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Research Article

Spectroscopic and Physicochemical Characteristic of Ozonated Rice Brand Oil as Antimicrobial

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ABSTRACT

Rice Brand Oil (RBO) is a vegetable oil, containing active substances that are very good for health and medicine. The use of RBO can be expanded by ozonation of unsaturated fatty acids in RBO to produce 1,2,4 trioxolane, aldehydes, hydroperoxides and peroxides as anti-viruses in patients with HIV, hepatitis, bird flu etc. The effect of the ozonation process on the characteristics of Rice Bran Oil (RBO) is estimated. Spectroscopic characteristics were analysed by GCMS and ^{13}C-NMR, while physical and chemical characteristics were studied by density, viscosity, pH, iodine number, peroxide number, and acid number. In addition, RBO color changes during the ozonation process also change. The carbon double bond in the RBO decreases with increasing ozonation time. 1,2,4 Trioxolane, aldehydes and peroxide are the main products in ozonation reactions. The presence of 1,2,4-trioxolane can be removed by gas chromatography and nuclear magnetic resonance spectroscopy analysis, where it arises during the 25-hour ozonation process, and the expanded field increases and ozonates for 175 hours. From ^{13}C-NMR spectrum, the presence of ozonides was confirmed by the signals at δ_C 104.5 ppm. Some saturated fatty acids formed during the ozonation processes from GCMS analysis, such as 1,1-dimethoxydodecane and methyl myristate proved that ozone as mediator reacted with the unsaturated fatty acid and broke down the double chain of C=C bond to become -C-C- bond. The amount of iodine decreases because the carbon double bonds change into a single bond and the amount of peroxide and acid increases because the component bonds have a single carbon bond, namely 1,2,4 Trioxolane, aldehydes and peroxide. The formation of 1,2,4-trioxolane and other single carbon bond compositions having higher molecular weight increases density and viscosity. Using existing ozone generators requires a longer ozonation time to place the RBO in saturated conditions and make 1, 2, 4-trioxolane unstable and degraded.

Keywords: Ozone, ^{13}C NMR, Peroxide Number, Iodine Number, 1,2,4-Trioxolane

INTRODUCTION

Rice Brand Oil (RBO) is a very special vegetable oil, containing active substances which are very good for health and medicines, such as rich in vitamin E (tocopherol and tootrienol), bioactive phytonutrients, phytosterols, γ-oryzanol, squalene alcohol and triterpene (Ali et al., 2017). According to Dougdoug (1999), the unsaturated fatty acid content in RBO is around 81,4% which consists of 50,1% oleic acid (C18: 1), 27,1% linoleic acid (C18: 2), and 2.2% linolenic acid (C18: 3), whereas the saturated fatty acids contained in this RBO consist of 0.9% stearic acid and 17.6% palmitic acid. The content of unsaturated fatty acids in RBO is much higher compared to Virgin Coconut Oil (VCO), which is only 6-8% (Ju and Vali, 2005).

The active substances contained in RBO can be as antioxidants (tocopherol and tocotrienol), anti-inflammatory (Gama-oryzanol), hypocholesterolemia (or-oryzanol), anti-aging (tootrienols) and anticancer properties (Poorna et al, 2016). The benefits of RBO as medicines can be increased through the ozonation process, where carbon-carbon double bonds from unsaturated fatty acids by ozone are converted to molozonida then become 1,2,4 Trioxolane and their stable derivatives such as aldehydes and peroxides (Criegee, 1975) as an anti-microbial (anti-bacterial, fungal and viral). Thus, RBO that has been ozoned has the potential be used to cure patients HIV/AIDS, hepatitis, bird flu and other diseases patients (Herman, 2013). Reaction of the formation of 1,2,4 Trioxolane and its derivatives resulting from the ozonation of unsaturated fatty acids in vegetable oils is presented in Figure 1 (Criegee, 1975).

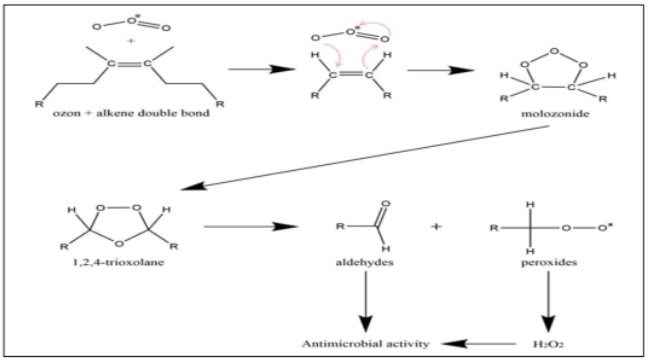

Fig.1: Criegee Mechanism for the Reaction of Ozone with Carbon-carbon Double bonds. (Criegee, R. 1975)

There are many ozonated vegetable oils that have been studied. For instance, Geweely (2006) did research about ozonated olive oil for antifungal properties. Díaz et al., (2005) studied the characteristics of ozonated sunflower oil, while Vallachi et al., (2011) investigated ozonated sesame oil characteristics for skin medicine purpose. Nonetheless, based on our literature searches so far, there are no researchers who have observed or studied ozonated RBO, whereas in fact, ozonated RBO can increase the value of a product, especially in medicine, cosmetics or personal care products, if it is used as one of the ingredients.

Ozonated vegetable oil is produced by an ozonation process from vegetable oil in a certain temperature and ozone dose. Ozonation can occur in unsaturated fatty acids or free fatty acids and produce many important products, such as ozonides or 1,2,4-trioxolane, peroxide and aldehydes (Zanardi et al, 2008; Bailey, 1978). According to Herman (2008), 1,2,4-trioxolane in ozonated vegetable oil can be used as an active ingredient for the treatment of diseases caused by microorganisms (antibacterial), fungi (anti fungicidal), and viruses (antivirus). So, it is suitable to be applied as an ingredient in the pharmaceutical and cosmetic industries. The content of ozonide or 1,2,4-trioxolane in ozonated vegetables oil is very dependent on process conditions, such as temperature, ozone dose, and the form of reactor used (Zanardi et al, 2008).

In this study, RBO was treated with an ozonation process to produce ozonated RBO, which has good properties for pharmaceutical or cosmetic needs. Spectroscopic characteristics were established by analyzing the RBO (before and after ozonation) using gas chromatography–mass spectrometry (GC-MS) and Nuclear magnetic resonance (NMR) spectroscopy. Physicochemical characteristics were obtained by analyzing kinematic viscosity, density, iodine number, peroxide number and acid number of RBO. Correlation between spectroscopy and physicochemical characteristic were also studied.

MATERIALS AND METHODS

Materials

Chemicals such as ethanol, phenolphthalein, NaOH, acetic acid glacial. chloroform, potassium iodide, sodium thiosulphate, were purchased from Merck and used without further purification. Starch and Rice Bran Oil were obtained from a local market in South Tangerang, Indonesia. The physicochemical characteristics of RBO can be shown in Table 1.

The Method Of Ozonation

The ozonated RBO was produced by a method described by Zanardi et al., (2008). Briefly, ozone/oxygen mixture was bubbled in a jacketed bubble column reactor containing 1000 mL of RBO for different times comprised between 5 and 175 hours (i.e. 5, 10, 15, 25, 50, 75, 125, 150, 175 hours). The operating temperature was kept at 5 °C and the RBO didn't freeze. Ozone was produced through a RS 09805 (60/50HZ) 110/220 Volt from ozone procesor Ressun (China) with dengan output ozon sebesar 0,025 g. L^{-1} per hour. The off-gas which contained non-reacted ozone was directed to two absorption containers, containing 100 ml acetic acid glacial and potassium iodide. A stirrer with a rotation rate of 750 rpm was set inside the reactor to make the RBO more homogenous. The ozonated RBO was then collected from the reactor to be

analyzed using NMR and GCMS, as well as for its physicochemical characteristic

NMR and GCMS analysis

The ^{13}C NMR spectra were collected from a JEOL JNMEX400 and JNM ECA500 single pulse spectrometer at 25 °C on untreated RBO and on RBO after 25 h, and 175 h of ozone treatment in $CDCl_3$. All the experiments were performed under the same experimental conditions and concentrations. The ^{13}C spectra were recorded with a relaxation delay of 2 s; a total of 1021 scans were collected for each sample with a 30◦ excitation pulse.

GCMS analysis of the untreated RBO and on RBO after 25 h, and 175 h of ozone treatment was performed using Shimadzu GCMS-QP2010 (Kyoto, Japan). Column temperature was programmed from 70-230°C at increasing rate of 10°C/min, held at initial for 1 min and equilibrium time for 3 min. The major peaks were analyzed by comparing its mass fragments patterns with standard spectra available in NIST library.

Iodine Number

Iodometric titration was used in this experiment to calculate the amount of ozone consumed by the oils/fatty acids. The reaction between iodide and ozone resulted in free Iodine as shown in Equation 1. Then Iodine was reacted with sodium thiosulphate, and starch acted as an indicator as shown in Equation 2. As an indication of equivalence point, the color would change from purple to colorless (Sadowska et al., 2008). The De-Ionized (DI) water was used as a blank solution.

$$O_3 + 2I^- + H_2O \rightarrow 2OH^- + O_2 + I_2 \quad (1)$$

$$I_2 + 2S_2O_32- \rightarrow 2I^- + S_4O_6^{2-} \quad (2)$$

Peroxide Number

According to British Pharmacopoeia, (2000a), Peroxide Number (PN) is the number which expresses the quantity of peroxide, in milli equivalents of active oxygen, which is contained in 1000 g of the substance. The PV of both untreated and ozonated samples were determined using the American Oil Chemists' Society (AOCS) official method, following the reaction mechanism in Equation (3) and (4):

$$2KI + 2CH_3COOH \rightarrow 2HI + 2CH_3COO-K^+ \quad (3)$$

$$R{\bullet}OO{\bullet}H + 2HI \rightarrow ROH + H_2O + I_2 \quad (4)$$

Peroxide ($R{\bullet}OO{\bullet}H$) will react with potassium iodide in the presence of acetic acid to result in iodine. Then, iodine is titrated with a sodium thiosulfate solution following Equation (2).

Acid Number

Acid Number (AN) according to British Pharmacopoeia, (2000b), is the number of base mass required (in mg) to neutralize the free acids per gram of the substance. Moreover, the acid value shows the degree to which the triglycerides in the oil sample have broken down to form free fatty acids. Sodium hydroxide was used as titrant to titrate the mixture solution between the oil sample and ethanol, while phenolphthalein acted as an indicator (Almeida Kogawa et al., 2015).

Ozonation Efficiency

The Ozonation Efficiency (OE) can be described as the ratio between the amount of peroxidation due to the ozonation process and the amount of total ozone applied to the system (Sega et al., 2010). It was calculated with Equation (8):

$$OE = \frac{PN_S - PN_0}{1000} \times \frac{mEq\ O_3}{AOD} \times 100\% \quad (8)$$

where PN_S and PN_0 are the Peroxide Number of the ozonated and untreated RBO, respectively; $mEqO_3$ is the ozone equivalent weight; and AOD represents the applied ozone dose (in mg g^{-1}).

Density, Kinematic Viscosity, and pH analysis

The density measurements were done by measuring the weight of pycnometer in the absence and presence of RBO samples. The kinematic viscosity was measured with Ostwald capillary viscometers at 25 °C, and pH was measured using a digital pH meter

RESULTS AND DISCUSSION

Spectroscopic Characterization

GCMS analysis

The GCMS analysis of untreated and ozonated RBO after 175 h is showed in Figure 2. There are two noticeable things found in that figure. First, the six peaks of spectra found in the GCMS analysis of untreated RBO indicating palmitic acid, methyl linoleate, methyl oleate, methyl stearate, montanic acid, and arachidic acid, with the highest spectra being methyl oleate. Fragmentation patterns were obtained from a spectrogram of each component, where there were special basic peaks in each fragmentation pattern (Silverstein, 2005). Second, nine peaks appeared in the GCMS analysis of RBO after 175h of ozonation process. The greater number of peaks show that some reaction occurred inside the RBO during ozonation process. The nine peaks in ozonated RBO refer to 1,1-Dimethoxydodecane, methyl myristate, palmitic acid, methyl linoleate, methyl oleate, methyl

stearate, methyl elaidate, arachidic acid methyl ester, and methyl behenate. These components are more or less the same as most components found in other vegetable oils. The methyl oleate still has the highest spectra compared to other peaks although the ozonation process occurred.

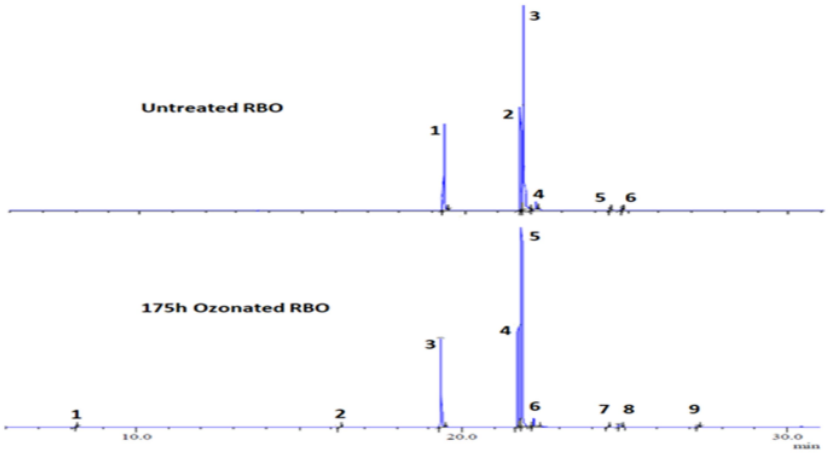

Fig.2: The GCMS analysis of untreated and ozonated RBO after 175 h

The complete data of retention time, area percentage, and ion mass from untreated and ozonated RBO is showed in Table 2 and Table 3. From the comparison between the chemical components in untreated and ozonated RBO, some saturated fatty acids formed during the ozonation processes, such as 1,1-Dimethoxydodecane, methyl myristate, and methyl behenate. It proves that ozone as mediator reacted with the unsaturated fatty acid and broke down the double chain of C=C bond to become -C-C- bond. Moreover, -C-C- bond bound with oxygen to form O=C-C=O as saturated fatty acid. Unfortunately, the cyclic 1,2,4-trioxolane couldn't be found because of its unstable nature (Almeida, 2012). This instability made trioxolane difficult to detect in GCMS. Another characterization such as NMR can reveal the presence of trioxolane in ozonated RBO.

Table 1: Physicochemical Characteristics of Rice Bran Oil as Raw Material

Parameter	Result	Unit
Color	Yellowish	-
Odor	Objectional	-
Density	0.921	g/mL
Kinematic viscosity	34.85	cP
pH	5.0	-
Iod Number	128	g I_2/100 g
Acid Number	2.311	mg NaOH/g
Peroxide Number	20	mEk/1000 g

Table 2: Chemical component of untreated RBO showed from GCMS analysis

Peak No.	%Area	Retention Time	Molecule name	Formula	Molar mass	Ion Mass (m/z)
1	20.26	19.351	*Palmitic acid*	$C_{17}H_{34}O_2$	270	$74[CH_2CO_2CH_3+H]^+$ $87[C_3H_7CO_2]^+$ $43[C_3H_7,CH_3C=O,C_2H_5N]^+$

						41[$CH_2C{=}N$, Ar]$^+$ 57[C_4H_9,$C_2H_5C{=}O$]$^+$
2	23.51	21.724	*Methyl linoleate*	$C_{19}H_{34}O_2$	294	55[C_4H_7,$CH_2{=}CHC{=}O$]$^+$ 67[C_5H_7]$^+$ 81[Side Chain Ring-CH_2,C_6H_9]$^+$ 41[$CH_2C{=}N$, Ar]$^+$ 95[Side Chain Ring-$C{=}O$]$^+$
3	53.62	21.827	*Methyl Oleate*	$C_{19}H_{36}O_2$	296	55[C_4H_7,$CH_2{=}CHC{=}O$]$^+$ 41[$CH_2C{=}N$, Ar]$^+$ 69[C_5H_9,CF_3,$CH_3CH{=}CHC{=}O$]$^+$ 97[C_7H_3,S-Side Chain Ring-CH_2]$^+$ 98[O-Side Chain Ring-$CH_2O{+}H$]$^+$
4	2.03	22.204	*Methyl stearate*	$C_{19}H_{38}O_2$	530	41[$CH_2C{=}N$, Ar]$^+$ 69[C_5H_9,CF_3,$CH_3CH{=}CHC{=}O$]$^+$ 83[C_6H_{11},$CH^{35}CL_2$]$^+$ 96[$(CH_2)_5{\equiv}N$]$^+$ 111[S-Side Chain Ring-$C{=}O$]$^+$
5	0.19	24.492	*Octacosanoic acid*	$C_{29}H_{58}O_2$	530	41[$CH_2C{=}N$, Ar]$^+$ 69[C_5H_9,CF_3,$CH_3CH{=}CHC{=}O$]$^+$ 83[C_6H_{11},$CH^{35}CL_2$]$^+$ 96[$(CH_2)_5{\equiv}N$]$^+$ 111[S-Side Chain Ring-$C{=}O$]$^+$
6	0.39	24.828	*Arachidic acid methyl ester*	$C_{21}H_{42}O_2$	490	74[$CH_2CO_2CH_3{+}H$]$^+$ 87[$C_3H_7CO_2$]$^+$ 43[C_3H_7,$CH_3C{=}O$,C_2H_5N]$^+$ 41[$CH_2C{=}N$, Ar]$^+$ 57[C_4H_9,$C_2H_5C{=}O$]$^+$

Table 3: Chemical Component of Ozonated RBO Showed from GCMS Analysis

Peak No.	%Area	Retention Time	Molecule Name	Formula	Molar mass	Ion Mass (m/z)*
1	0.23	8.138	*1,1-Dimethoxydodecane*	$C_{14}H_{30}O_2$	230	71[C_5H_{11},$C_3H_7C{=}O$] 41[$CH_2C{=}N$, Ar]$^+$ 55[C_4H_7,$CH_2{=}CHC{=}O$]$^+$ 58[$CH_3C(=O)CH_2{+}H$,$C_2H_5CHNH_2$] 96[$(CH_2)_5{\equiv}N$]$^+$
2	0.13	16.249	*Methyl myristate*	$C_{15}H_{30}O_2$	242	74[$CH_2CO_2CH_3{+}H$]$^+$ 87[$C_3H_7CO_2$]$^+$ 41[$CH_2C{=}N$, Ar]$^+$ 57[C_4H_9,$C_2H_5C{=}O$]$^+$
3	20.22	19.356	*Palmitic acid*	$C_{17}H_{34}O_2$	270	74[$CH_2CO_2CH_3{+}H$]$^+$ 87[$C_3H_7CO_2$]$^+$ 43[C_3H_7,$CH_3C{=}O$,C_2H_5N]$^+$ 41[$CH_2C{=}N$, Ar]$^+$ 57[C_4H_9,$C_2H_5C{=}O$]$^+$
4	23.8	21.730	*Methyl linoleate*	$C_{19}H_{34}O$	294	

	0			$_2$		55[C_4H_7,CH_2=CHC=O]$^+$ 67[C_5H_7]$^+$ 41[CH_2C=N, Ar]$^+$ 81[Side Chain Ring-CH_2,C_6H_9]$^+$ 55[C_4H_7,CH_2=CHC=O]$^+$ 109[Side Chain Ring-C=O]
5	51.97	21.833	*Methyl Oleate*	$C_{19}H_{36}O_2$	296	55[C_4H_7,CH_2=CHC=O]$^+$ 41[CH_2C=N, Ar]$^+$ 69[C_5H_9,CF_3,CH_3CH=CHC=O]$^+$ 97[C_7H_3,S-Side Chain Ring-CH_2]$^+$ 98[O-Side Chain Ring-CH_2O+H]$^+$
6	2.54	22.205	*Methyl stearate*	$C_{19}H_{38}O_2$	298	74[$CH_2CO_2CH_3$+H]$^+$ 87[$C_3H_7CO_2$]$^+$ 43[C_3H_7,CH_3C=O,C_2H_5N]$^+$ 41[CH_2C=N, Ar]$^+$ 57[C_4H_9,C_2H_5C=O]$^+$
7	0.25	24.480	*Methyl elaidate*	$C_{19}H_{36}O_2$	296	41[CH_2C=N, Ar]$^+$ 55[C_4H_7,CH_2=CHC=O]$^+$ 69[C_5H_9,CF_3, CH_3CH=CHC=O]$^+$ 97[C_7H_3, S-Side Chain Ring-CH_2]$^+$ 110
8	0.66	24.830	*Arachidic acid methyl ester*	$C_{21}H_{42}O_2$	326	74[$CH_2CO_2CH_3$+H]$^+$ 43[C_3H_7,CH_3C=O,C_2H_5N]$^+$ 87[$C_3H_7CO_2$]$^+$ 57[C_4H_9,C_2H_5C=O]$^+$ 41[CH_2C=N, Ar]$^+$
9	0.20	27.264	*Methyl behenate*	$C_{23}H_{46}O_2$	354	55[C_4H_7,CH_2=CHC=O]$^+$ 41[CH_2C=N, Ar]$^+$ 74[$CH_2CO_2CH_3$+H]$^+$ 57[C_4H_9,C_2H_5C=O]$^+$ 87[$C_3H_7CO_2$]$^+$

^{13}C NMR Analysis

Figure 3 shows the NMR spectra of untreated and ozonated RBO and assignments to all pertinent peaks were summarize in Table 4. NMR analyses further confirmed the structural changes undergone by untreated RBO methyl esters during ozonation. New signal at 104.5 ppm was assigned to the ring carbon of 1,2,4-trioxolane. The signal with ȝ = 130.139 ppm is the chemical shift of oleic acid, while the signal with ȝ = 128.022 ppm is a chemical shift of linoleic acid. From the picture it can be seen that the spectrum of oleic and linoleic acids signals is high; this indicates that the portion of the C=C double bond found in oleic and linoleic acids is also high. The presence of peaks between 10–50 ppm was attributed to sp^3-hydridized carbons which indicates that untreated RBO dominantly contains saturated hydrocarbons (Iorio et al., 2016).

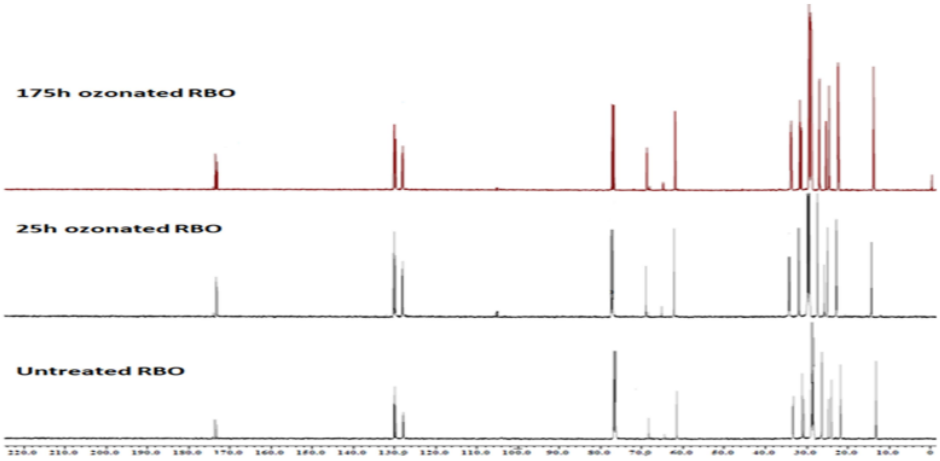

Fig.3: Full ^{13}C NMR Spectra of 175, 25 h Ozonated RBO and Untreated RBO

There were 10 primary signals found in the ozonated RBO after 25 h of ozonation process. In the process of ozonation, the reaction that occurred was an addition of ozone to double bonds in unsaturated fatty acids (oleic and linoleic). The ozonolytic reaction breaks the double bonds in oleic and linoleic acids and replaces them with O bonds to form cyclic -C-O- bond, so that a new ozonide compound is formed, namely 1,2,4-trioxolane (Soriano et al., 2003). The existence of 1,2,4-trioxolane in ozonated RBO can be seen in the new signals that appear on the spectrum, with ƍ = 104,5078 ppm. Unfortunately, 1,2,4-trioxolane peak disappeared when RBO was ozonated for 175 h. This shows that long-time ozonation process made the solution become saturated, and then 1,2,4-trioxolane degraded into aldehyde form and peroxide, due to its unstable nature, coherent with above mentioned GCMS data in Figure 1. The breakdown of unsaturated fatty acid by ozone is proved by a significant decrease in the intensity of the signals from oleic and linoleic acid in ƍ = 130 and 128 ppm, respectively. The other evidence of ozone attack on C double bonds is an increasing signal in chemical shifts around 24 and 31 which are related to methylene protons α and β to ring carbons, respectively (Sega et al., 2010). The formation of ozonides and the disappearance of unsaturated fatty acids are almost equal. The trioxolane ring is relatively stable since the proton signals from the aldehyde group at around 200 ppm are not visible in NMR spectra at each stage of the ozonation process. Some signal in chemical shift of ƍ =69.018-69.056, 65.174-65.210, and 62.227-62.263 had interesting behaviour, which is related to triglycerides and the alcohol chain. The behaviour showed that the intensity of the signal increased until a certain time, then decreased. It is related to the trioxolane production from ozone oxidation reaction with unsaturated fatty acids which is unstable (Almeida et al., 2013). The complete ^{13}C NMR chemical data of RBO before and after ozonation process for 25 and 175 hours can be shown in Table 4.

Table 4 : ^{13}C NMR data of RBO during ozonation process for 25 and 175 hours

Formula	Bonding name	Chemical shift (ppm)			Behavior
		untreated RBO	25 h ozonated RBO	175 h ozonated RBO	
C=C	Alkenes	130,139	130,185	130.192	decreasing
C=C	Alkenes	128,022	128,048	128.064	decreasing
ROOC-	Carboxyl	173,377	173,451	173.492	increasing
CH_2–OCOR	Triglycerides	69,018	69,035	69.056	increasing, then decrease
RCH_2O-	Alcohol	65,174	65,210	65.302	increasing, then

CH_2–OCOR	Triglycerides	62,227	62,263	62.289	decrease increasing, then decrease
$–(CH_2)_2–$	Acyl chains	31.021	31.045	31.057	increasing
$–(CH_2)_2–$	Acyl chains	29,815	29,842	29.881	increasing
RH_2COOH	Acyl chains	26.515	26.532	26.548	increasing
RCH_3	Acyl chains	14,220	14,256	14.277	increasing
$C_2H_4O_3$	1,2,4-trioxolane	-	104,508	-	increasing, then decrease
$CDCl_3$	Deuteratedchloroform	77.459	77.448	77.455	stable

Physicochemical Characterization

Density and Viscosity

Figure 4a-b shows the density and kinematic viscosity of ozonated RBO for various ozonation times. The density of ozonated RBO increased linearly as ozonation time increased, with a determination coefficient of 0.988. The formation of new molecules with a higher molar mass causes the density of ozonated RBO to increase (Uysal, 2014). This indirectly proves that the ozone broke down the double bond and produced 1,2,4-trioxolane, which has a relatively large molar mass (Crieege, 1975). An increase of the ozonation time also led to an exponential trend in the increase of ozonated RBO viscosity. Ozonated RBO reached viscosity values up to 56.59 cP under room temperature, or 65.47% from its initial value, after 175 h of ozonation time. These phenomena match the experiment conducted by Skalka et al., (2009). The increased viscosity for ozonated RBO indicates that the carbon double bonds reacted with ozone to form carbon single bond species.

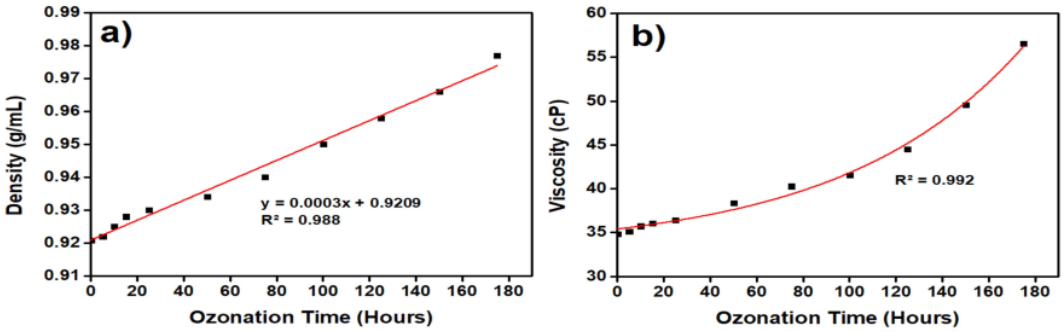

Fig.4: (a) Density and (b) Kinematic Viscosity of RBO during Ozonation Process.

Iodine, Peroxide, and Acid Number analysis

The analysis of iodine numbers in ozonated RBO aims to show a decrease in the amount of iodine with increasing ozonation time as shown in Figure 4a. Iodine numbers for ozonated RBO after 175 h of ozonation time decreased up to 91.14% from their initial value. The iodine numbers decreased rapidly during the first 25 h of ozonation time and then decreased slightly until the end of 175 h. This shows that almost all the double bonds in unsaturated fatty acids react with ozone as a strong oxidative agent, or in other words a decrease in the degree of unsaturation has occurred (Wasmi et al., 2015). The reaction with ozone leads to the gradual disappearance of the double bond, in line with the result of ^{13}C NMR in the previous section.

The Peroxide Number is the most important analysis in ozonated oil since it indicates the amount of peroxide compounds formed. The Peroxide number increases almost linearly with the increase of ozonation time, and reaches saturated condition after a certain time, as shown in Figure 4b. In these phenomena, the partial reaction of ozone in the RBO compartment is evidenced by the increase in the amount of ozone recovered in the iodide trap. This occurs if we assume that most of the unsaturated substance portion becomes slow to react with a certain time, due to probabilistic and steric conditions (Razumovskii and Zaikov, 1980). The formation

of polymeric peroxide responsible for viscous mass is achieved after a massive ozonation process. The Peroxide number for ozonated RBO with the ozonation time of 175 h was up to 340 mEk per 1000 g of RBO. The formation of peroxidic compounds, especially 1,2,4-trioxolane, is usually correlated by increasing peroxide numbers. Increasing the peroxide number can also be caused by the ozone changes to free oxygen (O_2).

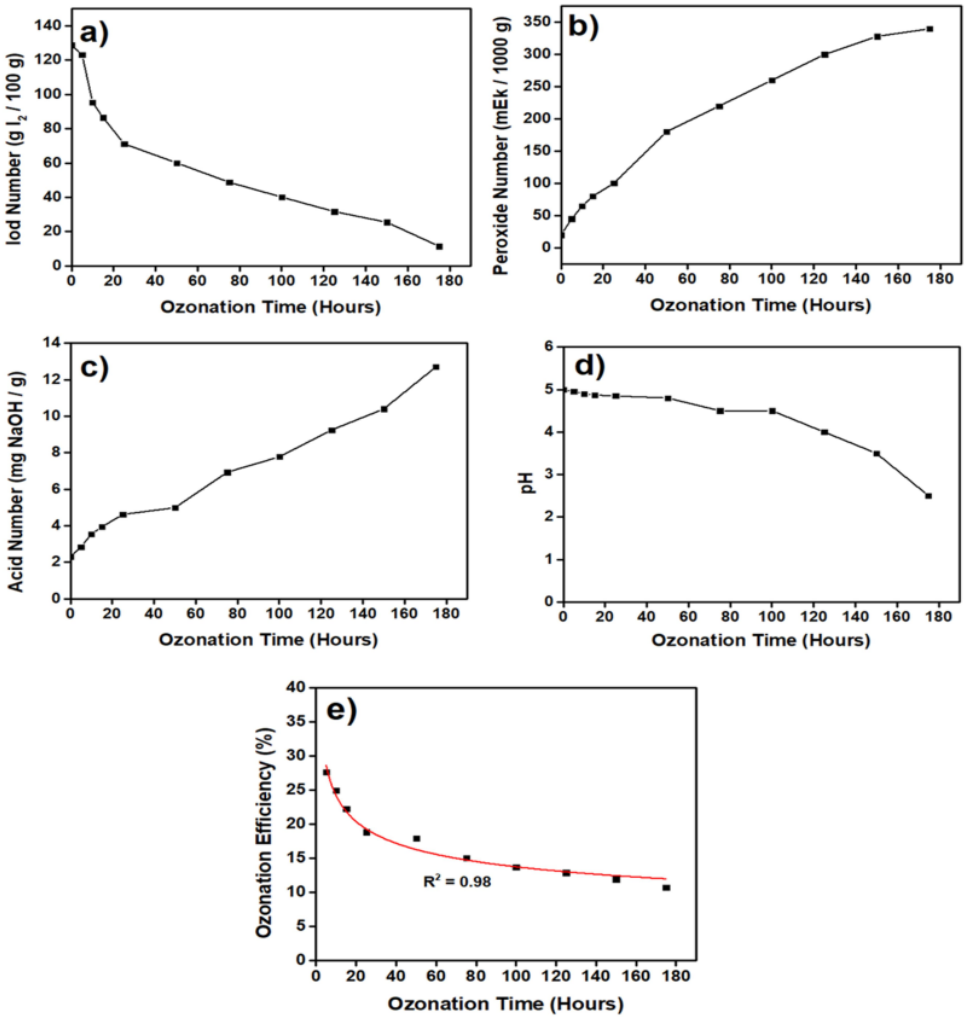

Fig.5(a) Iodine Number (b) Peroxide Number (c) Acid Number (d) pH and (e) OE of RBO during the Ozonation Process for 175 hour

Another parameter to characterize ozonated RBO is an Acid Number. The acid number increases with the increase of ozonation time, as shown in Figure 5c. Acid numbers are usually used to assess the freshness of an oil, and if stored properly. The success of the ozonization process is indicated by an increase in the acid number. During the ozonation process, partial hydrolysis occurs. and acid reaction occurs between unbound fatty acids and fat, even though fat shows a neutral reaction (Skalka et al., 2009).

The decrease in pH of ozonated RBO has a correlation with the increase in acid number (Figure 5d). It is clear because as the acid number increased, the amount of proton in the ozonated RBO also increased. The increasing proton made the pH of the solution decrease. There was a phenomenon related to the pH of ozonated RBO,

where the pH decreased insignificantly until ozonation time achieved 100 hours from 5 to 4.5. After that, the pH decreased significantly from 4.5 to 2.5. According to Langlais (1991), the ozonation effect is faster if the pH of the solution is low, so the profile of pH decrease in the low pH range gives a more significant decrease. Ozone is more stable at low pH so that the amount of ozone that reacts with doubel bonds in unsaturated fatty acids is more and then produces carboxylic acid.

OE represents the estimated number of ozonated compounds inside RBO, the same as described by the peroxide number, with the amount of ozone used. As expected, OE results decreased because of the disappearance of double bonds as time increased (longer ozonation period) and its R^2 value was 0.98.

The Color of Ozonated RBO

The color of RBO changed after the ozonation process from clear yellow to dark yellow. The yellow color pigment in RBO comes from oil-soluble carotenoids (Orthoefer and Eastman, 2005). The change in the color of oil after ozonation is because carotenoid pigments (α and β carotene) are oxidized to new carotenoid compounds with lower molecular weights (Rodriguez-Amaya, 2001). This color change is also caused by the oxidation process of ozone to carbon groups in the double bonds of unsaturated fatty acids found in the tocopherols contained in the RBO (Schwartz et al., 2008).

Fig.6: The Colour RBO During (a) 0 (b) 25 hour and (c) 175 hours ozonation time

Correlation between NMR spectra and physicochemical characteristic

The iodine number and peroxide number are more important characteristics compared to the acidic number and pH. Their correlation with NMR peaks is very important to study. In Figure 7a, as the iodine number increased the relative ion abundance value of 128 ppm (alkenes) also increased, whereas the relative ion abundance of 26.5 ppm (acyl chains) decreased. These phenomena are in agreement with the explanation of Figure 3, Figure 5a, and Table 4. With increasing ozonation time, the carbon double bond is broken by ozone to become a single bond.

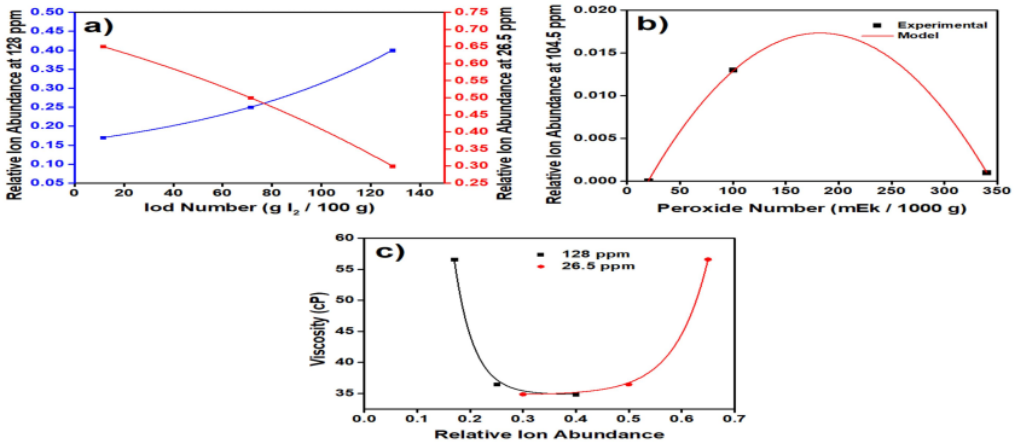

Fig.7: The Characteristic behavier between (a) Alkene and Iodine Number, as wel as acyl chains, (b) peroxide number and resulting 1,2,4, Trioxolane and (c)alkene and viscosity, as well as acyl chain

Figure 7b shows that the relative ion abundance value at 104.5 ppm (1,2,4-trioxolane) increased until a certain point before finally coming down, as the the peroxide number increased. The breakdown of carbon double bonds into single bonds resulted in a new valuable component inside RBO, i.e. 1,2,4-trioxolane. But, the long ozonation process made the system of ozonated RBO become saturated with ozone, and also caused the 1,2,4-trioxolane to become unstable, decrease, and then disappear. These phenomena are in harmony in Figure 1, Figure 2, and Figure 4b.

The correlation between viscosity and carbon double or single bonds is shown in Figure 6c. As the number of carbon double bonds or unsaturated fatty acid increased, the viscosity of RBO decreased. The decrease in viscosity occurred because there was no high molecular weight species in the RBO. This was confirmed by the increase in relative ion abundance of acyl chains, which are carbon single bonds that resulted from the ozonation breakdown process of carbon double bonds.

CONCLUSIONS

Ozonolysis of RBO was suggested to follow the Criegee mechanism. The formation of 1,2,4-trioxolane was seen in the RBO after ozonation process at 25 h and 175 h. The density and viscosity of ozonated RBO increases due to the breakdown of carbon double bonds by ozone to become carbon single bonds, which have relatively higher molecular weight. The decrease in iodine number by as much as 54.84%, from 3.748 to 1.693 g I2 per 100 g of sample, during the ozonation process of 175 hours shows that most of the double bonds in saturated fatty acids react with ozone. Increased peroxide value is observed where the value increases 16-fold during the 175 hours ozonation process. Moreover, the acid number increases 5.5-fold from 2.311 to 12.712 mg NaOH per g of sample. The maximum OE is 27.66% in the first 5 hours of ozonation and then decreases up to 10.72% after 175 hours of ozonation process.

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