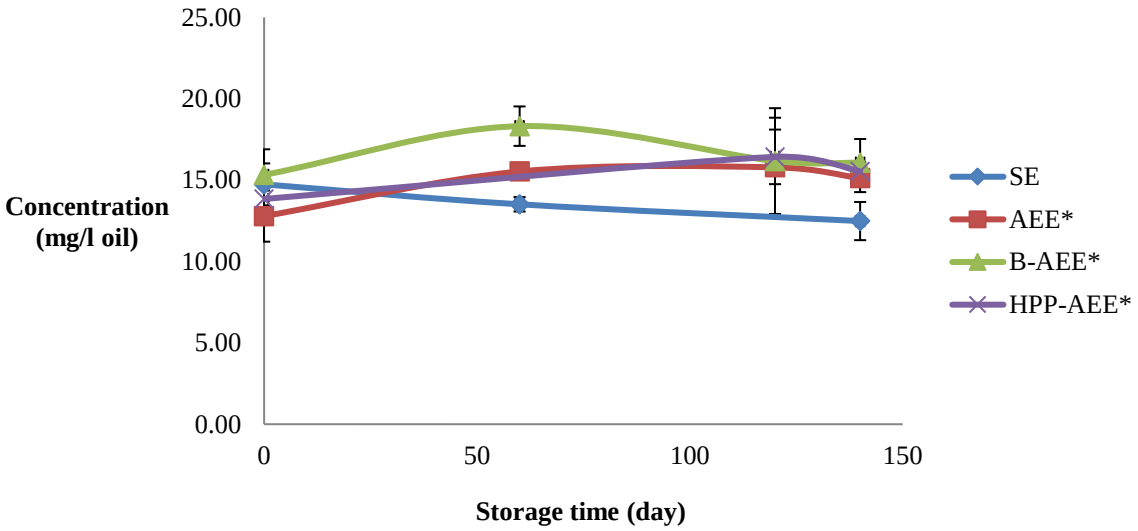
5.3.14 Effect of storage temperature on γ-tocopherol content in *Moringa oleifera* oil from different extraction methods



(a) storage temperature = 13 °C



(b) storage temperature = 25 °C



(c) storage temperature = 37 ºC

Figure 5.11 Gamma tocopherol concentration in *Moringa oleifera* oil from different extraction methods at different storage temperatures of (a) 13 ºC, (b) 25 ºC, and (c) 37 ºC on different storage time of 0 day to 120 days. SE, solvent extraction; AEE*, aqueous enzymatic extraction; B-AEE*, aqueous enzymatic extraction with boiling pre-treatment; HPP-AEE*, aqueous enzymatic extraction with high pressure processing pre-treatment.

5.3.15 Discussion on the effect of storage time and temperature on γ-tocopherol content in *Moringa oleifera* oil from different extraction methods

In this study, all MO oil samples exhibited lower amount of γ-tocopherol as compared to α-tocopherol. On day 0, all extraction methods resulted in oil samples with approximately similar γ-tocopherol content: 14.74±1.29 mg/l (SE), 12.79±1.26 mg/l (AEE*), 15.32±1.57 mg/l (B-AEE*), and 13.84±0.97 mg/l (HPP-AEE*). Differently, Tsaknis et al. (1999) reported higher γ-tocopherol content in oil from cold press (39.54 mg/kg) than that of solvent extraction methods (27.90-33.45 mg/kg) with the use of MO seed kernels of Kenya origin. Tsaknis and Lalas (2002) also reported higher γ-tocopherol content in cold pressed-oil (25.40 mg/kg) as compared to solvent extracted-oil (4.47-5.52 mg/kg) from MO seed kernels of India origin. In a study done by Rahman et al. (2009) using seed kernels of Bangladesh origin, different types of solvents resulted in oil samples with approximately similar γ-tocopherol content (62.2-77.4 mg/kg). To conclude, similar with α-tocopherol, the γ-tocopherol content varied in MO oil samples from seed kernels of different origins and is also dependent on extraction methods used.

On day 60 (Figure 5.10(a)), lower γ-tocopherol content in oil from SE method was observed at higher temperature of 37 °C (13.51±0.44 mg/l) than that at 25 °C (16.69±1.17 mg/l). On day 120 (Figure 5.10(b)), greater decrease in γ-tocopherol was observed with increase in temperature from 13 °C (16.86±0.27 mg/l) to 25 °C (6.96±0.22 mg/l), while no changes were detected in oil samples from enzymatic extraction methods. On day 140 (Figure 5.10(c)), the γ-tocopherol content in all oil samples was significantly higher ($P < 0.05$) at 37 °C (12.48-16.07 mg/l) as compared to lower storage temperatures of 13 °C (7.76-9.61 mg/l) and 25 °C (7.58-8.20 mg/l), and the reasons for this sudden increment was

not identified. In the case of oil from SE method, this trend was different from that of α-tocopherol which did not change upon different storage temperatures on day 140 (Figure 5.8(c)).

At 13 °C (Figure 5.11(a)), the γ-tocopherol content in all oil samples from all extraction methods decreased significantly ($P < 0.05$) after 120 days. Similarly at 25 °C (Figure 5.11(b)), the γ-tocopherol content in oil samples from enzymatic extraction methods decreased after 120 days, while in oil from SE method, the γ-tocopherol content started to decrease after 60 days. At 37 °C (Figure 5.11(c)), slight yet insignificant ($P > 0.05$) decrease in γ-tocopherol content in oil from SE method was observed, which was different from significant decrease ($P < 0.05$) in the case of α-tocopherol in the same storage condition. In overall at 37 °C, the storage time imparted no significant changes in the γ-tocopherol content in oil samples from all extraction methods.

5.4 Conclusions

In most MO oil samples, changes in oxidative properties and tocopherol contents started to take place after 120 days of storage, and the rate of changes increased with increase in temperature. The MO oil from SE method underwent greater oxidative deterioration as compared to other enzymatic extraction methods. The oil was not in good quality after 120 days at 37 °C, while it is still acceptable during storage at 13 °C up to 140 days of storage. The enzymatically extracted oils exhibited approximately similar oxidative properties throughout the whole storage conditions, except in the case of oil from HPP-AEE*. The high pressure pre-treatment is advantageous in terms of minimizing the cream emulsion formed after an AEE* process (Chapter 4), yet it caused high FFA content in the

MO oil after 120 days, even at as low as 13 °C storage temperature. This may be due to the high pressure applied which caused acceleration of hydrolytic reaction. On the other hand, the boiling pre-treatment was necessary to deactivate the hydrolytic enzymes in the seed kernels for better oil quality preservation during storage. Thus to conclude, within the storage conditions tested, B-AEE* method resulted in MO oil with greatest oxidative properties, followed by the AEE*, HPP-AEE*, and the SE method. No significant changes occurred in IV of all oil samples, indicating no changes in their degree of unsaturation throughout the storage condition. After 140 days at 37 °C, the concentration of both α- and γ-tocopherols in all oil samples were nearly 2 times higher than their concentrations at lower temperatures, and the reasons for this finding is not yet discovered. In overall, it is highly suggested to store MO oil at 13 °C or lower temperature if possible, for better preservation of its oxidative properties and tocopherol content. Both the boiling and HPP pre-treatments did not significantly affect the tocopherol contents of the MO oil. Moreover, the enzymatic extraction methods resulted in oils with better oxidative properties as compared to the use of solvent. This advantage assists in minimizing refinery loss and therefore should further be explored.

CHAPTER 6

CONCLUDING REMARKS

6.1 Conclusion

Moringa oleifera (MO) kernel cell is composed of up to 41% (w/w) oil as determined by solvent extraction method, which is enclosed with approximately 35% (w/w) protein content. In reference to this MO cell microstructure, it is necessary to disrupt the protein component in order to release the oil content. Thus in the aqueous enzymatic extraction (AEE) method conducted in this study, a mixture of protease and cellulase enzymes at 3:1 (w/w) ratio was used, while the starting material was the ground-sieved MO kernels of less than 710 μm particle size. The statistical optimization indicated higher MO oil recovery with the use of pH 4.0-4.5, higher water/kernel ratio up to 8:1 (w/w), and higher shaking speed of 300 stroke/min, with both the pH value and water content of the solution exhibits significant interaction effect on the oil recovery. Upon the use of the optimum parameters, other AEE parameters - the incubation time and temperature - did not significantly affect the oil recovery within the ranges used and resulted in approximately 70% (w/w) oil recovery with tiny oil-in-water cream emulsion. The resulted aqueous phase from this AEE process however is not recommended to be re-used for another AEE process of the same parameters, due to zero oil recovery and thick emulsion layer formed. On the other hand, in some AEE cases away from the optimum parameters - particularly Run 39 (pH 6.0, water/kernel ratio of 4:1 w/w, incubation at 50 °C for 12.5 hr at 300 stroke/min), the amount of oil recovered was approximately similar with that of the optimum AEE yet the cream emulsion was thicker. The resulted aqueous phase from this Run 39 was able to

be re-used which resulted in approximately 60% (w/w) oil recovery with thick cream emulsion. These findings suggested that the re-usability of enzymes in the resulted aqueous phase is highly dependent on the parameters used in the initial and the following AEE carried out. Additionally, the aqueous phase after an AEE may contain significant amount of MO protein, either in native or denatured form, which also have great effect on both the oil recovery and cream emulsion formed in the following AEE process.

Following studies on the AEE was the use of high pressure processing (HPP) as a pre-treatment prior to the AEE of Run 39. The ground-sieved MO kernels generally resulted in higher oil recoveries and thinner emulsion layers as compared to whole kernels within the HPP ranges been studied. With the use of 50 MPa at 60 °C for 35 min pressurizing time (Run 15), highest oil of 73.02% (w/w) was recovered from ground-sieved kernels. On the other hand, the use of 450 MPa at 40 °C for 10 min resulted in highest oil recovery of 57.46% (w/w) from the whole kernels. Statistical optimization of the HPP parameters indicated significant increase in the MO oil recovery with increased in both the pressurization temperature and time, but not the pressure level. Despite these outcomes, thin emulsion layers were formed in all cases as compared to the use of AEE alone. These observations highlighted the effect of HPP in altering the MO protein structure into a form of less emulsifying functional properties. Additional experiments were carried out to determine the effect of HPP pre-treatment in place of the gentle boiling step in the AEE method. To avoid confusion, the gentle boiling step was termed as boiling pre-treatment, while the following steps in the AEE method was termed as AEE*. The oil recoveries from both B-AEE* (AEE* with boiling pre-treatment) and HPP-AEE* (AEE* with HPP pre-treatment) methods were insignificantly higher than the AEE* alone, with thinner cream

emulsion layer observed in the case of HPP-AEE*. These findings highlighted the potential of HPP in reducing or eliminating the load of the following de-emulsification method.

The MO oil was finally extracted by using the AEE* (Run 39), the B-AEE*, the HPP-AEE* (HPP of Run 15), and the solvent extraction method (SE). The oil samples were stored for 140 days at different temperatures of 13 °C, 25 °C, and 37 °C. In most MO oil samples, changes in oxidative properties and tocopherol contents started to take place after 120 days of storage, and the rate of changes increased with increase in temperature. The MO oil from SE method underwent greater oxidative changes as compared to other enzymatic extraction methods. The oil was not in good quality after 120 days at 37 °C, while it is still acceptable during storage at 13 °C up to 140 days of storage. The enzymatically extracted oils exhibited approximately similar oxidative properties throughout the whole storage conditions, except in the case of oil from HPP-AEE*. The HPP pre-treatment is advantageous in terms of minimizing the cream emulsion formed after an enzymatic extraction process, yet it caused high free fatty acids (FFA) content in the MO oil after 120 days, even at as low as 13 °C storage temperature. This may be due to the high pressure applied which caused acceleration of hydrolytic reaction. On the other hand, the boiling pre-treatment was necessary to deactivate the hydrolytic enzymes in the seed kernels for better oil quality preservation during storage. Thus to conclude, within the storage conditions tested, B-AEE* method resulted in MO oil with greatest oxidative properties, followed by the AEE*, HPP-AEE*, and the SE method. No significant changes occurred in the iodine value (IV) and fatty acid composition (FAC) of all oil samples, indicating no changes in their degree of unsaturation throughout the storage condition. Both the oleic acid (C18:1) and behenic acid (C22:0) contents in the MO oil samples were

up to approximately 75% and 6%, respectively. After 140 days at 37 °C, the concentration of both α- and γ-tocopherols were nearly 2 times higher than their concentrations at lower temperatures, and the reasons for this finding is not yet discovered. Finally, it is highly suggested to store MO oil at 13 °C or lower temperature if possible, for better preservation of its oxidative properties and tocopherol content. Both the boiling and HPP pre-treatments did not significantly affect the tocopherol contents of the MO oil. Moreover, the enzymatic extraction methods resulted in oils with better oxidative properties as compared to the use of solvent, which assists in minimizing refinery loss.

In overall, the HPP pre-treatment is able to minimize the cream emulsion formed after an AEE process, which is of great advantage despite its inability to significantly enhance the MO oil recovery. Additionally, the HPP did not significantly affect the tocopherol contents of the MO oil. It is highly assumed that the shelf life of the oil, particularly in terms of its FFA, may be improved by ensuring absence of water in the recovered oil.

6.2 Recommendations for future work

Further investigations should be carried out on the following aspects in order to improve the process advantages:

i. The maximum MO oil recovered in this study using AEE (protease:cellulase, 3:1 w/w) was approximately 73% (w/w) which was lower in relation to the use of hexane. The use of more potent enzymes should therefore be explored which may have greater destruction effect on the MO kernels structure for higher oil release.

ii. The use of HPP pre-treatment within the parameters studied did not improve the recovery significantly. The potential of other types of pre-treatments prior to the AEE should be investigated. Similar with this study, the effect of other pre-treatments should be reported in terms of both the free oil recovered and cream emulsion formed after the AEE process.

iii. The potential of HPP in altering the MO protein structure was highlighted in this study, which was advantageous in minimizing the cream emulsion formed after an AEE process. Based on this finding, detailed studies are recommended on the changes in protein conformation and functionality upon different extraction processes which may provide other uses for the protein.

iv. Studies on AEE and HPP processes have never been done at higher than laboratory scale. In this study, the end product of the HPP pre-treatment, i.e. the mixture of sample and water, can directly be used in the following AEE process which also utilizes a mixture of sample and water as a starting material. Therefore, the process scale-up is highly recommended for both the HPP pre-treatment and the following AEE process, up to the economically viable scale where oil and a highly functional

protein will be the main products. The use of AEE at higher scale is highly recommended for oils of high commercial prices i.e. olive and avocado oils (Rosenthal et al., 1996). With reference to Chapter 5, the MO oil also bears great oxidative properties, particularly due to its approximately similar oleic acid content with that of olive oil and presence of significant amount of tocopherols. These findings further highlighted the potential of scaling up the HPP and AEE processes for extraction of MO oil. The process scale-up may also assist in cost estimation and production of by-products in comparison with conventional extraction process.

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Appendix 1. Part of the literature review (Chapter 2) in this thesis has been published as "Mat Yusoff, M., Gordon, M. H., Ezeh, O., and Niranjan, K. (2015). Aqueous enzyme assisted oil extraction from oilseeds and emulsion de-emulsifying methods: A review. *Trends in Food Science and Technology, 41(1)*, 60-82".

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Review

Aqueous enzyme assisted oil extraction from oilseeds and emulsion de-emulsifying methods: A review

Masni Mat Yusoff, Michael H. Gordon, Keshavan Niranjan

Department of Food and Nutritional Sciences, University of Reading, Whiteknights, PO Box 224, Reading RG6 6AP, United Kingdom

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Highlights

- Description of aqueous and enzymatic extraction media for oilseeds.
- Discussion of possible treatments prior to and/or post enzymatic extraction.
- Discussion of factors influencing oil yield with the use of enzyme containing media.
- Description of oil characteristics obtained under various process conditions.
- Exploring enzyme recovery and recycling to improve process economics.

Regulatory, safety, and environmental issues have prompted the development of aqueous enzymatic extraction (AEE) for extracting components from oil-bearing materials. The emulsion resulting from AEE requires de-emulsification to separate the oil; when enzymes are used for this purpose, the method is known as aqueous enzymatic emulsion de-emulsification (AEED). In general, enzyme assisted oil extraction is known to yield oil having highly favourable characteristics. This review covers technological aspects of enzyme assisted oil extraction, and explores the quality characteristics of the oils obtained, focussing particularly on recent efforts undertaken to improve process economics by recovering and re-using enzymes.

Appendix 2. Chapter 3 of this thesis has been published as "Mat Yusoff, M., Gordon, M. H., Ezeh, O., and Niranjan, K. (2016). Aqueous enzymatic extraction of *Moringa oleifera* oil. *Food Chemistry, 211*, 400-408".

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Aqueous enzymatic extraction of *Moringa oleifera* oil

Masni Mat Yusoff [a,b,*], Michael H. Gordon [a], Onyinye Ezeh [a], Keshavan Niranjan [a]

[a] Department of Food and Nutritional Sciences, University of Reading, Whiteknights, PO Box 224, Reading RG6 6AP, United Kingdom
[b] Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

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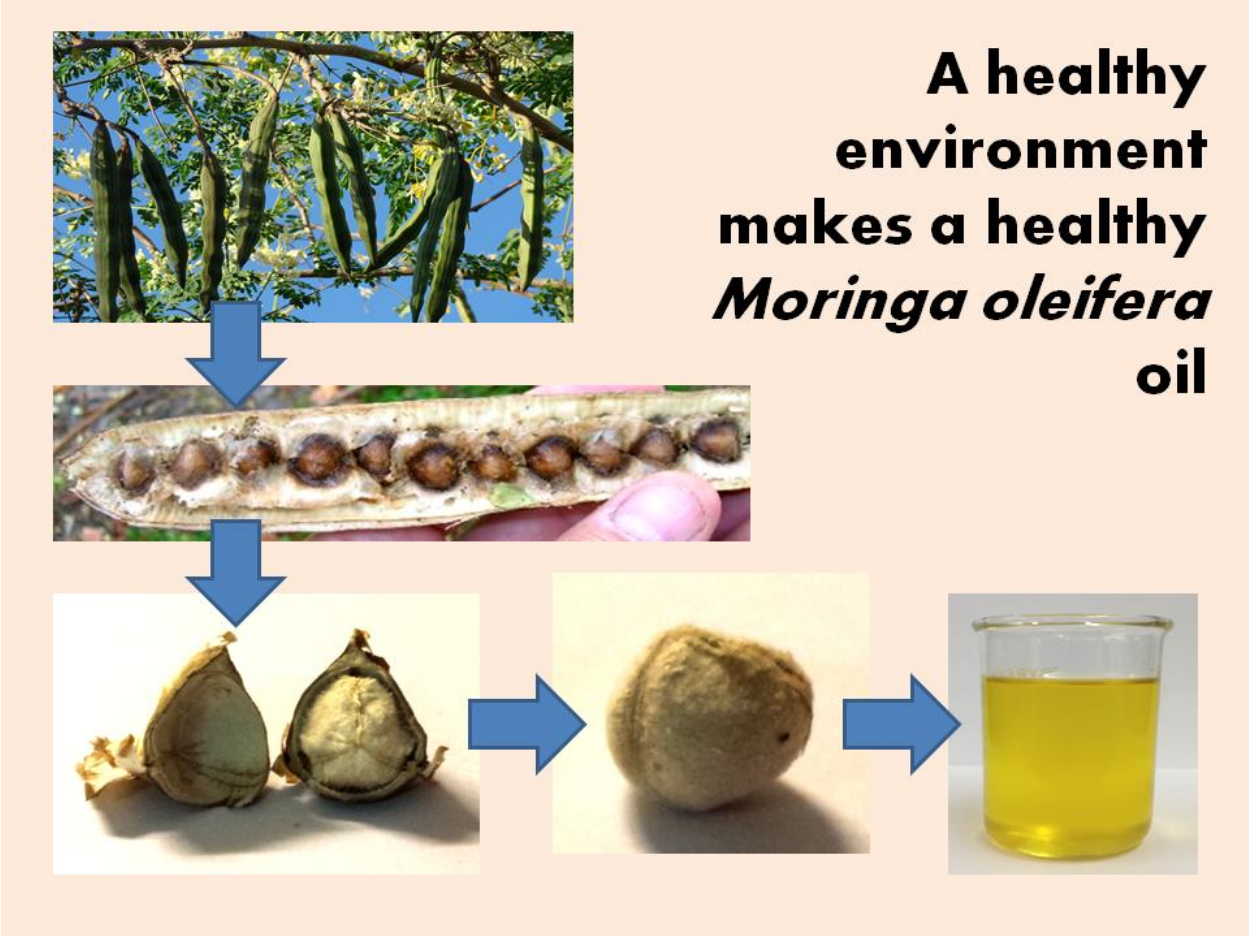
ABSTRACT

This paper reports on the extraction of *Moringa oleifera* (MO) oil by using aqueous enzymatic extraction (AEE) method. The effect of different process parameters on the oil recovery was discovered by using statistical optimization, besides the effect of selected parameters on the formation of its oil-in-water cream emulsions. Within the pre-determined ranges, the use of pH 4.5, moisture/kernel ratio of 8:1 (w/w), and 300 stroke/min shaking speed at 40 °C for 1 h incubation time resulted in highest oil recovery of approximately 70% (g oil/g solvent-extracted oil). These optimized parameters also result in a very thin emulsion layer, indicating minute amount of emulsion formed. Zero oil recovery with thick emulsion were observed when the used aqueous phase was re-utilized for another AEE process. The findings suggest that the critical selection of AEE parameters is key to high oil recovery with minimum emulsion formation thereby lowering the load on the de-emulsification step.

Appendix 3. This slide was presented during the Three Minute Thesis Competition in the Doctoral Research Conference on 18 June 2015, organized by the Graduate School, University of Reading.



Appendix 4. Acceptance letter for an oral presentation in the International Conference on Food Technology and Agricultural Engineering, 28-29 May 2015, Tokyo, Japan.

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ACCEPTANCE LETTER

December 10, 2014

PhD Candidate Masni Mat Yusoff
University of Reading
United Kingdom

Herewith, the international scientific committee is happy to inform you that the peer-reviewed draft paper code 15JP05000422 entitled (Effect of aqueous enzymatic extraction parameters on the Moringa oleifera oil yield and formation of emulsion by Masni Mat Yusoff, Michael H. Gordon, Keshavan Niranjan) has been accepted for oral presentation as well as inclusion in the conference proceedings of the ICFTAE 2015 : International Conference on Food Technology and Agricultural Engineering to be held in Tokyo, Japan during May, 28-29, 2015. The high-impact conference papers will also be considered for publication in the special journal issues at http://waset.org/Publications.

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Sincerely,

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Appendix 5. Acceptance letter for a poster presentation in the 29th EFFOST International Conference, Food Science Research and Innovation: Delivering sustainable solutions to the global economy and society. 10-12 November 2015, Athens, Greece.

10/17/2015 EFFOST International Conferce 2015 - Poster Accepatnce Email - Masni MatYusoff

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To: Masni MatYusoff <M.MatYusoff@pgr.reading.ac.uk>;

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Delivering sustainable solutions to the global economy and society

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Poster Acceptance Letter

ABSTRACT REF NO: EFFOST2015_0820

23 September 2015

m.matyusoff@pgr.reading.ac.uk

Dear M. Mat Yusoff,

Thank you for submitting an abstract to present at the **29th EFFoST International Conference.** On behalf of the Organising Committee I am delighted to inform you that your abstract entitled " **Aqueous enzymatic extraction of Moringa oleifera oil with ultra high pressure pre-treatment**" after review by expert members of the Scientific Committee has been accepted for **Poster presentation** at the Conference.

Title:	**Aqueous enzymatic extraction of Moringa oleifera oil with ultra high pressure pre-treatment**
Authors:	**M. Mat Yusoff, M.H. Gordon, O. Ezeh, K. Niranjan**
Presenting Author:	**M. Mat Yusoff**

Appendix 6. This article has been published in the American Oil Chemists' Society (AOCS) *Inform* magazine, May 2016, Vol. 27(5), page 14-16.

Aqueous enzymatic oil extraction as a green processing method

The commercial extraction of edible oils from oil-bearing seeds typically involves the use of chemical solvents. Hexane and petroleum ether are popular extraction mediums due to their oil-soluble characteristics. Although the use of such solvents results in the highest yields of extracted oil, further refining steps are required to remove undesirable compounds and odors. These additional refining steps remove other, beneficial compounds that are naturally present in the oil, such as antioxidants and oil-soluble vitamins. An abundance of studies has additionally highlighted the disadvantages of using evaporative solvents at high temperatures—particularly when it comes to the safety of operators and the environment.

Masni Mat Yusoff

- The advantages of aqueous enzymatic oil extraction (AEE) as a green processing method have been documented by many studies.
- In spite of these potential benefits, solvent-based extraction is still the main method for extracting edible oils from oilseeds.
- This article reviews the advantages of AEE as well as the disadvantages that have limited its use.

In aqueous enzymatic extraction (AEE), an aqueous phase (or water) serves as the extraction medium. The medium is adjusted to a specified pH value, followed by addition of enzymes, and incubation of this enzyme-containing medium at a specified time, temperature, and shaking speed. This causes oil from the seeds to be released into the water as free oil, which may go on to form an oil-in-water emulsion with the water (Fig. 1). In most of the studies that did not use enzymes, aqueous extraction resulted in lower oil recovery (Fig. 2). This highlights the important role enzymes play in hydrolyzing the cell wall and membrane of the oil-bearing cells for higher oil release.

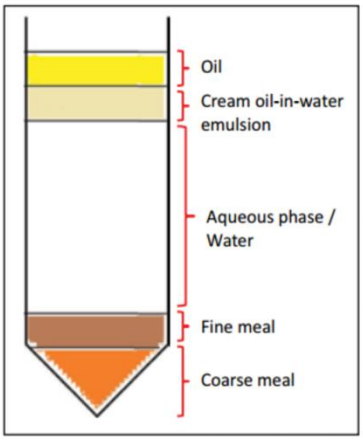

FIG. 1. Separation of different phases upon centrifugation after an aqueous enzymatic extraction method

Appendix 6 (continue). This article has been published in the American Oil Chemists' Society (AOCS) *Inform* magazine, May 2016, Vol. 27(5), page 14-16.

The use of water in place of solvent is highly desirable as a green processing method for oil extraction from various oil-bearing materials. Abundant studies have shown great interest in this method as compiled and discussed in review papers by Rosenthal *et al.* (1996) and Mat Yusoff *et al.* (2015). More ongoing studies concerning this approach were also reported in the case of pumpkin seeds (Jiao *et al.*, 2014), tiger nuts (Ezeh *et al.*, 2016), *Lophira lanceolata* seeds (Nonviho *et al.*, 2015), and sesame seeds (Ribeiro *et al.*, 2016). Recent studies also focused on the use of enzymes for the separation of the emulsified oil, as in the case of emulsified oils from maize germ (Matsakidou *et al.*, 2015). This de-emulsification method is discussed thoroughly in the review paper done by Mat Yusoff *et al.* (2015).

In light of the use of water as the extraction medium, the advantages of the AEE method are summarized as follows.

- Water is much safer and friendlier to the environment than solvents, which leads to safer and flexible operations.
- The use of water is economical, requires mild operational conditions, and the oil does not need to undergo further refining. These factors contribute to lower energy consumption, lower operational costs, lower capital investment, and consequently a lower overall cost of processing.
- AEE can be applied to a variety of oil-bearing materials, and the use of mild operating conditions results in valuable co-products such as non-toxic meal and value-added fiber and protein.

In addition to the green benefits gained from using water as an extraction medium, the use of enzymes imparts its own green benefits. As summarized by Simpson *et al.* (2012), the advantages of using enzymes are listed as follows.

- Enzymes always act only on their substrates due to their selective characteristics. Consequently, fewer co-products and waste products are formed during the production of food products.
- Enzymes are relatively nontoxic, which makes their use in foods and as processing aids acceptable to consumers.
- Enzymes function even at low concentrations and under mild processing or reaction conditions, which are easier on the environment and allow for the use of less expensive stainless steel vessels.
- Enzymes are composed of natural protein structures which are readily deactivated after use. They can also be immobilized for further recovery and re-use, which reduces processing costs.

Despite the advantages of AEE as a green processing method, AEE is only used commercially in the extraction of olive and avocado oil. Two key factors limiting large-scale commercial application of AEE to other oil-bearing materials have been identified.

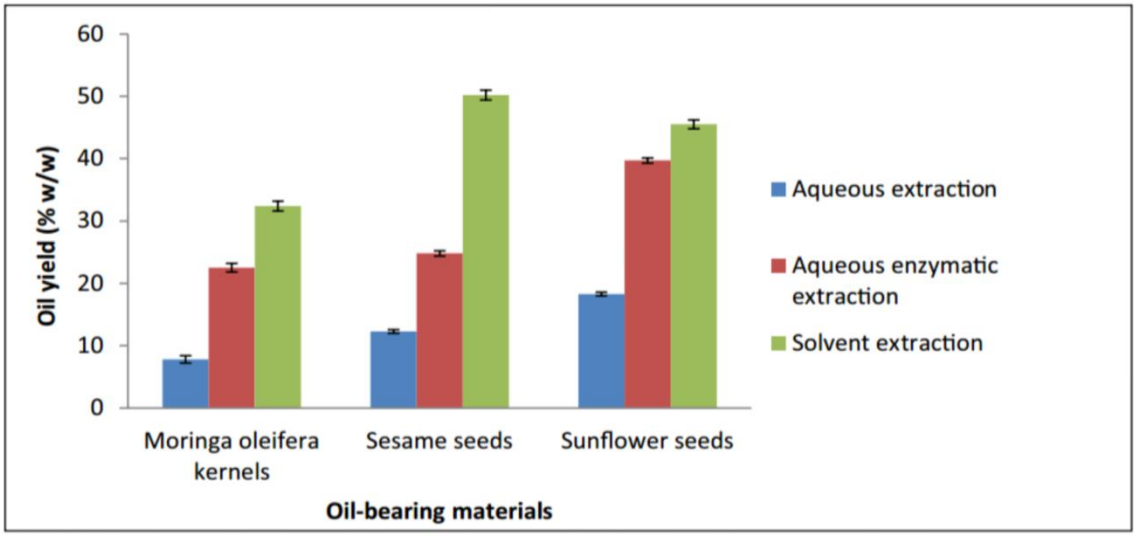

FIG. 2. The oil yields of different oil-bearing materials from aqueous extraction, aqueous enzymatic extraction, and solvent extraction methods. Although several enzymes were tested, the oil yields depicted are only those of the enzymes that achieved the highest yields for each material: protease (Protex 7L) for *Moringa oleifera* kernels (Latif *et al.*, 2011), protease (Alcalase 2.4L) for sesame seeds (Latif and Anwar, 2011), and a mixture of carbohydrases (*Viscozyme* L) for sunflower seeds (Latif and Anwar, 2009).

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- Most studies reported a lower oil recovery from an AEE process as compared to solvent extraction (Fig. 2, page 15). In most cases, the cell wall and membrane were not fully hydrolyzed by the enzymes, which prevented further oil release into the water.
- A portion of the released oil formed an oil-in-water emulsion with the water. This requires a further de-emulsification method for higher total oil recovery. This downstream process may results in higher operational costs.

As discussed by Dalsgaard and Abbotts (2003), in food industry, energy consumption is highly dependent on the types of food processed and the processing steps involved. AEE for oil-bearing seeds has only been done at laboratory scale due to the disadvantages highlighted above. Limited resources have made it difficult to evaluate overall energy costs and to identify any partial energy-savings that could potentially be incorporated into the process—aside from those related to the use of water as the extraction medium. Also, because solvent extraction has been the main oil-extraction method for oil seeds for decades, converting to a new extraction method would involve unknowns with respect to time, costs, energy expenditures, consultation experts, production losses, and rate of success.

In the case of palm oil, using water as an extraction medium could contribute to a reduction in the use of chemicals throughout the entire palm-oil-extraction process. This could potentially reduce the production of Green House Gas (GHG), potentially offsetting the lower oil extraction rate. Although further de-emulsification could require a longer dragging-time, the amount of gas released would probably be lower than that released during solvent extraction. Production of a lower GHG value is of great market demand, which could contribute to lower expenditures for maintaining the biogas plant in the future. Such advantages are encouraging. (Personal communication, 2016).

Despite its disadvantages, AEE offers great potential for significant long-term benefits to both the environment and health of operators. These potential benefits gave rise to several ongoing studies aimed at overcoming the limitations of AEE during the last few decades.

Masni Mat Yusoff is a PhD student in the Department of Food and Nutritional Sciences, University of Reading, Reading, United Kingdom. She is also a tutor in the Faculty of Food Science and Technology, Universiti Putra Malaysia, UPM Serdang, Malaysia. She can be contacted at m.matyusoff@pgr.reading.ac.uk., or masniyusoff@upm.edu.my

Information

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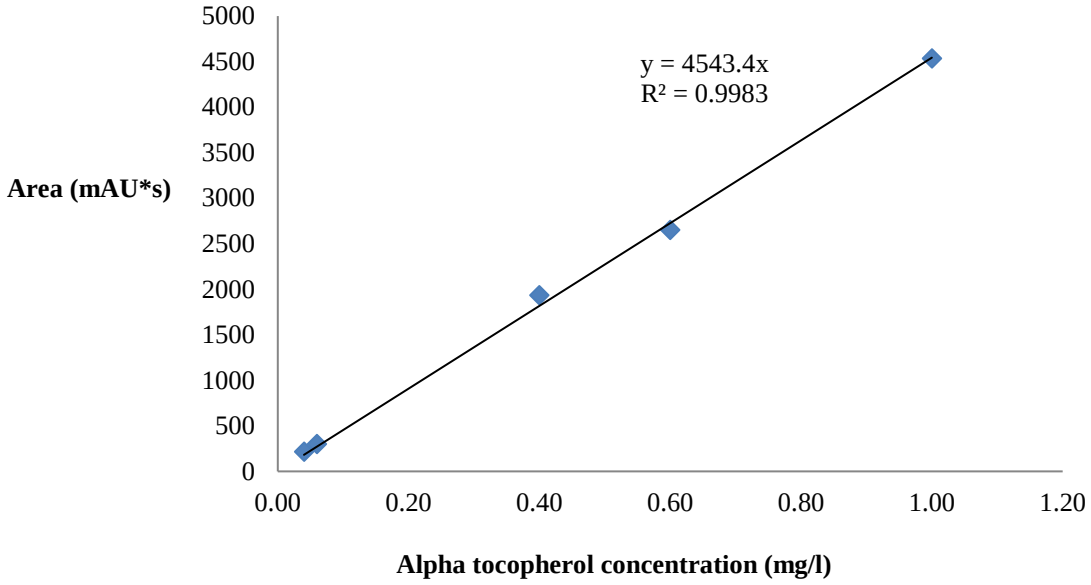
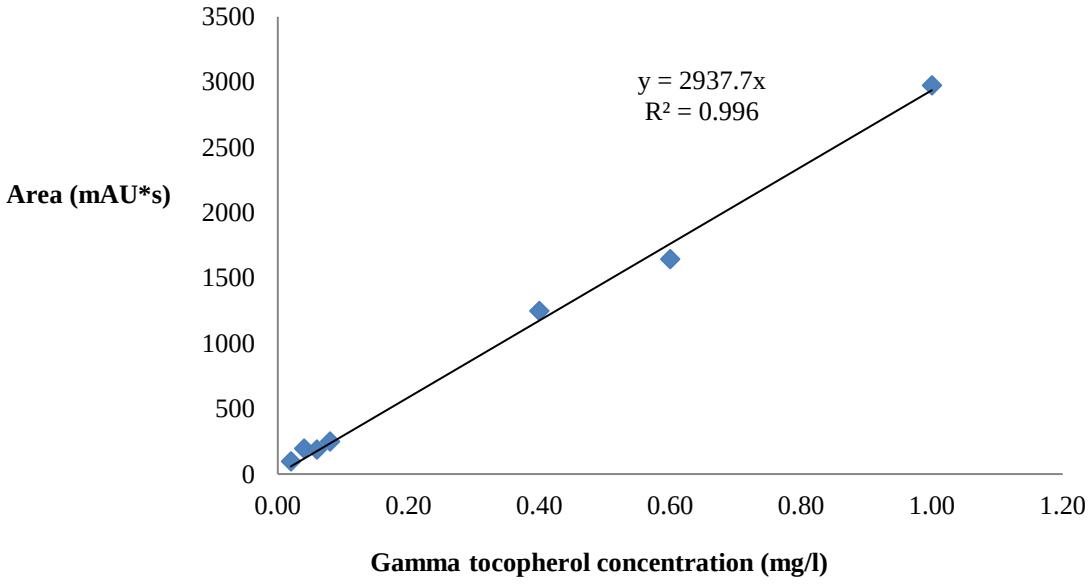
Appendix 7. Calibration curves of (a) alpha tocopherol and (b) gamma tocopherol standard solutions in methanol as detected by HPLC-UV.



(a)



(b)