Anti- and Pro-Oxidative Effect of α-Eleostearic Acid on the Autoxidation of Linoleic Acid

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ABSTRACT

In this study, fatty acid composition of tung oil and the remaining fatty acid contents after oxidation were determined. Moreover, the oxidative stability of α-ESA was compared with linoleic acid (LA), linolenic acid (LnA), arał chidonic acid (AA) and ecosapentaenoic acid (EPA) during auto-oxidation in the dark at 40°C. The results indicated that α-ESA content of tung oil was 79.45%. It was observed that the contents of remaining α-ESA, LA, LnA, AA and EPA after 24 h. were 0, 72.7, 53, 13 and 8.2%, respectively. Comparison between conjugated and non-conjugated fatty acids with the same number of double bonds showed that the conjugated fatty acid (CFA) declined more rapidly and fatty acid with more double bonds also degraded faster. The hydroperoxide content at 0.1, 0.2, 1 and 5% of α-ESA were 673, 762, 828 and 916 mM at 8 days and 40°C, respectively. Meanwhile, the peroxide content of methyl linoleate without α-ESA (control) was 563 mM at 8 days and 40°C.

Key words: Tung oil, α-eleostearic acid, oxidative stability, auto-oxidation.

INTRODUCTION

Tung oil tree (*Vernicia fordii*) is a natural woody oil plant in subtropical areas of China. This important economical tree has been cultivated in China for the production of tung oil for centuries. Tung seeds hold 50–60% oil with about 80% α-eleostearic acid (9 cis, 11trans and 13 trans octadecatrienoic acid). Tung oil is easily oxidized due to the presence of three conjugated double bonds in its structure (Tan *et al*., 2011).

As a kind of oil plant, the specific uses of tung oil in industry are attributed to α-eleostearic acid, a kind of unsaturated fatty acid found in it. After additional processing of tung oil, the value of tung oil rises sharply and the processed oil has numerous uses in many fields. In recent years, the yield of tung oil has increased quickly with a rise in cultivated areas of tung tree, which resulted in overstock at one time. Tung oil can be used in the chemical and medical manufactured (Ji *et al*., 2002, Min *et al*., 2005).

Some plant seed oils are known to contain conjugated fatty acids (CFA) such as α-eleostearic acid (α-ESA; 9c, 1lt, 13t-18:3), calendic acid (8t, l0t, 12c-18:3; n-6) and parinaric acid (9c, 11t, 13t, 15c-18:4). Additionally, CLA (9,11-18:2, conjugated dienoic acid) is well known to be a component of several dairy products including milk and cheese (Ha *et al*., 1987, Lin *et al.,* 1995). The CLA has been shown to have properties as anti-carcinogenic and anti-obesity agent in animal studies (Park *et al*., 1999). Furthermore, it was observed that tung oil fatty acids (mainly consisting of eleostearic acids) induce cytotoxic actions against human tumor cells as efficiently as the conjugated trienoic fatty acids (Igarashi & Miyazawa, 2000).

The conjugated linolenic acid (CLN) is a group of positional and geometric isomers of octadecatrienoic acids that hold three conjugated double bonds. The CLN can be present in tung oil, pomegranate seed oil, catalpa seed oil, balsam pear seed oil and cherry seed oil. Tung oil contains principally two CLN isomers, α-ESA (c9, t11, t13-18:3) and β- eleostearic acid (t9, t11, t13- 18:3) (Takagi & Itabashi, 1981).

Conjugated polyunsaturated fatty acids (PUFA) are exclusive fatty acid that contains conjugated double bonds in their molecules. Among these conjugated FA, CLA are well known, and many research articles have been published on the biological activities of CLA (Pariza *et al*., 1999, Pariza *et al.* 2003).

At present, CFAs have attracted substantial attention because of their potentially beneficial bio-

logical effects attenuating lifestyle-related diseases. The CFAs include a mixture of positional and geometric isomers of PUFA with conjugated double bonds. Theoretically, amounts of CFA isomers are comprise numerous combinations of numerical, positional and geometrical shapes of conjugation in double bonds. The α-eleostearic acid is a CFA, which can be extracted from tung (*Aleurites fordii*) oil (Rainer & Heiss, 2004).

The mechanism of autoxidation of methylene-interrupted fatty acid double bonds is fine established and includes a catalytic process which proceeds *via* a free radical mechanism. The initiation step consists of addition alkyl radical formation (R·) in the carbon adjacent to the double bond and the propagation step results in addition of oxygen to form alkyl peroxyl radicals (ROO·), hence the oxygen consumed is primarily converted to hydroperoxides (ROOH) (Frankel, 2005).

The aim of the present study was conducted to determine fatty acid composition of tung oil. The oxidative stability of methyl α-ESA was compared with methyl esters containing linoleic acid (LA), linolenic acid (LnA), arachidonic acid (AA) and ecosapentaenoic acid (EPA) which were auto-oxidized in the dark at 40°C. To evaluate the oxidative stability, fatty acid composition and the remaining FA content were determined.

MATERIALS AND METHODS

Materials:

Stearic acid (18:0), linoleic acid (LA) (18:2n-6), linolenic acid (LnA) (18:3n-3), arachidonic acid (AA) (20:4n-4), ecosapentaenoic acid (EPA) (20:5n-5) and BHT were obtained from Sigma Chemical Co. (St. Louis, MO). α-ESA (79 % purity) was isolated from tung oil. Tung oil was provided by Nippon Oil and Fats Co. Ltd. (Tokyo, Japan).

Methods:

Preparation and purification of methyl fatty acids from tung oil.

Fatty acid methyl esters (FAMEs) were obtained from tung oil (triacylglcerol) by transesterification using sodium methoxide as the catalyst. After placing tung oil (ca. 10 g) and toluene (20 ml) into a 100 ml screw- capped flask, 70 ml of methanol and 10 ml of 28% sodium methoxide in methanol were added, and transesterification was completed by heating the mixture with stirring at 60 °C for 1 hr. The reaction was stopped by the addition of acetic acid (5 ml). The reaction mixture was put into a separatory funnel, added hexane (200 ml) and water (100 ml), and mixed. The lower layer was re-extracted with hexane (100 ml). The hexane layer was combined and washed with water for several times. The hexane solution was dried under vacuum using a rotary evaporator. The recovered FAMEs were refined on a silicic acid column (silica-gel, ca. 50 g) by eluting them with hexane (100 ml) and a solution of hexane / ethyl acetate v /v (96:4, 200 ml; 94:6, 200 ml). The elutions were collected each 50 ml and checked by silica – gel TLC developed with hexane / diethyl ether / acetic acid (70:30:1, by vol). The detection of the spot by TLC was done by spraying the plate with aqueous H_2SO_4 and heating it on a hot plate. The FAME fractions were collected and the solvent was removed under vacuum. The isolated FAMEs were dissolved in ethanol and stored at -30 °C. The yields were obtained directly by dividing the resulting weight of fatty acid methyl esters on the weight of the original oil (Suzuki, *et al*., 2004).

Analysis of fatty acid composition of methyl esters

Fatty acid composition of the methyl esters were determined by GC. The analysis was performed on a Shimadzu GC-14B chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with an FID and a capillary column (Omegawax 320, 30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA) at a column temperature of 200°C.The injector and detector temperatures were held at 250 and 260°C, respectively. Helium was used as carrier gas, with a flow rate of 20 ml/ min. Component peaks were identified by comparison with standard FAMEs (Suzuki, *et al.,* 2004).

Isolation of methyl α-eleostearate (MeES)

Methyl α-eleostearate (MeES, $C_{19}H_{32}O_2$= 292.5) was prepared from tung oil and isolated using preparative HPLC. The isolated MeES was weighed in ethanol to make the known amount solution of MeES and stored at −25°C (Suzuki, *et al.,* 2004).

Autoxidation of fatty acid methyl esters (FAMEs).

Each FAME (MeL, MeLn, MeA, MEP and Me α-ES) diluted solution (100 µL = 0.50 mg) was placed into a 10-ml screw-capped test tube. Then, the diluted MeS solution (100 µL = 0.25 mg) was

added into the test tube. The solvent in each test tube was removed by a centrifuged-evaporator and then by staying under vacuum-chamber for 30 min. Each test tube was incubated in a dark room 40 °C for 0,8,16 and 24 hr. After incubation, 0.5 ml of BHT solution (1mM in hexane) was added to terminate the oxidative reaction. The remaining FAME content was determined using liquid chromatography (Suzuki, *et al.,* 2004).

Autoxidation of methyl linoleate in present of α-MeES:

The first experimentation (hydroperoxide) MeL contained 0.1, 0.2, 1.0, and 5 % α-MeES. Each 0.5 g of sample oil (MeL without or with MeES) is placed into a glass vial. Each test tube was incubated in the dark at 40 °C for 8 days. Periodically, 25 µL of each oil was collected and dise solved in 1.0 ml of ethanol. This sample solution was stored at −25°C until analysis. The second experimentation (epoxide), MeL and methyl myrisate (Mem) containing 5 % of α-MeES were incubated at the conditions same of the first experimentation.

Measurement of peroxide value

The bulk oil sample (25 µL) was dissolved in acetonitril (1.0 ml). This sample solution (100 µL) or ethanl (100 µL, as a blank) was placed into a test tube, then 25 µL of 0.1 M Na_2EDTA in water and 1.0 ml of acetic acid/chloroform (3:2, v/v) were added. Finally, each 0.1 ml of saturated KI solution (this solution was prepared just before the experiment) was quickly added in the sample solution. The tube was left at 30 min in the dark. After the reaction, 4.0 ml of water were added in each tube, mixed by vortex mixer, and centrifuged at 2000 rpm for 5 min. The resulting upper yellow phase (1.0 ml) was withdrawn to another test tube. Water (4.0 ml) was added to this tube and mixed. The produced I_2 was measured by the absorbance at 350 nm. The blank test (acetonitril was used instead of the sample solution) before and after measured samples (Wills, 1971).

Measurement the residual amounts of α-MeES

Fatty acid methyl esters in the remaining substrate were determined by GLC using a CP-Sil 88 fused-silica capillary column (100 m×0.25 mm i.d. × 0.2µm film thickness, Chrompack, Middelburg, Netherlands) on a Perkin–Elmer chromatograph (Model Clarus, Beaconsfield, UK) equipped with a flame ionization detector. The column was held at 100 ˚C for 1 min after injection, temperature-programmed at 7 ˚C/min to 170 C, held there for 55 min, then temperature programmed at10 ˚C/min to 230 ˚C and held there for 23 min. Helium was the carrier gas with a column inertsil ODS (4.6 x 150 mm), detection: UV at 273 nm (absorbance of conjugated triene structure of MeES) and a splitless injection system. Injection volume was 1.0 µL (50 mg/ml). The areas of peaks were calculated using known amounts of MeES as internal standard (Luna *et al*., 2007).

Statistical analysis

The data were expressed as mean ± SD. A one-way ANOVA was also used for statistical analysis between groups. The F ratio of one-way ANOVA is significant when the P value ≤ 0.05. Turkey's multiple range method (Scheffe, 1961) was used for comparison. The statistical program was Minitab release 13.31 (Minitab, State College, PA).

RESULTS AND DICUSSION

The yields of conjugated and non- conjugated fatty acids of tung oil

The yields of α-elestearic, linoleic, linolenic, arachidonic and ecosapentaenoic acids were 0.521, 0.848, 0.705, 0.301 and 5.18 g/100 g, respectively as shown in Table (1).

Fatty acid composition of tung oil

The fatty acid composition of tung oil is presented in Table (2). It was observed that the saturated fatty acids present in the oil are palmitic acid (1.92%), stearic acid (1.96%). While, the unsaturated fatty acids present in the oil are oleic acid (4.75%), linoleic acid (6.94%), α-elestearic acid (79.45%) and β-eleostearic acid (4.97 %). The content of unsaturated fatty acids in tung oil amounting to 96.11% of the total fatty acids. These results are almost compatible with Koji & Teruyoshi (2005).

Table 1: The yields (g/100 g) of conjugated and non- conjugated fatty acids

Fatty acids	α-eleostearic acid	Linoleic acid	Linolenic acid	Arachidonic acid	Ecosapentaenoic acid
Yields (g/100 g)	0.521	0.848	0.705	0.301	5.18

Table (2): Relative percentage of fatty acid composition of tung oil

	Fatty acids					
	Palmitic acid	**Stearic acid**	**Oleic acid**	**Linoleic acid**	**α-eleostearic acid***	**β-eleostearic acid***
Relative %	1.92	1.96	4.75	6.94	79.45	4.97

*: α-eleostearic acid and β- eleostearic acid are conjugated linolenic acids.

The remaining content of conjugated and non-conjugated methyl esters:

Measurement of the remaining substrate may be necessary when comparing the oxidation of lipids that produce different oxidation products (Luna, *et al*., 2007). The lipid oxidation products are responsible for the deterioration of lipid-containing foods. Therefore, clarifying the difference in the oxidation products between non-conjugated and conjugated poly unsaturated fatty acids is also important. The oxidative stability and oxidation products of each ester could be characterized by the oxidation of each conjugated or non-conjugated fatty acids. Fig. (1) showed that, methyl α- eleostearate (α-MeES), methyl linoleate (Mel), methyl linolenate (Meln), methyl arachidonate (MeA) and methyl eicosapentaenate (MeE), were oxidized in the dark at 40°C. Also, it is clear that the time course of the remaining substrates during the oxidation period was 24 hr. It was observed that the contents of remaining α-MeES, Mel, Meln, MeA and MeE after 24 h were 0, 72.7, 53, 13 and 8.2%, respectively (Fig. 1). While, the contents of remaining α-MeES, Mel, Meln, MeA and MeE after 16 hr were 10.7, 81, 74.1, 65.6 and 28.2%, respectively. Comparison between conjugated (α-MeES) and non-conjugated methyl esters (Mel and Meln) with the same number of double bonds showed that the conjugated fatty acids declined more rapidly, and fatty acids with more double bonds (MeA and MeE) also degraded faster. These results agreed with those reported by Jiang & Kamal-Eldin (1998) and Luna *et al*., (2007) who mentioned that conjugated linoleic acid absorbs more oxygen per mole of oxidized substrate than linoleic acid and produces mainly polymeric products.

Ha *et al*., (1990) mentioned that CLA was more oxidatively stable than LA at room temperature. On the contrary, Zhang & Chen, (1997) reported that CLA was oxidized more rapidly than LA.

Effect of methyl α–eleostearate on the auo toxidation of methyl linoleate:

The data in Fig. (2) show the effect of methyl α–eleostearate on the autoxidation of methyl linoleate by using replacement rates (0.1, 0.2, 1 and 5% of α-ES). It was cleared that, the peroxide content increased with increasing storage time (1 to 8 days). Also, the peroxide content increased by using the high percentage of α–eleostearate (5%). In

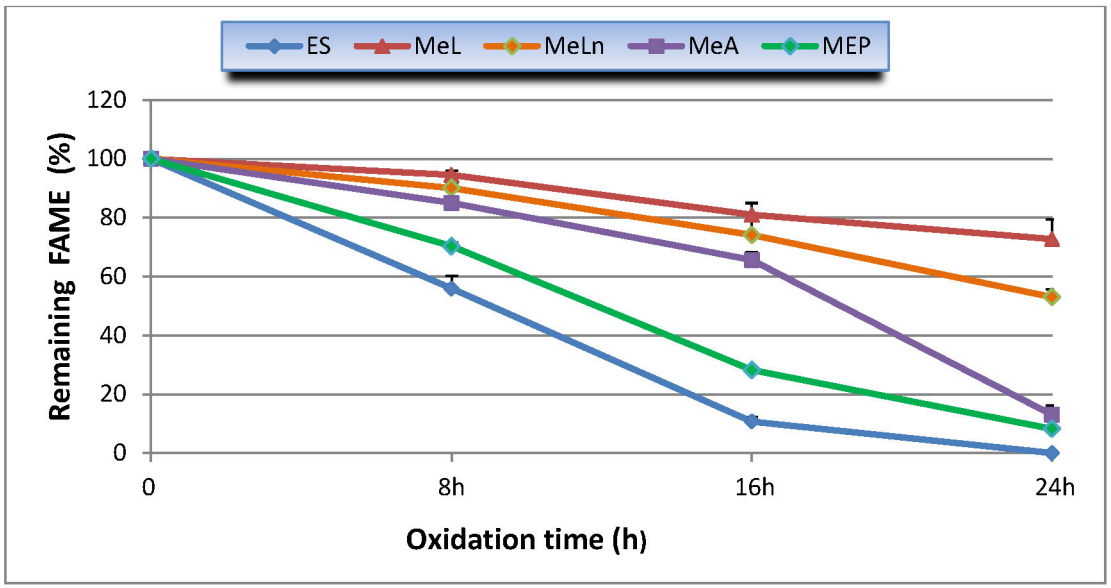

Fig. 1: Time course of the remaining methyl α- eleostearate (α-ES), methyl linoleate (MeL), methyl linolenate (MeLn), methyl arachidonate (MeA) and methyl eicosapentaenate (MeEP), oxidized at 40°C

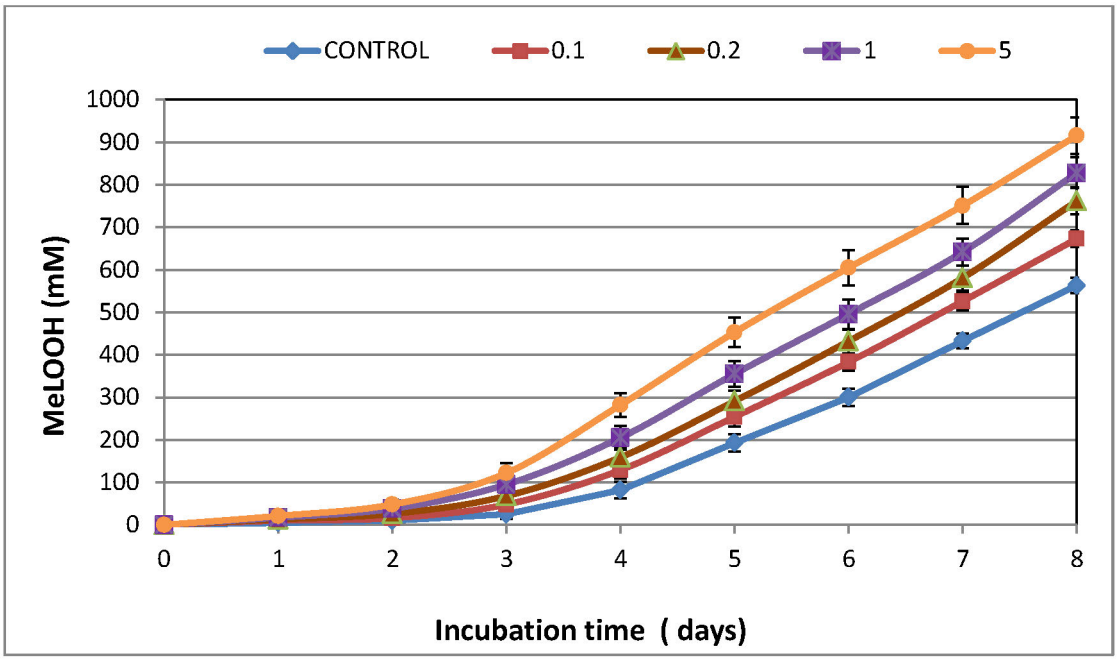

Fig. 2: Effect of methyl α–eleostearate on the autoxidation of methyl linoleate, changes in peroxide value by HPLC during the autoxidation in the dark at 40 °C

addition, the peroxide content at 0.1, 0.2, 1 and 5% of α-ES were 673, 762, 828 and 916 mM at 8 days and 40°C, respectively. Meanwhile, the peroxide content of methyl linoleate without α-ES (control) were 563 mM at 8 days. The oxidative rate of Mel increased with increasing concentrations of α–MeES. These results are in agreement with the data published by Kazuo & Tom (1986) who reported that during autoxidation, increasing of unsaturated fatty acid increased hydroperoxides resulting from oxidation process.

The remaining methyl α–eleostearate during the autoxidation of methyl linoleate

The pro-oxidative effect of α-MeES on the au toxidation of Mel was confirmed by the determination of the residual contents. The results presented in Fig. (3) indicated the changes in the amount of the residual of α-MeES during the autoxidation in

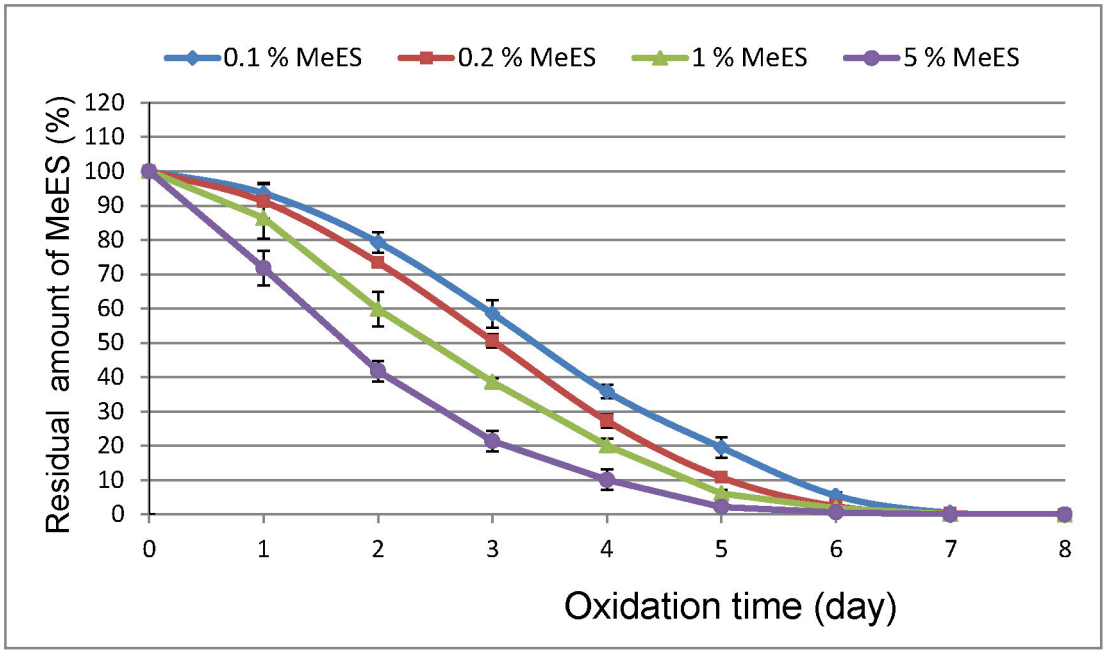

Fig. 3: Effect of methyl α–eleostearate on the autoxidation of methyl linoleate, changes in the amount of the residual of α-MeES during the autoxidation in the dark at 40°C

the dark at 40 °C. It was observed that the residual of α-MeES (%) decreased with elongation of the storage time. The residual of α-MeES (%) for 0.1, 0.2, 1 and 5% were 5.5, 2.4, 1.8 and 0.6 % at 6 days, respectively. Meanwhile, it reached to zero% at 8 days at all percentage of α-MeES.

The remaining methyl α–eleostearate in MeM and Mel:

Secondary oxidation products consist of aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids, and epoxy compounds, among others. The results in Fig. (4) indicated that the formation of MeES epoxides resulting from autoxidation of methyl linolate (Mel) contained 5% MeES as compared with that of methyl myrstate (MeM) that contained 5% MeES. The remaining content of α–MeES in Mel was less than that of the remaining content of α–MeES in MeM. On the other hand, epoxied content of α–MeES in Mel was less than that of epoxied content of α–MeES in MeM during storage period (8 days).

CONCLUSION

Tung oil hold on the higher percentage of α-elestearic acid as CFA and also it is much less stable, not only in comparison with the non-conjugated fatty acid, but also more PUFA including LnA and AA, when exposed to air oxidation at 40°C. Future studies on antioxidants studies of α-elestearic acid as CLN and its use in food as unsaturated acid.

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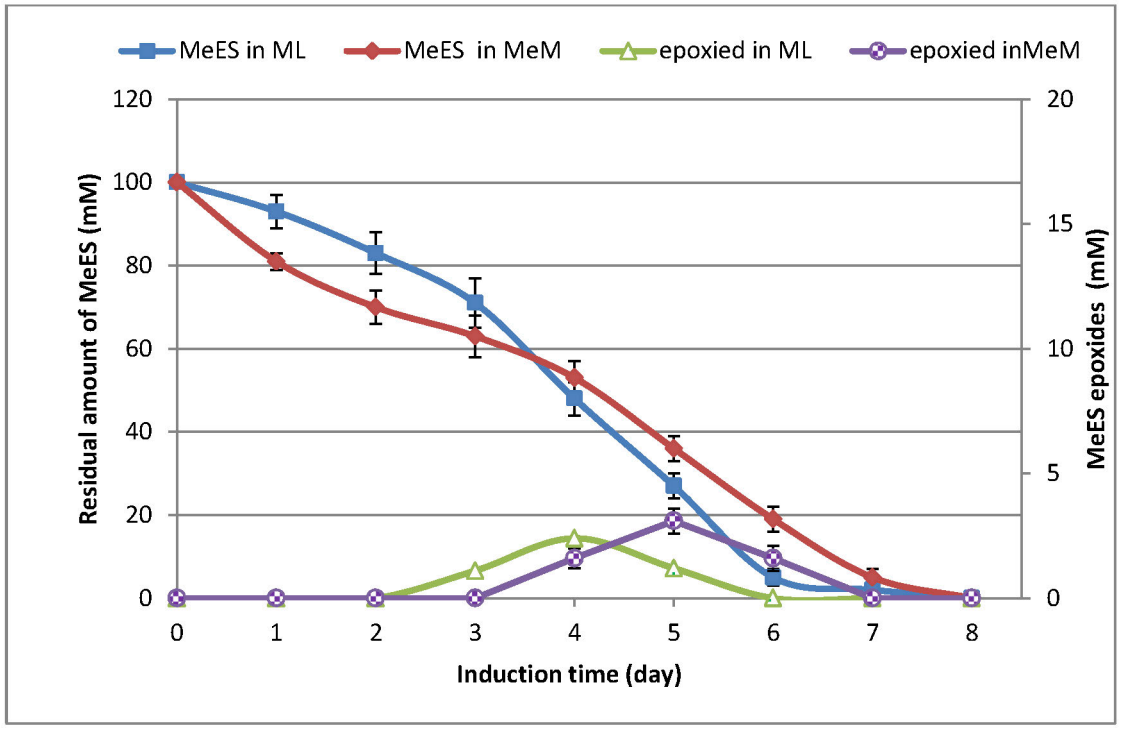

Figure 4: The formation of MeLOOH and MeES epoxides and deletion of MeES at 5% MeES in MeL autoxidized at 40 °C

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التأثير المانع والمسرع للأكسدة لحامض ألفا إيلوستيارك على الأكسدة الذاتية لحامض اللينوليك

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يعتبر حامض ألفا إيلوستيارك من أحماض اللينولينك المترافقة والذى له تأثير قوى مضاد للتورم مقارنة بأحماض اللينوليك المترافقة. اجريت هذه الدراسة لتقدير تركيب الأحماض الدهنية لزيت التنج والمحتوى المتبقى من الأحماض الدهنية بعد عملية الأكسدة ، بالإضافة الى مقارنة الثبات التأكسدى لحامض ألفا إيلوستيارك بأحماض اللينوليك ، اللينولينك ، الأراكيدونيك والأيكوزابنتانويك أثناء الاكسدة فى الظلام على درجة حرارة ٤٠ م°. وأوضحت النتائج أن محتوى حامض ألفا إيلوستيارك فى زيت التنج كان ٧٩,٤٥٪ . ولوحظ أن المتبقى من أحماض ألفا إيلوستيارك ، اللينوليك ، اللينولينك ، الأراكيدونيك والأيكوزابنتانويك بعد ٢٤ ساعة كان ٠ ، ٧٢,٨ ، ٥٣ ، ١٣ و ٨,٢٪ على الترتيب . بمقارنة الأحماض الدهنية المترافقة وغير المترافقة والتى تحتوى على نفس عدد الروابط المزدوجة تبين أن الأحماض الدهنية المترافقة تتدهور بسرعة وينطبق ذلك على الأحماض الدهنية المحتوية على أكثر من رابطة مزدوجة . من ناحية أخرى ، فإن محتوى الهيدروبيروكسيدات عند تركيزات ٠ ، ٠,١ ، ٠,٢ ، ١ و ٥٪ حامض ألفا إيلوستيارك كانت ٦٧٣ ، ٧٦٢، ٨٢٨ و ٩١٦ مليمكافئ على الترتيب مقارنة بالكنترول والذى كان له قيمة هيدروبيروكسيد ٥٦٣ مليمكافئ عند التخزين لمدة ٨أيام وعند درجة حرارة ٤٠ م°.