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Oxidative properties of *Moringa oleifera* kernel oil during storage

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Abstract

BACKGROUND

Moringa oleifera (MO) kernel oil is categorized as high-oleic oil which resembles olive oil. However, different from olive, MO trees are largely available in most subtropical and tropical

countries. Therefore, in these countries, the benefits of oleic acid can be obtained at a cheaper price through consumption of MO kernel oil. This study reports on the effect of different extraction methods on oxidative properties of MO kernel oil during storage for 140 days at 13 ºC, 25 ºC, and 37 ºC.

RESULTS

All aqueous enzymatic extraction (AEE)-based methods generally resulted in oil with better oxidative properties and higher tocopherol retention than the use of solvent. Prior to AEE, boiling pre-treatment deactivated the hydrolytic enzymes and preserved the oil quality. In contrast, high pressure processing (HPP) pre-treatment accelerated hydrolytic reaction and resulted in higher free fatty acids after 140 days at all temperatures. No significant changes were detected in the oils' iodine values and fatty acid compositions. The tocopherol contents decreased significantly at both 13 ºC and 25 ºC after 60 days in the oil from SE method, and after 120 days in oils from AEE-based methods.

CONCLUSION

These findings are significant in highlighting the extraction method resulting in crude MO kernel oil with greatest oxidative stability in the storage conditions tested. Subsequently, the suitable storage condition of the oil prior to refining can be determined. Further studies are recommended in determining the suitable refining processes and parameters for the MO kernel oil prior to application in variety food products.

Keywords horseradish, drumstick, murunggai, seed, lipid, fat

Introduction

Moringa oleifera (MO), also known as horse-radish, kelor, or drumstick tree, are widely distributed in Pakistan, India, Nigeria, Philippines, Kenya, Carribean Island, Cambodia, Malaysia, and Bangladesh. One of the important parts of the tree is its fruit pods which turn into brown color upon maturation and contain mature brown MO seeds. The kernels inside the mature seeds contain the edible MO oil, also known as Ben or Behen oil [1-9]. This oil is applicable in perfume industry, hair-care products, in medicinal practices, and act as a good lubricant for fine machineries [7]. As an edible oil, MO oil is generally applied as culinary and salad oil in Haiti and other countries [10, 11]. Tsaknis and Lalas (2002) [12] and Abdulkarim et al. (2007) [13] 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 tocopherols content [7, 12, 14]. These properties contributed to the oil's oxidative stability [6], and consumption of oleic acids was always related to reduced-risk of developing coronary heart disease [13, 15].

A number of techniques are available for oil extraction from MO kernels, which include solvent extraction (SE) and aqueous enzymatic extraction (AEE) methods, among others. The AEE of oil from oil-bearing materials was reviewed by Mat Yusoff et al. (2015) [16] and Rosenthal et al. (1996) [17], while studies specifically on AEE of MO oil have been reported by Mat Yusoff et al. (2016) [18], Latif et al. (2011) [19], and Abdulkarim et al. (2005, 2006) [4, 20]. According to these studies, MO kernel cells contain approximately 35% (w/w) protein and 40% (w/w) fat content, and the protein is the major component in the MO cell wall surrounding the oil. These findings proved the need to add a proteolytic enzyme to hydrolyze the protein component for higher oil release. At the end of an AEE process, two types of oils are produced – free oil which can be recovered, and emulsified oil in a cream emulsion which is formed due to the use of water as an extraction medium in the presence of

MO kernel protein. The earlier studies only succeeded in extracting up to 70% (w/w) total MO oil – measured as the mass of oil extracted using enzymes against the total oil extracted using solvent.

Similar issues of lower extracted oil in an AEE process as compared to the use of solvent were also reported in most studies involving other types of oils as reviewed by Mat Yusoff et al. (2015) [16]. Many studies attempted to overcome this problem by conducting a pre-treatment in order to assist cells rupture for higher oil release in the following AEE process. One of the pre-treatments conducted was the use of high-pressure processing (HPP) on soybean seeds which resulted in 3.20% (w/w) and 1.30% (w/w) higher free oil recovery at 200 MPa and 500 MPa, respectively. In the case of MO kernels, Mat Yusoff et al. (2017) [21] reported that the HPP pre-treatment successfully modified the MO protein structure into a form of less emulsifying ability, thus smaller amount of oil got emulsified which resulted in thinner cream emulsion layer and 4.19% (w/w) higher free oil recovery as compared to the use of AEE alone [21]. Additionally, the study also reported 4.98% (w/w) increase in free oil recovery when the AEE was pre-treated with boiling (100 °C, 5 min) – this boiling pre-treatment was conducted in earlier studies [4, 18, 19, 20] prior to an AEE of MO oil to inactivate the natural hydrolytic enzymes in the seed kernels [22].

Fotouo et al. (2016) [23] demonstrated the effect of storage of MO seeds on the quantity and quality of the MO oil for its potential in biodiesel production. However, the effect of storage of MO oil at different temperatures on its quality attributes had never been reported, which is of great importance in determining the shelf life of the oil. Furthermore, to the best of our knowledge, the study on HPP pre-treatment prior to AEE of MO oil was conducted for the first time [21], and no study has reported on the quality attributes of the MO oil extracted from this process.

The main objective of this study was 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 ºC, 25 ºC, and 37 ºC, 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.

Materials and Methods

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.

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.

Preparation of *Moringa oleifera* Kernels for Oil Extraction

The MO kernels were randomly collected and conditioned at 50 ºC for 8 hr followed by grinding (De'Longhi KG49 Electric Coffee Grinder, Hampshire, UK) and sieving using a vibratory sieve shaker (Fritsch, Analysette 3E) to ≤710 µm particle size. According to Mat Yusoff et al. (2016) [18], the use of ground-sieved MO kernels at this particle size resulted in highest amount of MO oil (410.3 g kg^{-1}) as extracted using hexane. All oil extraction methods conducted in this study were based on studies done by Mat Yusoff et al. (2016, 2017) [18, 21] and were performed on the ground-sieved MO kernels.

Solvent Extraction (SE) Method

Soxhlet method was used to extract the MO oil with the use of hexane for 6 hr extraction time. A total of six refluxes were used each time. The hexane was evaporated from the extracted oil in a round bottom flask of pre-determined weight by using a rotary evaporator (60 ºC, 10 min), followed by heating in an oven (100 ºC, 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.

Aqueous Enzymatic Extraction (AEE) Method

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 ºC 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 'Recovery of free oil'.

Aqueous Enzymatic Extraction with Boiling Pre-treatment (B-AEE)

Similar AEE as in the previous section was conducted, with addition of boiling pre-treatment in a water bath (100 ºC, 5 min), followed by cooling to room temperature prior to adjusting the mixture's pH, Recovery of the free oil is explained in section 'Recovery of free oil'.

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. According to Mat Yusoff et al. (2017) [21], some preliminary tests were carried out to determine this solid-to-liquid ratio. The use of smaller amount of water caused formation of a very thick paste which adhered to the polyester bag, thus wasted some of the sample. In another way, addition of higher water content resulted in a very dilute mixture which thus allowed only small amount of ground-sieved MO kernel to be processed at one time.

The mixtures at 1:1 (w/w) ratio were treated with high pressure (Stansted Fluid Powder Ltd., Stansted, UK) at 50 MPa and 60 ºC for 35 min, followed by addition of distilled water up to 4:1 water/kernel (w/w) ratio for the subsequent AEE as in the earlier section. Recovery of the free oil is explained in section 'Recovery of free oil'.

Recovery of Free Oil

The centrifuged mixtures obtained in all the AEE-based methods explainer earlier were kept at -20 ºC for 24 h. The solidified oil was transferred to a crucible of pre-determined weight and heated in an oven at 100 ºC 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:

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

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

Storage of *Moringa oleifera* Oil

MO oil samples extracted using the SE method and all AEE-based methods explained in previous sections 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. The storage temperatures used in this study were in

198 reference to Pristouri et al. (2010) [24]. At 13 ºC, the MO oil was stored to simulate the
199 temperature of the cellar commercially used for storing olive oil. Storage at 25 ºC and 37 ºC
200 were selected for simulating room temperature and elevated ambient temperature normally
201 occurred during the summer, respectively. All oil samples from different extraction methods
202 were stored in these temperatures for 140 days, and the analysis of their PV, *p*-AV, TOTOX,
203 FFA, and tocopherol content were performed on day 0, day 60, day 120, and day 140.
204 Additionally, differences in the IV and FAC between the extracted oils before (i.e. day 0) and
205 after the whole storage period (i.e. day 140) were also examined. Determination of these
206 oxidative properties was performed on each oil sample in triplicate.
207
208 **Determination of Peroxide Value (PV)**
209
210 Peroxide value (PV) of the oil samples was determined in reference to AOCS Official
211 Method Cd 8-53 (2000) [25] and MPOB Test Method p2.3 (2004) [26] with modification. A
212 mixture of 5.0 g oil sample and 20 ml glacial acetic acid/chloroform (1.5:1 v/v) was prepared
213 and swirled until completely dissolved. Excess of saturated potassium iodide solution was
214 added to the mixture, followed by swirling for 1 min. The mixture was combined with 30 ml
215 distilled water and few drops of starch indicator, before being titrated with 0.01 N sodium
216 thiosulphate until the blue-gray color disappeared. The above steps were repeated without
217 adding the oil sample for blank purpose. The following formula was used to calculate the PV
218 of the oil sample:
219
220 $$\text{Peroxide value } \left(\text{mEq}\frac{\text{O}_2}{\text{kg}}\right) = \frac{(\text{Vb} - \text{Vs}) \text{ x } 0.01 \text{ x } 1000}{\text{W}}$$
221
222 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)

Determination of *p*-Anisidine Value (*p*-AV)

p-Anisidine value (*p*-AV) of oil samples was slightly modified according to AOCS Official Method Cd18-90 (2000) [25]. 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)

Determination of Total Oxidation (TOTOX) Value

Total oxidation (TOTOX) value of the oil samples was determined according to AOCS Official Method Cg 3-91 (2000) [25]. 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

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 AOCS Official Method Cd 3d-63 (2000) [25]. 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

Determination of Iodine Value (IV)

Iodine value (IV) of the oil samples was determined according to MPOB Test Method p3:2 (2004) [26] and AOCS Official Method Cd 1d-92 (2000) [25] with slight modification. Earlier studies revealed IV of MO oil which ranged from 60-70 g I_2 / 100 g [7, 14, 19]. 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 were 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

Determination of Fatty Acid Composition (FAC)

Fatty acid composition (FAC) of MO oil was determined according to Ezeh et al. (2016) [27], 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 ºC and 260 ºC, respectively, while the oven temperature was 230 ºC (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.

Determination of Tocopherol

Following the method used by Ezeh et al. (2016) [27] and Costa et al. (2010) [28] 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 ºC 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.

Results and Discussion

Effect of storage condition on peroxide value, *p*-Anisidine value, and total oxidation value of *Moringa oleifera* kernel oil

In the following discussion, the following terms will be used: SE-oil, AEE-oil, B-AEE-oil, and HPP-AEE-oil which indicate the MO kernel oil extracted using SE, AEE, B-AEE, and HPP-AEE methods, respectively.

Oxidation of lipids takes place by a free radical chain mechanism which can be explained in terms of initiation, propagation, and termination processes. These processes generally comprised of complex sequences and overlapping reactions. Figure 1 revealed changes in PV in the MO oil samples during storage at different temperatures of (a) 13 °C, (b) 25 °C, and (c) 37 °C. PV indicates formation of peroxides and hydroperoxides resulted from propagation reaction. This reaction describes the first oxidation stage involving formation of hydroperoxides as fundamental primary products. These compounds do not have significant effect on the oil's flavor deterioration. Propagation is also the most widely oxidation reaction that takes place in an oil and fat [29-33]. According to Figure 1(a), on day 0, there was no significant difference ($p > 0.05$) between PV of MO kernel oil extracted using

different methods (approximately 0.49 mEq O_2/kg). However, after 140 days storage at 13 °C, PV of the SE-oil, AEE-oil, and HPP-AEE-oil increased significantly ($p < 0.05$) (0.75-0.98 mEq O_2/kg). These findings indicated that at 13 °C, oxidation reaction started to take place in the oil samples after 60 days of storage in SE-oil and AEE-oil. The PV started to increase later after 120 days in HPP-AEE oil, while the PV of B-AEE oil remained unchanged. At 25 °C, Figure 1(b) shows significant increase ($p < 0.05$) in PV of SE-oil from day 0 (0.49 mEq O_2/kg) to day-120 (2.19 mEq O_2/kg) as compared to other oil samples. At 37 °C (Figure 1(c)), faster increase in PV was observed in the SE-oil. On day-60, the PV of SE-oil at 25 °C was 0.99 mEq O_2/kg, and the PV was significantly higher ($p < 0.05$) at 37 °C; 1.97 mEq O_2/kg. Furthermore, on day-120, the PV of SE-oil at 25 °C was 2.19 mEq O_2/kg, and the PV was significantly higher ($p < 0.05$) at 37 °C; 2.96 mEq O_2/kg. These findings revealed the significant effect of higher storage temperature in increasing the oxidation rate of SE-oil as compared to other oil samples.

After 120 days at both 25 °C and 37 °C, the PV of SE-oil started to decrease which indicated the start of initiation reaction. In this reaction, decomposition of hydroperoxides into free radicals took place which is an endothermic reaction promoted by the higher temperatures of 25 °C and 37 °C. Moreover, this thermal oxidation of unsaturated lipids is normally an autocatalytic reaction and is metal-catalyzed. The SE-oil was a crude oil which most likely contained trace metals. These trace metals are very difficult to be eliminated, thus they may act as potent catalysts which catalyzed the initiation reaction [33]. Figure 1 (a-c) also shows that the B-AEE-oil exhibited higher oxidative stability upon 140 storage days at 13 °C, since its PV did not increase at this temperature as compared to other oil samples. On the other hand, the maximum PV of HPP-AEE-oil was 0.75 mEq O_2/kg under all storage condition.

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) [7, 12]. According to O'Brien (2009) [29], the quality of SE-oil may be lower than that of pressed oil due to simultaneous extraction of non-triglycerides and other undesirable minor components in the former case. Therefore, in this study, the SE-oil 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 [29, 34]. 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 [30, 31]. Moreover, according to Codex (1999) [35], the maximum PV for refined oil is 10 mEq O_2/kg, while for cold pressed and virgin oils, the maximum PV is 15 mEq O_2/kg. Despite the high PV of SE-oil, all values in all storage conditions were less than 3 mEq O_2/kg. Thus, in terms of PV, the MO kernel oil samples from SE and enzymatic extraction methods are categorized as oil samples with low oxidation rate within the storage conditions used.

With reference to Figure 1(c) at 37 °C, the PV of SE oil started to decrease after 120 days which indicated decomposition of primary oxidation products into secondary oxidation products as explained earlier, besides indicating the faster oxidation reaction in the SE-oil as compared to other oil samples. This phenomenon is reinforced by the sudden increase in the *p*-AV of SE oil up to 10.72±1.41. The *p*-AV represents the formation of secondary oxidation products in the form of 2-alkenals and 2,4- alkadienals. In this same storage condition, the *p*-AV of other oil samples remained as low as 0.70-1.44. Latif et al. (2011) [19] reported approximately similar *p*-AV of MO kernel oil extracted using solvent and enzymes which

ranged 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) [36], or less than 2.0 according to Subramaniam et al. (2000) [37]. Therefore, to conclude, SE-oil is considered unacceptable after 120 days of storage at 37 ºC due to the high *p*-AV, and TOTOX value of 16.64.

Effect of storage condition on free fatty acids of *Moringa oleifera* kernel oil

FFA is responsible for the off-flavor and off-odor in fats and oils products [29, 38]. Prolong storage time causes decomposition and oxidation of secondary oxidation products into FFA as tertiary oxidation product [31]. 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 moisture which initiates a hydrolysis reaction, causing formation of FFA [29].

Figure 2 shows the FFA (as oleic acid) of oil samples stored at different temperatures of (a) 13 °C, (b) 25 °C, and (c) 37 °C. On day 0, the SE-oil exhibited significantly higher ($p < 0.05$) FFA (2.02±0.14%) than the B-AEE-oil (1.09±0.32%). Abdulkarim et al. (2005) [4] also reported higher FFA in SE-oil (2.48%) as compared to B-AEE-oil (1.13%). The higher FFA in SE-oil may be due to simultaneous extraction of other non-triglycerides and minor components by the solvent which also contributed to higher PV as explained earlier.

Additionally, on day 0, the B-AEE-oil exhibited nearly 4 times lower FFA (1.09±0.32%) than the AEE-oil (3.85±0.26%). This finding proved the importance of boiling pre-treatment on the ground MO kernels to inactivate hydrolytic enzymes prior to oil extraction. Along the storage period at all temperatures, the FFA in B-AEE-oil remained at low level of 0.96-1.71%, while the FFA in AEE-oil decreased to 1.34-2.08%. These values were significantly lower ($p < 0.05$) as compared to the FFA in HPP-AEE-oil which

significantly increased ($p < 0.05$) from day 0 to day 140 at all temperatures of 13 ºC (6.66±0.19%), 25 ºC (7.19±0.32%), and 37 ºC (5.96±0.12%). These significantly higher FFA in HPP-AEE-oil samples were most likely due to possible presence of minute moisture content in the oil.

According to O'Brien (2009) [29], presence of moisture in combination with high pressure may results in acceleration of hydrolytic reaction, therefore resulted in higher FFA as compared to other enzymatic extraction methods in this study. Increase in FFA was also observed in the SE-oil along the storage period, yet was still lower (2.47-3.62%) than that of HPP-AEE-oil samples.

Codex (1999) [35] indicates maximum FFA level in oil 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, highest FFA was observed in HPP-AEE-oil on day 140: 13.26 mg KOH / g (13 ºC), 14.30±0.64 mg KOH / g (25 ºC), and 11.86 mg KOH / g (37 ºC). The B-AEE-oil exhibited lowest AV of below 4.0 mg KOH / g throughout the storage conditions (1.34-3.40 mg KOH / g). These findings further highlighted the significant effect of boiling pre-treatment in inactivating the hydrolytic enzymes, prevents enzymatic hydrolysis, and thus preserving the oil's oxidative stability.

Effect of storage condition on iodine value and fatty acid composition of *Moringa oleifera* oil

There was no significant difference ($p > 0.05$) in 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 ºC and 37 ºC. Abdulkarim et al. (2005) [4] also reported similar IV of SE-oil (65.4 g I_2 / 100 g) and B-AEE-oil (66.1 g I_2 / 100 g). Additionally, there was no difference in IV between SE-oil

(66.6-66.8 g I_2 / 100 g) and cold-pressed oil (66.8 g I_2 / 100 g) in a study done by Tsaknis et al. (1999) [7]. These findings indicated that the MO 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 by insignificant changes in FAC of the oil samples (Table 1(a-d)) 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 [13, 15]. 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 the Introduction.

Effect of storage condition on α-tocopherol content in *Moringa oleifera* oil

Figure 3 shows the α-tocopherol content in oil samples stored at different temperatures of (a) 13 °C, (b) 25 °C, and (c) 37 °C. On day 0, highest α-tocopherol content was discovered in B-AEE-oil (31.17±3.52 mg/l) which was insignificantly different ($p > 0.05$) with the AEE-oil (28.04±1.26 mg/l) and HPP-AEE-oil (28.77±1.05 mg/l). As compared to these enzymatic extraction methods, significantly lower ($p < 0.05$) α-tocopherol content was observed in SE-oil (23.33±0.99 mg/l). In a study done by Tsaknis et al. (1999) [7] 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) [14] 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) [12] on seed kernels of India origin, the SE-oil contained higher α-tocopherol (15.38 mg/kg) as compared to cold-pressed oil (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) [12] (5.06-15.38 mg/kg) were far too low than that of reported by Tsaknis et al. (1999) [7] (98-105 mg/kg) and Rahman et al. (2009) [14] (121-154 mg/kg). These findings highlighted 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 kernel oils extracted using solvents, enzymes, cold press, and supercritical fluid extraction method [6, 7, 12, 14], 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.

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

Effect of storage condition on γ-tocopherol content in *Moringa oleifera* oil

Figure 4 shows the γ-tocopherol content in oil samples stored at different temperatures: (a) 13 °C, (b) 25 °C, and (c) 37 °C. In this study, all MO oil samples exhibited lower γ-tocopherol content 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) [7] reported higher γ-tocopherol content in cold-pressed oil (39.54 mg/kg) than that of SE-oil (27.90-33.45 mg/kg) with the use of MO seed kernels of Kenya origin. Tsaknis and Lalas (2002) [12] also reported higher γ-tocopherol content in cold pressed-oil (25.40 mg/kg) as compared to SE-oil (4.47-5.52 mg/kg) from MO seed kernels of India origin. In a study done by Rahman et al. (2009) [14] 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 140, the γ-tocopherol content in all oil samples was significantly higher ($p < 0.05$) at 37 ºC (12.48-16.07 mg/l) as compared to lower storage temperatures of 13 ºC (7.76-9.61 mg/l) and 25 ºC (7.58-8.20 mg/l). The reasons for this sudden increment was not identified. In the SE-oil, this trend was different from that of α-tocopherol which did not change upon different storage temperatures on day 140.

At 13 ºC, the γ-tocopherol content in all oil samples from all extraction methods decreased significantly ($p < 0.05$) after 120 days. Similarly, at 25 ºC, the γ-tocopherol content in oil samples from enzymatic extraction methods decreased after 120 days, while in SE-oil, the γ-tocopherol content started to decrease earlier which was after 60 days. At 37 ºC, insignificant ($p > 0.05$) decrease in γ-tocopherol content in SE-oil 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 ºC, the storage time imparted no significant changes in the γ-tocopherol content in oil samples from all extraction methods.

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 increased in temperature. The SE-oil underwent greater oxidative deterioration as compared to other AEE-based oils. The SE-oil was not in good quality after 120 days at 37 ºC, while it is still acceptable during storage at 13 ºC up to 140 days of storage. The AEE-based oils exhibited approximately similar oxidative properties throughout the whole storage conditions, except in the case of HPP-AEE-oil which exhibited high FFA content after 120 days, even at as low as 13 ºC 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 inactivate the hydrolytic enzymes in the seed kernels for better oil quality during storage. Thus, to conclude, within the storage conditions tested, B-AEE-oil exhibited greatest oxidative properties, followed by the AEE-oil, HPP-AEE-oil, and the SE-oil. 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 ºC, the concentration of both α- and γ-tocopherols in all oil samples were nearly two times higher than their concentrations at lower temperatures, and the reasons for this finding is not yet discovered. Both the boiling and HPP pre-treatments did not significantly affect the tocopherol contents of the MO oil. Moreover, the AEE-based 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.

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Table Caption

Table 1 Fatty acid composition (%) of *Moringa oleifera* oil from (a) solvent (hexane) extraction method (SE), (b) aqueous enzymatic extraction (AEE) method, (c) aqueous enzymatic extraction method with boiling pre-treatment (B-AEE), and (d) 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

Figure Captions

Fig. 1. Effect of different extraction methods on the peroxide values of *Moringa oleifera* kernel oil stored for 140 days 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.

Fig. 2. Effect of different extraction methods on the free fatty acids of *Moringa oleifera* kernel oil stored for 140 days 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.

Fig. 3. Effect of different extraction methods on the alpha tocopherol content in *Moringa oleifera* kernel oil stored for 140 days 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.

Fig. 4 Effect of different extraction methods on the gamma tocopherol content in *Moringa oleifera* kernel oil stored for 140 days 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.