ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

FORMATION OF EPOXY FATTY ACIDS DURING AUTOXIDATION OF LINSEED, SUNFLOWER AND OLIVE OIL

M.Sc. THESIS

Selin Hande BAŞARAN

Department of Food Engineering

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

KETEN TOHUMU YAĞI , AYÇİÇEĞİ YAĞI VE ZEYTİNYAĞININ OTOOKSİDASYONU SIRASINDA EPOKSİ YAĞ ASİTLERİNİN OLUŞUMUNUN İNCELENMESİ

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Selin Hande Başaran, a M.Sc. student of ITU Graduate School of Science Engineering and Technology student ID 506121541, successfully defended the thesis entitled "FORMATION OF EPOXY FATTY ACIDS DURING AUTOXIDATION OF LINSEED, SUNFLOWER AND OLIVE OIL", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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FOREWORD

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October 2015 Selin Hande BAŞARAN

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ABBREVIATIONS

CD	**:** Conjugated diene
CT	**:** Conjugated trienes
DAGs	**:** Diacylglycerols
EFAs	**:** Epoxy fatty acids
ES	**:** Epoxy stearate
EO	**:** Epoxy oleate
EOL	**:** Epoxy linoleate
FAME	**:** Fatty acid methyl ester
FFA	**:** Free fatty acids
GC-FID	**:** Gas chromatography-flame ionization detector
IS	**:** Internal standard
L•	**:** Lipid radical
LOO•	**:** Peroxyl radical
LO•	**:** Alkoxyl radical
LOOL	**:** Peroxyl dimers
LOD	**:** Limit of determination
LOQ	**:** Limit of quantification
MAGs	**:** Monoacylglycerols
MDA	**:** Malonaldehyde
OOH	**:** Hydroxyl groups
p-AV	**:** p-anisidine
PV	**:** Peroxide value
SLO	**:** Stripped linseed oil
SOO	**:** Stripped olive oil
SSO	**:** Stripped sunflower oil
SPE	**:** Solid phase extraction
TAGs	**:** Triacylglycerols
tBME	**:** Tert butyl methyl ether
VOO	**:** Virgin olive oil

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$^1O_2$	**:** Singlet oxygen
$^3O_2$	**:** Triplet oxygen
C17:0	**:** Methyl-*cis*-10,11-epoxy heptadecanoate
C18:0	**:** Stearic acid
C18:1	**:** Oleic acid
C18:2	**:** Linoleic acid
C18:3	**:** Linolenic acid
C19:0	**:** Nanodecanoic acid
m	**:** Slope of the calibration curve
M	**:** Molarity (mol/L)
meq	**:** Miliequavalent
W	**:** Sample mass
A	**:** Corrected absorbance
ε	**:** Molecular extinction coefficient
l	**:** Width of cuvette
C	**:** Concentration
A_b	**:** Corrected absorbance of the solution without addition of p-anisidine reagent
A_s	**:** Corrected absorbance of the solution with of p-anisidine reagent
L	**:** Thickness of the spectrophotometer cell
LOOH	**:** Hydroperoxides
BF_3	**:** Boron trifluoride
MeOH	**:** Methanol

FORMATION OF EPOXY FATTY ACIDS DURING AUTOXIDATION OF LINSEED, SUNFLOWER AND OLIVE OIL

SUMMARY

Lipids are essential components for providing high quantity of energy and being the main storage source. Light, temperature and oxygen parameters may initiate oxidation and leading to not only taste and odor alteration but also formation of toxic end-products.

Oxidation reactions are grouped under four different types of mechanisms; autoxidation, thermoxidation, photoxidation and enzymatic oxidation. Lipid autoxidation, is a free-radical chain reaction which has initiation, propagation and termination steps. Apart from light, temperature and oxygen which are the main factors affecting autoxidation, fatty acid composition, presence of transition metals and antioxidants have an important impact.

Hydroperoxides, primary oxidation products, are formed by the abstraction of hydrogen atom from the acly carbon. Due to the saturation degree of fatty acids, hyroperoxide formation rate differs. Linoleic acid formes hyroperoxides 250 times than oleic acid.

Epoxy fatty acids (EFAs) formed during hyroperoxide decomposition are one group of toxic end-products of oxidation reactions. They have cyclic structure consisting three atoms. However, knowladge about their formation mechanism is limited which make them to be a study area.

Control and non-control models of linseed oil, olive oil and sunflower oil blended repectively with stripped/virgin olive oil at a ratio of 2:1 were defined. The objective of these two models were to see the effect of antioxidants, mainly chlorophyll, on autoxidation. Oil models were subjected to an incubation period of 56 days in total, at dark to obtain the autoxidation conditions. Samples were analyzed fresh and at days 4, 7, 14, 21, 28, 42 and 56.

Gas chromatography with flame ionization detector (GC-FID) equipped with a polar CP-Sil 88 column was used for quantification of EFAs in oil models. Peroxide value (PV), p-anisidine value (p- AV), conjugated diene (CD) and conjugated triene (CT) analysis were performed to identify the correlation between EFAs and other oxidation products. Fatty acid composition with respect to oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) was also determined for monitoring the relation with the type of EFAs and rate of oxidation. Chlorophyll content was also measured as it was one of the main parameter to be studied.

Chlorophyll pigment of the models blended with virgin olive oil was 6.8 mg/kg oil averagely. This value was approximetly same at the begininng and at the end of

incubation period. In the samples blended with stripped olive oil, no chlorophyll pigment was measured.

Linseed blends were rich in oleic and linolenic acid whereas sunflower blends included higher amounts of linoleic and oleic acid. Finally, models composed of only olive oil showed the highest amounts of oleic acid.

Epoxy fatty acid formation was not observed at significant amounts in stripped models of sunflower and olive oil. At the end of incubation process, total epoxy fatty acid content were ranged between 112.1 to 533.0 μg/g of sample. The stripped linseed and olive oil blend showed the highest amount of EFA formation. This was followed by stripped linseed and virgin olive oil blend (122.92 μg/g of oil).

When epoxy fatty acid isomers were considered, it was seen that epoxy stearate was formed from oleic acid, epoxy oleate was formed from linoleic acid and lastly epoxy linoleate from linolenic acid.

Within 12 identified EFAs, both *cis* and *trans* isomers were existed. Non-control model samples which were blended with virgin olive oil were showed higher formation of *cis* EFAs. On the other hand, in control model *trans* formation was higher.

No correlation was obtained between p-anisidine value and epoxy fatty acid content by Perason's correlation. Models including linseed oil showed a positive correlation against peroxide value. Additionaly, formation amount of EFAs and hydroperoxides were compared. Results showed, EFA formation was higher even hydroperoxide formation was not high. Conjugated diene and triene amounts were found to be related with epoxy fatty acid formation in stripped linseed-olive oil blend and stripped olive-virgin olive oil blend.

This study shows, eventhough peroxide value was not high, epoxy fatty acid formation was showed to be considerably high. EFA formation in high amounts may pose health risks. Further studies should be carried out to determine the epoxy fatty acid formation mechanism and parameters effecting the formation rate.

KETEN TOHUMU YAĞI , AYÇİÇEĞİ YAĞI VE ZEYTİNYAĞININ OTOOKSİDASYONU SIRASINDA EPOKSİ YAĞ ASİTLERİ OLUŞUMUNUN İNCELENMESİ

ÖZET

Yağlar, vücutta önemli enerji ve depo kaynağı olmaları sebebiyle kilit bir role sahiptir. Ancak ışık, sıcaklık ve oksijen gibi faktörler sebebiyle oksidasyon reaksiyonları başlayabilmektedir. Oksidasyon reaksiyonları sonucunda oluşan bileşenler, tat ve kokuda bozukluk ile besin değerinin azalmasının yanı sıra toksiksisite sebebi olabilmektedir.

Mekanizmalarına göre oksidasyon reaksiyonları; otooksidasyon, termooksidasyon, fotooksidasyon ve enzimatik oksidasyon olmak üzere 4 çeşittir.

Bu çalışmanın temel aldığı otooksidasyon, oda sıcaklığında serbest radikal zincir reaksiyonuna dayalı oksidasyon çeşididir. Başlangıç, yayılma ve terminasyon olmak üzere üç aşamada gerçekleşmektedir. Başlangıç aşamasında, katalizör varlığında doymamış yağ asitlerinden hidrojen ayrılmasıyla serbest radikaller oluşmakta, yayılma aşamasında ise oluşan bu serbest radikaller ile moleküler oksijen reaksiyona geçerek hidrokperoksitler oluşmaktadır. Ortamda hidroperoksitlerin birikmesi, birbirleriyle reaksiyona girmelerine ve böylelikle radikal olmayan bileşenlerin oluşmasıyla sonuçlanır. Bu aşama terminasyon olarak adlandırılmaktadır.

Fotooksidasyon, ışık, ve klorofil gibi ışığa duyarlı bileşenler varlığında karasız oksijen ile gerçekleşen oksidasyon reaksiyonudur. Kararsız oksijen, moleküler oksijene göre daha reaktif bir bileşen olduğundan fotooksidasyon reaksiyonları otooksidasyon reaksiyonlarından daha hızlı gerçekleşmektedir.

Termooksidasyon, yüksek sıcaklıklarda otooksidasyon mekanizmasına benzer şekilde gerçekleşir ancak bu tip oksidasyonda sıcaklığa bağlı reaksiyonlar ve oksidasyon eş zamanlı gerçekleşmektedir.

Enzimatik oksidasyon, işlenmemiş gıdalarda lipoksigenaz enzimi tarafından katalizlenmekte olup, yemeklik yağlarda meydana gelmez.

Oksidasyonu etkileyen temel faktörler; ışık, sıcaklık, oksijen, yağ asidi komposizyonu ve yağın içerisinde eser miktarda bulunan bileşenlerdir.

Işık; klorofil ve benzeri ışığa duyarlı molekülleri aktive ederek moleküler oksijenin daha aktif kararsız oksijene dönüştürülerek fotooksidasyon reaksiyonlarının başlaması sağlamaktadır. Otooksidasyon ve hidroperoksit bozunması sırasında ise hidrojen ayrılmasını sağlamaktadır. Sıcaklık ile oksidasyon hızı doğru orantılı olarak artmaktadır. Yağın doymamışlık oranı arttıkça oksidasyon hızı da artmaktadır. Ayrıca, yağın içerisinde bulunan antioksidanlar, metaller ve ışığa duyarlı moleküller

de az miktarda bulunmalarına rağmen oksidasyon hızını etkileyen önemli parametrelerdir. Antioksidanlar, çeşitlerine göre farklı mekanizmalarla oksidasyonun önlenmesini sağlarlar. Bitkisel yağlarda yüksek miktarlarda bulunan klorofil pigmenti, oksijen ve ışık varlığında fotooksidasyonu hızlandırırken, karanlıkta antioksidan olarak etki etmektedir. Metaller ise otooksidasyonun başlangıç aşamasında ve hidroperoksitlerin diğer kimyasal bileşiklere bozunmasında etkilidirler.

Birincil oksidasyon ürünleri olan hidroperoksitler, açil karbonlardaki hidrojenin ayrılmasıyla oluşmaktadır. Oleik asit, linoleik asit ve linolenik asit içeren yağlarda açil karbon sayısına bağlı olarak farklı kimyasal yapıda konjuge hidroperoksitler oluşmaktadır. Linolenik asitte daha fazla açil karbon olması sebebiyle, otooksidasyon oleik asite göre 250 kat hızlı gerçekleşmekte ve daha fazla miktarda peroksit oluşumu gözlenmektedir.

Birincil oksidasyon ürünleri olan hidroperoksitlerin bozunması ile ikincil oksidasyon ürünleri oluşmaktadır. Okside olmuş monomerler, aldehitler, ketonlar ve polimerik bileşikler bunların bir kısmıdır. Epoksi yağ asitleri, 3 atomlu halkalı yapıya sahip okside olmuş monomerlerdir. Bilinen oluşum mekanizmaları peroksi radikallerin, hidroperoksitlerdeki çift bağa katılımı şeklindedir.

Epoksi yağ asitleri ile ilgili literatürde kısıtlı bilgi bulunması sebebiyle bu çalışmada, çeşitli bitkisel yağlarda otooksidasyon reaksiyonları sonucunda oluşan epoksi yağ asitlerinin incelenmesi hedef alınmıştır. Deney modellemesi yapılırken, oksidasyonu etkileyen iki temel parametre baz alınmıştır; yağ asidi kompozisyonu ve klorofil içeriği. Yağ asidi kompozisyonundaki farklılıkların etkisini gözlemlemek amacıyla, farklı doymuşluk oranına sahip içersinde bulunan tüm bileşenlerinden ayrılmış üç çeşit yağ kullanılmıştır; zeytinyağı (C18:3), ayçiçek yağı (C18:2) ve keten tohumu yağı (C18:1). Klorofil içeriğinin etkisi ise, kontrol ve deney grupları oluşturularak belirlenmiştir. Deney grupları her üç çeşit yağın sızma zeytinyağı ile sırasıyla 2:1 oranında karıştırılmasıyle oluşturulmuştur. Kontrol grupları ise her yağ çeşidinin, içersindeki tüm bileşenlerden arındırılmış zeytinyağı ile deney gruplarındaki oranla aynı olacak şekilde karıştırılmasıyla elde edilmiştir. Modeller hazırlandıktan sonra, örnekler 1, 4, 7, 14, 21, 28, 42 ve 56 günlerde analiz edilmek üzere inkübasyona bırakılmıştır. Otooksidasyon koşullarının sağlanması için örnekler karanlıkta bekletilmiş, tüplerin kapağı oksijen geçişini sağlamak için pamuk ile kapatılmıştır. Epoksi yağ asitleri GC-FID'de polar CP-Sil 88 kolon kullanılarak nicel ve nitel olarak tanımlanmıştır. Sonuçlar 'SPSS Statistics 22' kullanılarak tek yollu ANOVA ile değerlendirilmiştir. Oksidasyonun göstergeleri olması sebebiyle peroksit değeri, konjuge dien ve trien ile p-anisidin analizleri de yapılmıştır. Epoksi yağ asitleri oluşumunun herhangi biriyle ilişkili olup olmadığını gözlemlemek amacıya Pearson korelasyonu uygulanmıştır. Ayrıca ana parametreler olan klorofil içeriği ve yağ asidi komposizyonu (GC-FID ile) da analizlenmiştir.

Sızma zeytinyağı kullanılan karışımlarda klorofil miktarı ortalama olarak 6.8 mg/kg olarak hesaplanmıştır. Bu değerde, örneklerin karanlıkta bekletilmeleri sebebiyle 56 günlük inkübasyon süresi sonunda önemli miktarda bir azalış gözlenmemiştir. Zeytinyağının içerisindeki klorofil dahil bütün minor bileşenlerin uzaklaştırıldığı kontrol gruplarında ise klorofil miktarı 0 mg/kg olarak saptanmıştır. Bu durumda, sadece klorofil miktarı baz alındığında, sızma zeytinyağı kullanılan örneklerde bulunan klorofilin antioksidan olarak etki göstermesi ve oksidasyonun diğer modele göre daha yavaş gerçekleşmesi beklenmektedir.

Yağ asidi kompozisyonları karşılaştırıldığında, literatürde belirtildiği gibi keten tohumu yağı örneklerinin linolenik asitce, ayçiçek yağı örneklerinin linoleik asitce ve zeytinyağı örneklerinin oleik asitçe zengin olduğu GC-FID ile yapılan analizde bir kez daha kanıtlanmıştır. Bütün gruplar, zeytinyağı ile karıştırıldığı için önemli miktarlarda oleik asit her örnekte mevcuttur. Dolayısıyla, sadece yağ asidi kompozisyonu karşılaştırıldığında oksidasyona en hassas örnek grubu keten tohumu yağı içeren grup olarak belirlenmiştir.

Oksidatif stabilite ve birincil oksidasyon ürünlerinin oluşumunu gösteren peroksit değeri inkübasyon süresi boyunca, 1 meq O_2/kg yağ değerinden genel olarak bütün gruplarda düşük olduğundan, oksidasyon hızının yavaş olduğu görülmektedir. Sadece, klorofil içermeyen keten tohumu yağı örneklerinde 42. günden itibaren bu değerin üzerinde peroksit değeri gözlense de, örneklerin çok fazla okside olduğu söylenememektedir. Konjuge dien ve trienler hidroperoksitler ile çok yakın yapıda olduklarından, benzer trend göstermişlerdir. İkincil uçucu oksidasyon ürünlerinin oluşumunu belirten p-anisidin değeri ise inkübasyon süresi boyunca dalgalı bir trend göstermiştir.

Epoksi yağ asitleri oluşumu en fazla miktarda yapısında üç çift bağ bulunduran keten tohumu yağında gözlenmiştir. Ancak klorofil pigmenti içermeyen ayçiçek yağı karışımı ve zeytinyağı karışımı örneklerinde 56 gün sonunda epoksi yağ asidi oluşumu gözlenmemiştir. Deney modellemesine bağlı olarak, klorofil içeren örneklerde oksidasyonun daha hızlı olacağı öngörülmüştü. Tam tersi durumun oluşmasının sebebi, klorofil içermeyen kontrol grubu örneklerinin aynı zamanda oksidasyonu hızlandırıcı etkisi olan metal gibi eser miktarda bulunan bileşenleri de içermemesi olarak görülmektedir. Bu durumun diğer bir sonucu keten tohumu modelleri karşılaştırıldığında da anlaşılmaktadır. Sızma zeytinyağı ile karıştırılan yağ örneklerinde epoksi yağ aside oluşumu metal içeriğine bağlı olarak hemen başlarken, diğer grupta oluşumun daha geç ancak daha hızlı meydana gelmesidir. Ancak yalnızca 56 gün inkübasyon sonundaki sonuçlara bakılacak olursa, ayçiçeği ve zeytinyağı gruplarından farklı olarak, kontrol grubunda yaklaşık 5 kat fazla epoksi yağ aside formasyonu görülmüştür.

Cis ve *trans* isomere sahip 11 çeşit epoksi yağ asidi tanımlanmıştır. Sızma zeytinyağı içeren modeldeki örneklerde *cis* yapısı daha fazla miktarda oluşurken, diğer modelde *trans* epoksi yağ asidi oluşumu baskın olarak görülmektedir.

Epoksi yağ asitleri oluşumu ile p-anisidin değerleri arasında korelasyon bulunanamamıştır. Peroksit değeri ile epoksi yağ asitleri arasında ise sadece keten tohumu yağı kullanılan modellerde güçlü bir korelasyon bulunmuştur. Epoksi yağ asitlerinin hidroperoksitlerin bozunmasıyla oluşan bileşenler olduğu literatürde belirtildiğinden, oluşan peroksit miktarı ile epoksi yağ aside miktarı karşılaştırılmıştır ve şaşırtıcı olarak epoksilerin peroksitlerden çok daha fazla miktarda oluştuğu görülmüştür. Benzer düşünceyle, peroksitler ile konjuge dien ve trien yapısı arasındaki ilişki Pearson korelasyonu kullanılarak kanıtlanmıştır.

Sonuç olarak, epoksi yağ asitlerinin oksidasyon çok hızlı olmadığı zaman bile yüksek miktarlarda oluştuğu bu çalışmada görülmüştür. Oluşum mekanizmalarının net olarak anlaşılması ve bu konuda yapılan çalışmaların arttırılması, cevabı şu an için kesin olmayan bir çok soruya ışık tutması açısından önem teşkil etmektedir.

1. INTRODUCTION

Lipids are important food components for the human body. They are the main energy source and carriers of fat soluble vitamins. Participating in body structure, lipids additionally have regulatory functions (Vercoletti et al.,1992). The human body cannot synthesize all of the fatty acids, thus some of them have significant importance in nutritional aspects. In addition, some fatty acids like omega-3 fatty acids (e.g. α-linolenic acid) and conjugated linoleic acid have been suggested to have health promoting effects. However, when oxidation reactions take place, all these health promoting effects are altered to detrimental effects (Zamora et al., 2013).

Four mechanisms were described for lipid oxidation: autoxidation, photoxidation, thermoxidation and enzymatic oxidation. Autoxidation is a free radical reaction catalyzed by triplet oxygen ($^3O_2$). Photoxidation, on the other hand, is catalyzed by singlet oxygen ($^1O_2$) which can directly react with the double bonds of fatty acids (Maruquez-Ruiz et al., 2013).

Despite the different mechanisms, similar families of products are formed. Epoxy fatty acids, the main oxidation products focused on this study, are defined as secondary oxidation products derived from decomposition of hydroperoxides (LOOH). Epoxy compounds have been reported to be protoxins (Mubiru et al., 2013). Accordingly, their formation in oxidized oils showed to be an important issue for human health. However, formation mechanisms of epoxy compounds in vegetable oils and parameters effecting their formation rate are not clearly defined. In addition, quantification of epoxy compounds is rarely studied. Coelution of different isomers during GC-FID analysis has been also the challenging point of epoxy fatty acid quantification. Mubiru et al., (2014), developed a method including a three step solid phase extraction (SPE) together with gas chromatography analysis to overcome this problem.

The objective of the study was determination and quantification of epoxy fatty acids formed in linseed oil, sunflower oil and olive oil blends during autoxidation based on

the fatty acid composition and antioxidant parameters. Additionally, relation with other oxidation products and epoxy fatty acids was identified by Pearson correlation.

2. LITERATURE REVIEW

Lipids have an important role in human metabolism by being the main energy sources and storage components (Vercoletti et al.,1992). Hydroperoxides, primary oxidation products, are decomposed to form secondary oxidation products such as ketones, aldehydes, alcohols and acids (Şimşek, 2008). These products are responsible for the undesirable flavor, odor and also toxicity. Moreover, due to the oxidation nutritional losses occur which makes the product undesirable for the consumer (Kim and Min, 2008).

2.1 Mechanisms of Lipid Oxidation

2.1.1 Autoxidation

Fats and oil oxidation by oxygen at room temperatures, is known as autoxidation or free radical oxidation. The autoxidation mechanism consists of three main steps: initiation (1), propagation (2) and termination (3) as shown in Figure 2.1. Oxidation reactions may be initiated by free radical formation by hydrogen abstraction from fatty acids, thermal/photochemical cleavage of R-H bond of hydroperoxides or metal-catalyzed decomposition of hydroperoxides. Lipid radicals (L•) lead to peroxyl radical (LOO•) formation by reacting with oxygen in the propagation step. Termination is observed when two radical species react with each other and form non-radicals (Kolakowska, 2010; List et al., 2005).

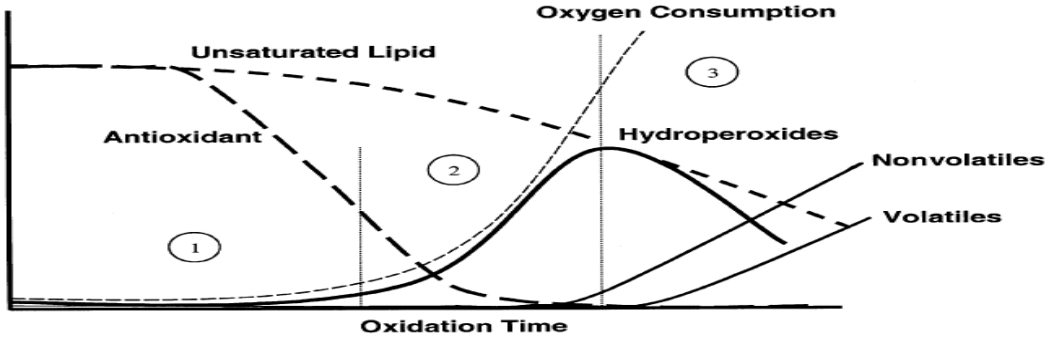

Figure 2.1 : Kinetic curve of autoxidation (Kamal-Eldin et al., 2003).

2.1.1.1 Initiation

Unsaturated fatty acids lose hydrogen to form free lipid radicals in the presence of initiators. Type of lipids, degree of unsaturation and presence of natural antioxidants determine the duration of initiation (Kiokias, 2009).

$$L_1H \rightarrow L_1H\bullet \tag{2.1}$$

Hydroperoxide decomposition is a chain breaking reaction leads to the formation of more free radicals (Marquez-Ruiz et al, 2013). Thermal/metal-catalyzed decomposition of hydroperoxides and photoxidation are three reactions producing alkoxyl (LO•) and peroxyl radicals (Juita et al., 2011).

$$LOOH \rightarrow LO\bullet + OH^- \tag{2.2}$$

$$LOOH \rightarrow LOO\bullet + H^+ \tag{2.3}$$

$$LOOH \rightarrow LOO\bullet + OH\bullet \tag{2.4}$$

When the amount of hydoperoxides present in oil is low, hydroperoxide decomposition is named as monomolecular (equation 2.4) which refers to decomposition of only one LOOH molecule. At higher concentrations of hydroperoxides, two LOOH molecule breaks down at once namely bimolecular (Equation 2.5). Bimolecular chain breaking reactions result in accelerated rate of oxidation and more complex reactions. (Schaich, 2006).

$$2LOOH \rightarrow LO\bullet + LOO\bullet + H_2O \tag{2.5}$$

2.1.1.2 Propagation

Lipid radicals formed during the initiation step, rapidly react with triplet oxygen to produce peroxyl radicals. Equation 2.7, the hydrogen transfer reaction, is always slower than equation 2.6 (Frankel, 2005a). At higher temperatures, reactions become more destructive by the production of HO•, which is extremely reactive and unselective. It may abstract hydrogen atoms all along the acyl chain and add readily into double bonds (Schaich, 2006).

$$L\bullet + O_2 \rightarrow LOO\bullet \quad (2.6)$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \quad (2.7)$$

$$L_1OO\bullet + LOOH \rightarrow LOOH + L_nOO\bullet \quad (2.8)$$

$$LO\bullet + LOOH \rightarrow LOH + LOO\bullet \quad (2.9)$$

The radicals formed from hydroperoxide decomposition may follow the below reactions.

$$LO\bullet + LH \rightarrow LOH + L\bullet \quad (2.10)$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \quad (2.11)$$

$$OH\bullet + LH \rightarrow HOH + L\bullet \quad (2.12)$$

2.1.1.3 Termination

When hydroperoxides accumulate, they start to interact with each other to form non-radical components. Peroxyl dimers (LOOL) may formed while condensation of peroxyl, alkoxyl or alkyl radicals (Frankel, 2005a).

$$2LOO\bullet \rightarrow LOOL + O_2 \quad (2.13)$$

Ether-containing dimers and carbon–carbon linked dimers may also act as a termination end-products at low temperatures or elevated temperatures (Frankel, 2005a).

$$Mn + LOOH \rightarrow LO \quad (2.14)$$

$$2L\bullet \rightarrow L - L \quad (2.15)$$

Scission reactions which require proton source such as water and generates malonaldehyde (MDA) is another type of termination reactions (Schaich et al., 2012).

2.1.1.4 Primary oxidation products: Hydroperoxides

General hydroperoxide formation mechanism valid for oleate, linoleate and linolenate autoxidation is shown in Figure 2.2.

In methyl oleate free radical oxidation, hydrogen abstraction at the allylic carbon-8 and carbon-11 enables the oxygen attack at the end- carbon positions. Four allylic hydroperoxides are produced in equal amounts (Frankel, 2005b):

9-hydroperoxy-*trans*-10-octadecenoate (*trans*-9-OOH)

11-hydroperoxy-*cis*-9-octadecenoate (*cis*-11-OOH)

10-hydroperoxy-*trans*-8-octadecenoate (*trans*-10-OOH)

8-hydroperoxy-*cis*-9-octadecenoate (*cis*-8-OOH)

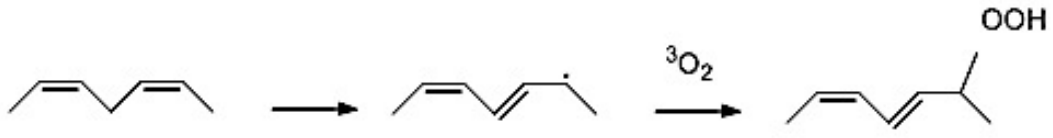

Figure 2.2 : Formation of hydroperoxides by triplet oxygen (Min and Lee, 1999).

Due to bis-allyclic methylene group between two double bonds on carbon-11 in linoleate, abstraction of hydrogen atom is 40 times more rapid from oleate. Loss of the hydrogen atom at carbon-11 produces a hybrid pentadieneyl radical.

Interaction with carbon-9 and carbon-13 results in two conjugated diene 9- and 13-hydroperoxides (Marquez-Ruiz et al., 2013):

9-hydroperoxy-*trans*-10, *cis*-12-octadecadienoate (*cis,trans*-9-OOH)

9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoate (*trans,trans*-9-OOH)

13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate (*cis,trans*-13-OOH)

13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoate (*trans,trans*-13-OOH)

Linolenate has two bis-allyclic methylane group which makes the hydrogen abstraction even more rapid than linoleate. By the same mechanism, two pentadieneyl radicals are formed by the hydrogen abstraction between 1,4 diene systems on carbon-9, carbon-13 and carbon-12, carbon-16.

Reaction with oxygen at the end-carbon positions produces four peroxyl radicals (Marquez-Ruiz et al., 2013):

9-hydroperoxy-*trans*-10, *cis*-12, *cis*-15-octadecatrienoate (*trans,cis,cis*-9-OOH)

13-hydroperoxy-*cis*-9, *trans*-11, cis-15-octadecatrienoate (*cis,trans,cis*-13-OOH)

12-hydroperoxy-*cis*-9, *trans*-13, cis-15-octadecatrienoate (*cis,trans,cis*-12-OOH)

16-hydroperoxy-*cis*-9, *cis*-12, *trans*-14-octadecatrienoate (*cis,cis,trans*-16-OOH)

2.1.1.5 Secondary oxidation products

Decomposition of primary oxidation products, hydroperoxides, enables the formation of numerous type of secondary oxidation products (Figure 2.3). According to their molecular weight three types are products may formed: volatile compounds, oxidized monomers (epoxy, hydroxy and keto functions) and polymeric compounds (Marquez-Ruiz et al., 2013).

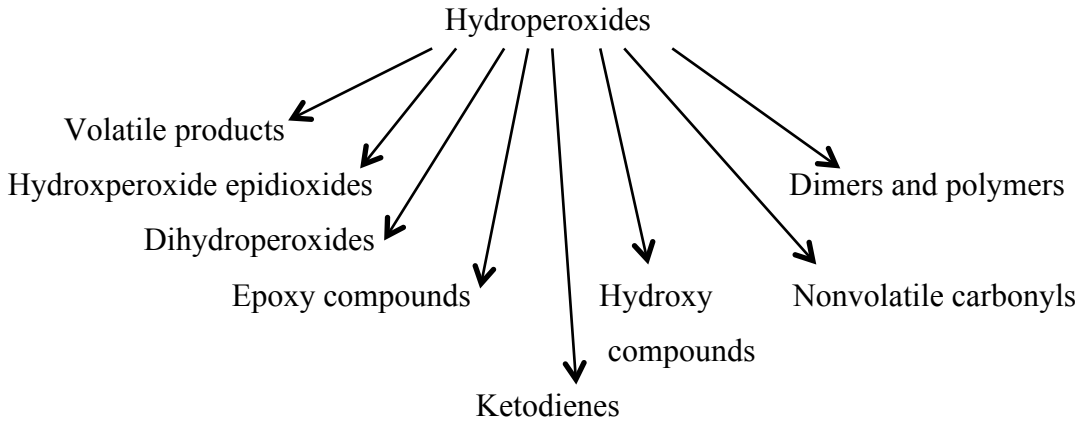

Figure 2.3 : Secondary oxidation products of hydroperoxides (Kamal-Eldin, 2003).

The main reason for flavor deterioration of oxidized lipids is the formation of volatile compounds (Omwamba et al, 2010). Volatiles such as short-chain aldehydes, alcohols, and hydrocarbons are formed according to the homolytic β scission of C-C bonds (Kolakowska, 2010). By this reaction, alkoxy radicals produced from hydroperoxides are cleaved to produce oxo compounds and alkyl or alkenyl radical (Kim and Min, 2008).

Polymerization compounds are formed from alkoxy radicals by condensation reactions at the end of the induction period. Polymers are generally produced in frying process due to the high temperatures. (Marquez-Ruiz et al, 2013).

Hydroxy- fatty acids are composed of a series of straight-chain carboxylic acids that contain one or more hydroxyl groups substituted on the hydrocarbon portion of the molecule. Keto- fatty acids have the same definition to that hydroxyl fatty acids with the keto position prefixing the fatty acid (Lobb and Chow, 2007).

Epoxides having a ring structure of three atoms including an ether group, are secondary oxidation products. Two epoxy fatty acid mechanisms are described in Figure 2.4. First mechanism as in Figure 2.4a is 1,2 addition to the nearest double bond resulting in epoxyallyclic radical formation (Mubiru et al., 2014). Addition of peroxyl radical to a nonconjugated double bond and then release of alkoxy radical is the second way of epoxy fatty acid formation, is shown in Figure 2.4b. This mechanism does not exist in autoxidation due to the formation of conjugated hydroperoxides only (Choe, 2008).

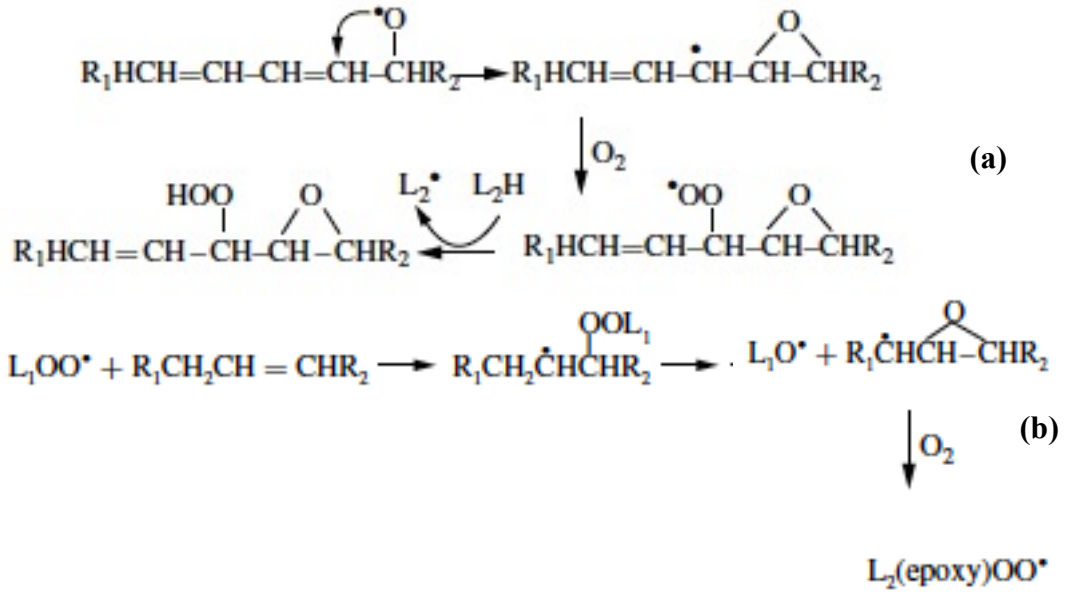

Figure 2.4 : Epoxide ring formation during lipid oxidation: (a) 1,2 addition (b) Peroxyl radical addition (Schaich, 2006).

In a study about camellia seed oil which is rich in oleic acid, hydroperoxides, epoxy epidioxides, and mono-epoxides were found to be the compounds produced by autoxidation (18^{o}C 12 months). It was mentioned that other oils containing high amounts of oleic acid may form the same compounds as formation of oxidation products are characteristic to dominant fatty acid. (Zeb, 2012).

Mubiru et al. (2014), has screened the epoxy fatty acids formed during the autoxidation of oils rich in oleic, linoleic and linolenic acid (Table 2.1). Structures of epoxy fatty acids are visualized in Figure 2.5.

Table 2.1 : Oxidized monomers from oleic, linoleic and linolenic acids (Mubiru et al., 2014).

	Oleic acid	Linoleic acid	Linolenic acid
Epoxy fatty acids	Methyl trans-9,10-epoxystearate, Methyl cis-9,10-epoxystearate	Methyl-trans 12,13-epoxyoleate, methyl cis-12,13-epoxyoleate, methyl trans-9,10-epoxyoleate methyl cis-9,10-epoxyoleate	methyl trans-12,13-epoxy-9,15-octadecadieneoate, methyl cis-12,13-epoxy-9,15-octadecadieneoate, methyl trans-15,16-epoxy-9,12-octadecadieneoate, methyl cis-15,16-epoxy-9,12-octadecadieneoate, methyl trans-9,10-epoxy-12,15-octadecadieneoate methyl cis-9,10-epoxy-12,15-octadecadieneoate

Non-volatile oxidation products in rapeseed oil and linseed/safflower oil were found to be hydroperoxy, hydroxy, epoxy, oxo and di-hydroxy triacylglycerides in different concentrations. Linseed/safflower oxidized oil contains epoxy and oxo triacylglycerols (TAGs) in higher amounts whereas hydroxyl TAGs are the main oxidation products (Steenhorst-Slikkerveer et al., 2000).

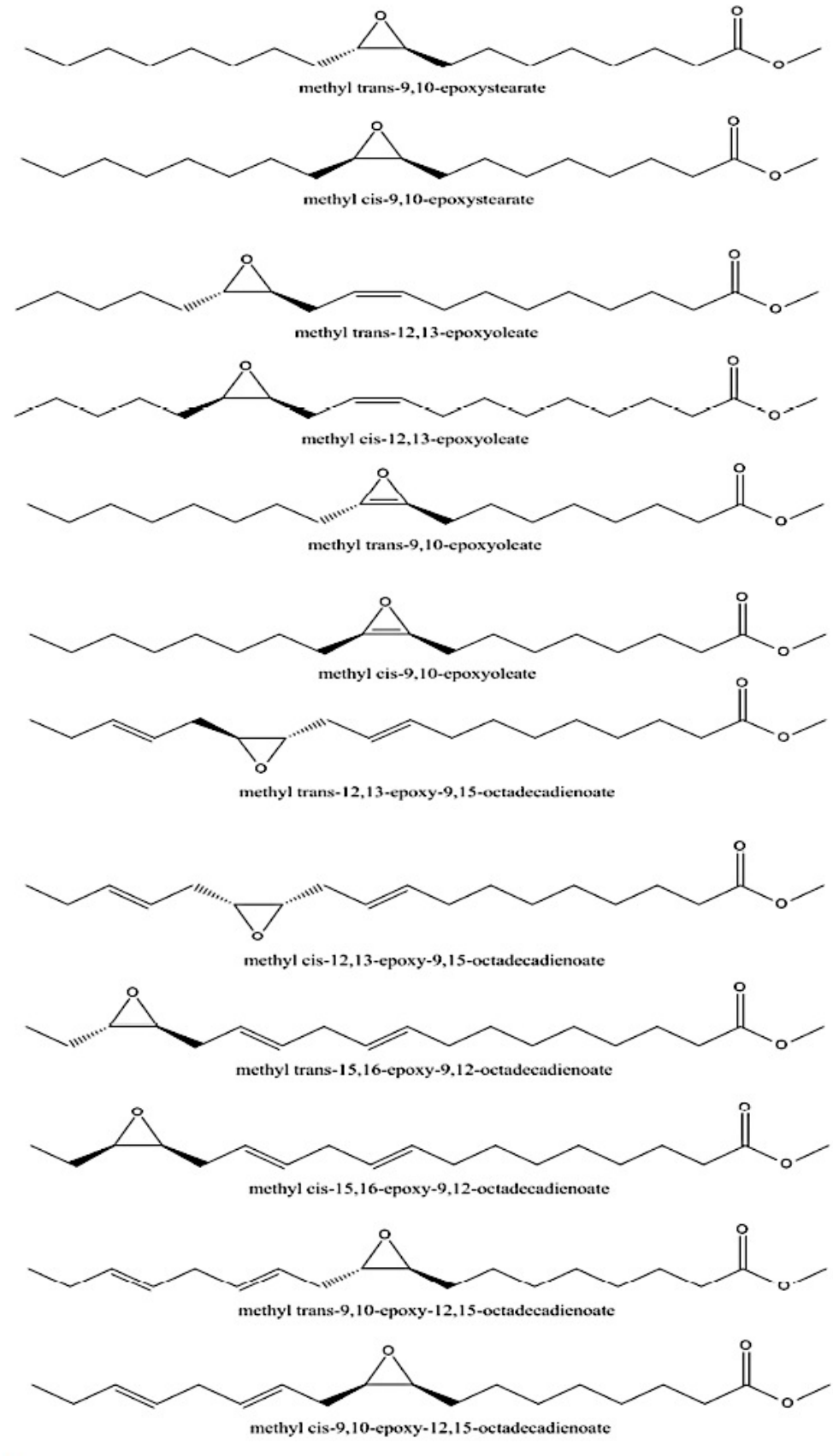

Figure 2.5 : Structures of epoxy fatty acids (Dobarganes, 2009).

2.1.2 Photooxidation

Oxidation may occur by a reaction of unsaturated fatty acids directly with singlet oxygen in the presence of light and photosensitizers. Activation energy of singlet oxygen is very low (0-25 kJ/mol), thus temperature does not have much effect on oxidation rate, as well as conjugated double bonds in dienes and trienes. Light is the most effective factor for singlet oxygen oxidation, in shorter wavelengths oxidation rate is higher rather than longer wavelengths (Choe, 2008). Photosensitizer is activated by the absorption of light energy. Triplet oxygen is not highly active compared to singlet oxygen so it does not interact with unsaturated fatty acids directly, which makes free radical reaction rate slower than photoxidation. When hydroperoxides are formed by photosensitized oxidation, free radical mechanism takes place and becomes the predominant oxidation (Kim and Min, 2008).

2.1.3 Thermoxidation

Thermoxidation, by means of oxidation related with higher temperatures has the same mechanism with autoxidation. However, due to the temperature effect, oxidation and thermal reactions occur simultaneously. In the frying process, moisture in food induces hydrolysis. Esther bonds are broken, free fatty acids (FFAs) and diacylglycerols (DAGs) are released. These free fatty acids are more sensitive to oxidation than triacylglycerols. Another factor accelerating thermoxidation is exposure to air (Sánchez-Muniz et al., 2008).

2.1.4 Enzymatic oxidation

Lipoxygenases catalyzes enzymatic reactions only in unprocessed foods due to their denaturation at higher temperatures which normally does not occur in edible oils. Although same hydroperoxides are formed with autoxidation, amounts and stereochemistry of the reaction is different due to the regiospecifity and stereospecifity of the enzymatic reaction (Márquez-Ruiz et al, 2013).

2.2 Factors Effecting Oxidation

Oxidation rate of oils may depends on some factors such as; light, oxygen, temperature, nature of lipid, and minor components. (Şimşek, 2008).

2.2.1 Temperature

Oxidation rate gradually increases as the temperature increases. Products formed at low and high temperatures are different. Below 100℃, mainly oxidized triglyceride monomers are formed due to the higher production rate of hydroperoxides compared with their decomposition. At higher temperatures, hydroperoxide decomposition is faster so that secondary oxidation products are the main products (Sanchez-Muniz et al., 2007). Additionally, temperature has an inverse impact on oxygen solubility (Márquez-Ruiz et al, 2013).

2.2.2 Oxygen

The type of oxygen is the main factor affecting lipid oxidation mechanism and the formation of oxidation products (Table 2.2). Triplet oxygen cannot directly react with fatty acids as it is a diradical compound which can only react with radicals. However, singlet oxygen is a radical compound, it can react directly with fatty acids (Hahm and Min, 1995). Oxidation catalyzed by triplet oxygen (autoxidation) needs energy to form free radicals in the initiation step, thus, requires longer time (Min and Lee, 1999). Relative oxidation rates of singlet and triplet oxygen are shown in Table 2.3.

Table 2.2 : Hydroperoxides of fatty acids formed by triplet and singlet oxygen oxidation (Lee, 2002).

	Oleate	Linoleate	Linolenate
Triplet oxygen	*cis*-8-trans-9-OOH	*cis, trans*-9-OOH	*trans, cis, cis*-9-OOH
	trans-10-OOH	*trans, trans*-9-OOH	*cis, trans, cis*-12-OOH
	cis-11-OOH	*cis, trans*-13-OOH	*cis, trans, cis*-13-OOH
		trans, trans-13-OOH	*cis, cis, trans*-16-OOH
Singlet oxygen	*trans*-9-OOH	*trans, cis*-9-OOH	*trans, cis, cis*-9-OOH
	trans-10-OOH	*cis, trans*-13-OOH	*cis, trans, cis*-12-OOH
		trans,cis-10-OOH	*cis, trans, cis*-13-OOH
		cis, trans-12-00H	*cis, cis, trans*-16-OOH
			trans, cis, cis-10-OOH
			cis, cis, trans-15-OOOH

Photosensitizers, naturally occurring in foods such as chlorophyll, riboflavin, myoglobin, absorb the light. The energy in the light is transferred to triplet oxygen to form singlet oxygen. (Kim and Min, 2008).

Table 2.3 : Relative oxidation rates of triplet and singlet oxygen with oleate, linoleate, and linolenate (Min and Lee, 1999).

Oxygen	Oleate	Linoleate	Linolenate
Triplet Oxygen	1	27	77
Singlet Oxygen	30,000	40,000	70,000

2.2.3 Nature of Lipids

Degree of saturation is one of the most important factors for determining the oxidation rate. Higher amounts of double allyclic hydrogens accelerate propogation and chain breaking reactions. Thus, oils which have higher number of double bonds (Table 2.4) are more sensitive to oxidation (Schaich, 2006).

Table 2.4 : Oxidation ratios of important fatty acids (Şimşek, 2008).

Acid code	Common name	Oxidation ratio
C18:0	Stearic acid	1
C18:1	Oleic acid	100
C18:2	Linoleic acid	1200
C18:3	Linoleic acid	2500

According to the fatty acid composition of sunflower, olive oil and linseed oil, (Table 2.5) linseed oil is the most sensitive type of oil to oxidation, followed by sunflower and olive oil.

Table 2.5 : Fatty acid composition of linseed, sunflower and olive oil (Zamora and Hidalgo, 2004).

	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1
Linseed oil	4-6	2-3	10-22	12-18	56-71	<0.5	<0.6
Sunflower oil	5-7	4-6	15-25	62-70	<0.2	<1	<0.5
Olive oil	8-14	3-6	61-80	3-14	<1	<0.5	<0.4

Besides the number of double bonds, positional and geometrical configuration of the double bonds also have an impact. Fatty acids at *sn*-1 and *sn-3* accelerate the oxidation, while fatty acids at *sn*-2 position stabilizes triacylglycerols. In addition, *cis* isomers oxidizes more faster than *trans* isomers because of the exposure to allyclic hydrogens (Márquez-Ruiz et al, 2013).

Oxidation rate increases with the degree of esterification of aclyglycerols (Table 2.6). In pure systems, free fatty acid oxidation rate is slower compared with free esters. In many cases, oil systems includes small amounts of metals resulting in higher oxidation rate than esters. (Schaich, 2006).

Table 2.6 : Effect of degree of esterification on oxidation (Schaich, 2006).

Lipid	Oxidizability (x 10^2 $M^{-1/2}$ $sec^{-1/2}$)
Monoacylglycerols (MAGs)	2.83
Diacylglycerols (DAGs)	5.89
Triacylglycerols (TAGs)	7.98

2.2.4 Light

Light accelerates autoxidation in two ways: acting as a catalyst of hydrogen abstraction in the initiation step and decomposition of hydroperoxides (Márquez-Ruiz et al, 2013). In photosensitized oxidation, light is the main energy source to convert triplet oxygen to singlet by activating photosensitizers present in food or oil (Lee, 2002).

2.2.5 Minor components

Chloropyll, phenolic compounds, tocopherols, metals, phospholipids, free fatty acids, diacylglycerols and monoacylglycerols are the minor components of edible oils (Choe et al, 2005).

Transition metals serves as main prooxidants (Márquez-Ruiz et al, 2013). Oils generally contain transition metals such as cobalt, iron, copper either naturally or contamination from processing conditions. Virgin olive oil contains metals up to 9.8 ppb copper and 0.7 ppm iron. Refining process decreases the metal content in edible oils. Effect of metals on accelerating the lipid oxidation is due to lowering the activation energy in the initiation step (Choe, 2008).

Hydroperoxide decomposition starts by the transition metal even if small amounts of hydroperoxide are produced (Kiokias, 2009).

$$Mn^{n+} + LOOH \rightarrow LO\bullet + OH^{-} + Mn^{(n+1)+} \quad (2.16)$$

$$Mn^{(n+1)} + LOOH \rightarrow LOO\bullet + H^{+} + Mn^{n+} \quad (2.17)$$

Antioxidants are the compounds responsible for the inhibition of oxidation mechanism thus, they extend the shelf life and improve the quality. Depending on their functions, antioxidants are classified in three groups; chain breakers, oxygen scavengers (e.g. carotenoids) and hydroperoxide reducers (e.g. ascorbic acid) (Kiokias et al., 2009). Chain breaking antioxidants which are phenolic antioxidants such as tocopherols, reacting with peroxyl radicals (LOO•) to interrupt propagation step of autoxidation and leading to less reactive species (Schaich et al., 2012). In the study of Fuster et al (1998), it is found that α- and γ-tocopherols may increase peroxide value of autoxidized sunflower oil up to 90%. Prooxidant effects were not observed at higher concentrations until 2000 ppm. (Fuster et al., 1998).

In vegetable oils, chlorophyll compounds are very common. Virgin olive oil contains 10 ± 4.9 ppm chlorophyll and 4.1–15.1 ppm pheophytin, whereas in rapeseed oil chlorophyll content is 5–35 ppm. In the presence of light and atmospheric oxygen, chlorophylls and pheophytins produce singlet oxygen (Choe et al., 2005). However, in the dark they act as an antioxidant (Go´mez-Alonso et al., 2007).

By decreasing surface tension, thus, increasing oxygen diffusion from the headspace into the oil, phospholipids accelerates oxidation rate. However, in the presence of metals they become metal chelators and decrease the rate of oxidation (Choe, 2008).

Impact of FFA, DAG and MAG were summarized in section 2.1.3.

3. MATERIALS AND METHODS

3.1 Chemicals and reagents

Silica gel 60 (particle size 0.063-0.100 mm) was purchased from Merck Chemicals (Overijse, Belgium). Iso-octane, sodium sulfate , sea sand (acid washed and calcinated), diethylether, barium chloride, 37% hydrochloric acid dichloromethane and ammonium thiocyanate (NH_4SCN) were obtained from Chem-Lab NV (Zedelgem, Belgium). Aluminium oxide (Brockmann I activated), sodium methoxide (25%w/v) and iron sulfate were supplied by Sigma-Aldrich (St. Louis, MO, USA). Methyl cis-10,11-epoxyheptadecanoate (C17:0) and methyl nonadecanoate (C19:0) were obtained from Nu-Chek-Prep., Inc (USA). Petroleum ether, hexane (95%+), methanol (MeOH), sulfuric acid (95%+) and chloroform were bought from Fisher Scientific (Tournai, Belgium). Lastly, methyl-tert-butylether (tBME) was obtained from Acros Organics (Geel, Belgium).

3.2 Samples

Olive, sunflower and linseed oil were bought from a Bio-planet supermarket in Belgium. Three models including control and non-control groups and were prepared (Table 3.1). Non-control models include stripped oils (linseed, olive or sunflower oil) mixed with virgin olive oil with a ratio of (2:1) (w/w). Whereas in control models, stripped olive oil was used instead of virgin olive oil in the same ratio. The oils were prepared homogenously by mixing with an Ultra-Turrax blender (Janke & Kunkel,IKA-Werk, Staufeb, Germany) for 5 min at 14000 rpm.

Virgin olive oil contributes to oxidation mechanism as antioxidant at dark. Therefore, difference between stripped and non-stripped models were expected to determine the antioxidant effect in autoxidation reactions.

Table 3.1 : Sample models.

Non-control model		
Samples	Composition	Abbreviation
A	Stripped linseed oil +virgin olive oil	SLO+VOO
B	Stripped sunflower oil +virgin olive oil	SSO+VOO
C	Stripped olive oil+virgin olive oil	SOO+VOO
Control model		
Samples	Composition	Abbreviation
D	Stripped linseed oil +stripped olive oil	SLO+OO
E	Stripped sunflower oil +stripped olive oil	SSO+OO
F	Stripped olive oil+stripped olive oil	SOO+OO

3.2.1 Sample preparation

3.2.1.1 Oil stripping

All types of oils (linseed, sunflower and olive oil) used for the experiments were stripped to remove the minor components and obtain pure oil. Stripping process consisted of two steps. For the first step, 25 g of silica was mixed with hexane to pack the glass column (2 cm x 40 cm). Then 50 g oil dissolved in 50 mL hexane has loaded on the column. The sample has eluted 3 times with 50 mL hexane and collected in a round flask. Hexane was removed by rotary evaporator at 30^{o}C under vacuum (Heidolph, Germany) and was dried under nitrogen.

Approximately 100 g of aluminum oxide activated at 200^{o}C overnight was weighed and mixed with petroleum ether. An amount of 50 g oil obtained after stripping with silica was mixed with 60 mL petroleum ether and loaded onto the column (2cm x 40 cm). The column was rinsed with 2 times 100 mL hexane to elute the sample. Solvents were removed by rotary evaporator at 30^{o}C under vacuum and dried under nitrogen.

To prevent photoxidation, all glass wares and columns were covered with aluminum foil.

3.2.2 Incubation of the samples

After model preparation as described in Table 1, 4 mL of each oil blend except fresh samples (day 0) were transferred to amberlit SPME glass vials which were subsequently plugged with cotton and stored in a cold room (6 ± 1^{o}C). Samples were protected from light by putting them inside the boxes. Samples were homogenized by using an orbital shaker (Edmund Bühler, Hechingen, Germany) at a rate of 175 round/min during storage. Samples were stored for 1, 4, 7, 14, 21, 28, 42 and 56 days. All the analysis were carried out in triplicate.

Samples were also subjected to photoxidation by storage at dimmed light in the cold room (6 ± 1^{o}C). However, the data of photoxidation and autoxidation were separated as present work only includes autoxidation of the described oil samples.

3.3 Measurement of lipid oxidation level

3.3.1 Epoxy determination

The epoxy fatty acid determination in oils consisted of two steps: transmethylation and solid phase extraction (SPE). Finally, epoxy fatty acids were analyzed using gas chromatography with flame ionization detector.

3.3.1.1 Base-catalyzed transmethylation

The transmethylation method used for the epoxy fatty acids was the base-catalyzed transmethylation with sodium methoxide at room temperature which was decribed by Mubiri et al (2013). Briefly, 30 µg of methyl-*cis*-10,11-epoxy heptadecanoate (C17:0) was added as an internal standard (IS) and dried under nitrogen. An amount of 200 mg oil sample was accurately weighed into 25 mL test tube. After the addition of 5 mL tert butyl methyl ether and 2.5mL of 0.2M sodium methoxide solution (in methanol), samples were vortexed for 1 minute and allowed to stand at room temperature for 2 minutes. For neutralization and preventing saponification, 0.17mL of 0.5 M sulphuric acid was added and vortexed for few seconds. Finally 5 mL of water was added and mixed for 30 seconds. Samples were left for phase separation. In case of unclear phase separation, samples were centrifuged at 3076xG for 5 minutes. The top layer of the phases, which is organic the layer, was collected.

Extraction was repeated by the addition of 5 mL tBME. Samples were dried under a gentle flow of nitrogen.

3.3.1.2 Solid phase extraction

Silica gel was activated for 12 hours at 450^{o}C in a muffle furnace (Heraeus Instruments, Germany) and later cooled in a desiccator. Then, the moisture content was adjusted to 10% and equilibrated on a shaker for 2 hours. Finally it was kept in desiccator until use. Subsequently, 1 g silica gel (10% water) was added to SPE columns (6 mL, 6.5 cm × 1.3cm, Waters, Zellik, Belgium) and packed with 5 mL of hexane-diethylether (98:2, v/v). Care was taken to avoid air by tapping the column slightly to ensure uniform packing. Finally a small amount of sea sand was added to protect the column. The resultant fatty acid methyl esters (FAMEs) from transmethylation step were dissolved in 2 mL of hexane-diethyl ether (98:2, v/v) and loaded into the column. In the first seperation step, nonpolar fractions consisting of the non-altered FAMEs were eluted by 15 mL of hexane-diethylether (98:2, v/v). The epoxy fraction was collected in the second step with 15 mL of hexane-diethylether (90:10, v/v) and dried under nitrogen to remove the solvents. Samples were dissolved in 200 µL of iso-octane, vortexed and transferred into vials ready for GC injection.

3.3.1.3 Gas chromatography and flame ionization detection

Method described by Mubiru et al (2013) was used to analyze EFAs by using gas chromatograph with flame ionization detection (Agilent 6890N series gas chromatograph, Agilent Technologies, USA). 0,1 μL of sample in the vials which were prepared before were directly injected into the column using a cold on column injector. CP-Sil 88 column for FAME (60 mx0.25 mm i.d.) capillary column coated with 0.2 μm film was used for separation. Protection of the column was performed by the use of a deactivated fused silica precolumn (3 mx0.25 mm i.d., Agilent Technologies, USA). The temperature program was set as follows: 50^{o}C hold for 4 minutes, then ramp to 225^{o}C after 4 min at a rate of 20^{o} C/min, and hold for 25 min. The flame ionization detector temperature was set at 330^{o}C. The detector flow rates for hydrogen, air and helium were 40, 400 and 20 mL min^{-1}, respectively. Helium was used a carrier gas at a flow rate of 1 mL min^{-1}. EFAs were identified by comparing their retention times with epoxy standard. An example for one sample

was shown in Table B.1. A correction factor value (1.22) was adapted from Mubiru et al (2013).

EFA amount (μg g^{-1} oil) was calculated as follows:

$$EFA = \frac{peak\ area\ of\ (\frac{epoxy\ compound}{IS})}{correction\ factor \cdot sample\ weight(g)} \cdot IS\ amount(\mu g) \quad (3.1)$$

3.3.2 Peroxide value

3.3.2.1 Preperation of reagents

The peroxide value was determined according to the ferric thiocyanate method (IDF, 1974). An iron (II) solution was prepared by dissolving 0.4 g barium chloride in 50 mL water and slowly pouring into the iron (II) sulphate solution which was made by dissolving 0.5g iron (II) sulphate in 50 mL distilled water. Finally, 2 mL 10N hydrochloric acid was added to the mixture. The mixture was left to settle. Then, the clear solution was decanted into a brown bottle. A fresh solution was prepared before every measurement and it was not stored for more than one week.

30 g ammonium thiocyanate was dissolved in 100 mL distilled water for preparing the ammonium thiocyanate solution. The solution should be colorless. Lastly, methanol/dichloromethane solvent with a ratio of 30:70 v/v was prepared.

3.3.2.2 Standard Fe(III) solution preparation

The standard iron chloride solution was prepared by dissolving 0.5g iron powder in 50 mL of 10N hydrochloric acid and addition of 1-2 mL of hydrogen peroxide solution (30%, w/w). The solution was boiled for 5 minutes to remove the excess hydrogen peroxide, cooled to room temperature and diluted with water to 500mL. Then, 1 mL iron chloride solution was diluted with methanol/dichloromethane (3:7 v/v) to 100 mL in a volumetric flask.

Standard Fe (III) solutions 5-40 μmL^{-1} solution for calibration were prepared as described by the table below (Table 3.2).

Table 3.2 : Preparation of calibration samples.

Fe(III) amount ($\boldsymbol{\mu g}$)	0	5	10	15	20	25	30	35	40
Stock solution (mL)	0	0.5	1	1.5	2	2.5	3	3.5	4
Solvent (mL)	10	9.5	9.0	8.5	8.0	7.5	7.0	6.5	6.0

3.3.2.3 Procedure

A Cary UV-visible spectrophotometer (Varian, Australia) was set at 500 nm. Absorbance of the prepared standard solutions were measured after exact 5 minutes of 50 μl ammonium thiocyanate solution addition. The measured absorbance was then plotted against the added amounts of Fe (III).

An exact amount of 10 mg fat sample was dissolved in 10 mL of solvent. 50 μL NH_4SCN solution was added, vortexed for few seconds. Then, 50 μL Fe(II) solution was added, vortexed for few seconds. Absorbance was measured exactly 5 minutes at 500 nm. Absorbance was corrected with reagent blank which was measured similarly but without samples.

3.3.2.4 Calculation

The peroxide value of the samples was calculated as milliequivalents of peroxide per kilogram oil (mEq peroxide/ kg) using this formulation:

$$PV = \frac{corrected\ absorbance \cdot m}{55.84 \cdot W \cdot 2} \tag{3.2}$$

Where:

Corrected absorbance = Absorbance of sample – absorbance of reagent blank

m = Slope of calibration curve

W = Mass of the samples (gram)

55.84 = Atomic weight of iron

3.3.3 Conjugated dienes and trienes

The conjugated dienes and conjugated trienes were determined by dissolving 10 and 50 mg oil sample in 10 mL iso-octane, mixing and measuring absorbance at 230 nm and 268 nm respectively. Absorbance was corrected with iso-octane. The Lambert-Beer law was used for calculations:

$$A = \varepsilon \cdot l \cdot C \tag{3.3}$$

Where:

A = Corrected absorbance= Absorbance of sample – absorbance of iso-ocatane

ε = Molecular extinction coefficient: 29000 $M^{-1}cm^{-1}$

l = Width of cuvette=1 mm

C = Concentration (mol/l)

CD and CT were expressed as micromoles CD/CT per gram of oil (μmol CD/CT g^{-1} oil).

3.3.4 p-anisidine Value

The p-anisidine value was evaluated by the AOCS official method Cd 18-90 (AOCS, 1990). 0.1 g oil sample was weighed in a 25 mL volumetric flask and dissolved in iso-octane. Absorbance was measured at 350 nm using iso-octane as blank. An volume of 5 ml solution of oil and 5 ml of iso-octane were transferred to a test tube. Then, 1 mL p-anisidine reagent was added. After exactly 10 minutes absorbance was measured at 350 nm. Absorbance of iso-octane measured at exactly 10 minutes after adding p-anisidine was used as blank. p-anisidine value of the samples were calculated using following equation:

$$p - AV = 25 \cdot (1.2A_s - A_b) / W \tag{3.4}$$

Where:

A_b= Corrected absorbance of the solution without addition of p-anisidine reagent

A_s= Corrected absorbance of the solution with p-anisidine reagent

W=Weight of the sample (g)

3.3.5 Chlorophyll content

3.3.5.1 Procedure

Chlorophyll content in oil models was determined according to the AOCS method described by Pokorny et al (1999). Chlorophyll content was calculated based on pheophytin A, which is the main chlorophyll pigment in vegetable oils by measuring absorbance at 610, 670 and 710 nm against air (International Union of Pure and Applied Chemistry, 1995). Plastic cuvettes of 10 mm were used for the measurements.

3.3.5.2 Calculation

Chlorophyll content was calculated by the below formulation:

$$C = \frac{345.3 \cdot (A_{670} - 0.5 \cdot A_{630} - 0.5 \cdot A_{710})}{L} \quad (3.5)$$

Where:

C = Content of chlorophyll pigments in mg of pheophytin A in 1 kg of oil

A = Absorbance

L = Thickness of the spectrophotometer cell (mm)

3.4 Fatty acid composition

AOCS official method Ce 1b-89 was used for determination of fatty acid composition (1990). Exactly 1 mL of internal standard, prepared by dissolving 250 mg of nanodecanoic acid (C19:0) in iso-octane in a 50 mL volumetric flask, dried under nitrogen. Briefly, 50 mg of oil sample was weighed in a glass test tube including internal standard. Triacylglycerols were saponified by the addition of 2 mL of 0.5N sodium hydroxide solution in methanol. This mixture was heated in a boiling water bath for 7 minutes, cooled subsequently. 2 mL of BF_3/MeOH was added and the tube was placed in a boiling water bath for 5 minutes and cooled with water. 3 mL of iso-octane was added and vortexed for 30 seconds followed by 5 mL saturated sodium chloride solution addition. Final mixture was vortexed again for 30 seconds and left for phase separation. Iso-octane phase was transferred to other test tube. The extraction was repeated with 3 mL of iso-octane. After separation iso-octane phase

was pipetted to second test tube. Dry sodium sulphate was added to the test tube with the organic layer, vortexed and left to allow anhydrous sodium sulphate to settle down. Exact volume of 20 μl FAME solution was transferred to GC vials filled with 980 μL iso-octane. The resulting FAMEs were analyzed on the GC-FID using the same method as described in section 3.1.1.3 and by comparing them with gas liquid chromatography (GLC) standard as shown in Table B.2.

Fatty acid contentrations were calculated according to the following equation:

$$Fatty\,acid\,concentration = \frac{peak\,area\,of\,(\frac{fatty\,acid}{IS})}{sample\,weight(g)} \cdot IS\,amount(g) \tag{3.5}$$

3.5 Data processing

Sample preparation and analysis were done in triplicates. Data collected was statistically analyzed using SPSS Statistics 22 (IBM Corp., New York, USA). Correlations between epoxy fatty acid (EFA) content and peroxide value, CD and CT was determined by Pearson's correlation. One-way ANOVA with Tukey's honesty significance test was used to compare the means of all analysis in every model.

4. RESULTS AND DISCUSSION

4.1 Chlorophyll Content

Chlorophyll pigments are commonly found in vegetable oils, especially in virgin olive oil at high amounts. Chlorophyll pigments could act as antioxidants and photosensitizers depending on the storage conditions (Go´mez-Alonso et al., 2007). Samples were kept in dark and low temperatures for performing autoxidation, therefore, chlorophyll pigments have showed antioxidant properties in this study.

Chlorophyll content of stripped linseed, sunflower and olive oil blended with virgin olive oil models was estimated on an average of 6.8 mg/kg fresh oil. Some studies indicated VOO have 10-11 mg/kg oil chlorophyll pigments (Choe et al., 2005). However, chlorophyll content of fresh virgin olive oil may vary between 2.6-43 mg/kg oil depending on the quality and type of oils (Go´mez-Alonso et al., 2007). Chlorophyll concentration at the beginning (6.8 mg/kg oil) and the end of incubation was mostly remained unchanged (6.6 mg/kg oil) in samples subjected autoxidation. However, in all these three models, chlorophyll content showed a sharp decrease to approximately 3mg/kg oil. Additionally, in stripped linseed oil blended with virgin olive oil, chlorophyll content decreased to 0.2 mg/kg oil. During following days, it increased back to 6.6 mg/kg oil. These fluctuations were not expected due to the fact that pigments could not degrade fast in dark. Moreover, even if some decrease might be observed, it should not be increasing in the following days. Fluctuations could be explained by unknown defect in spectrophotometer used.

In control models, chlorophyll pigments amount was nearly 0 mg/kg oil, expectedly due to the stripping process which removes all minor components in the oil (Table A.1).

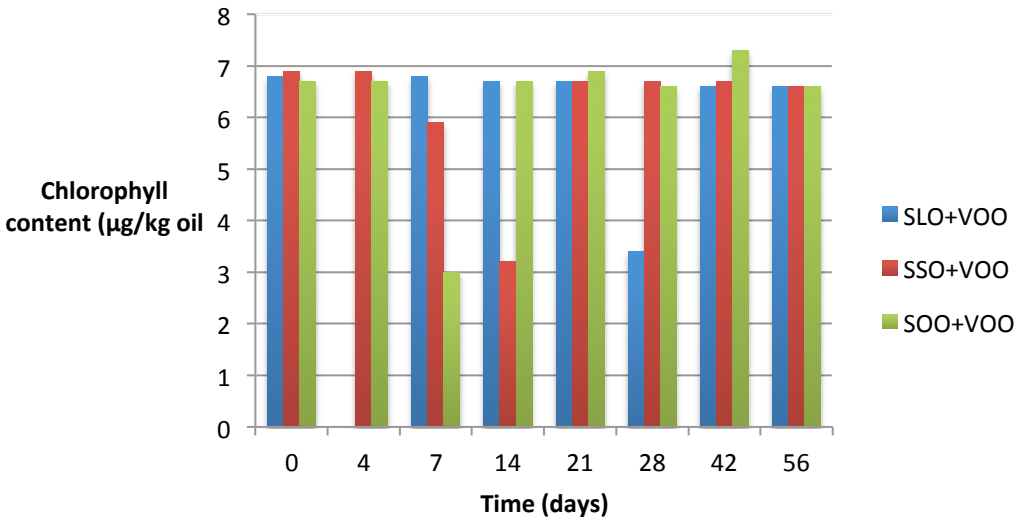

Figure 4.1 : Chlorophyll content of non-control models during 56 days of incubation.

4.2 Fatty Acid Composition of the Oil Models

The fatty acid composition by means of number of double bonds of oils is one of the main factor effecting the formation rate of oxidation products (Schaich, 2006). Therefore, focusing on profiling fatty acid composition is an important parameter for understanding epoxy fatty acid formation pathways. Oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) were determined and shown in Table 4.1.

Table 4.1 : Fatty acid composition of oil models.

	Fatty acid composition (g/100g oil)				
Model	18:0	18:1c9	18:2	18:3n3	Others
A	3.09	28.05	13.68	33.56	9.42
B	2.47	34.77	38.02	0.25	9.50
C	2.55	60.30	14.27	0.64	19.07
D	2.99	26.97	12.96	32.64	8.62
E	2.40	33.89	36.87	0.20	12.49
F	2.32	54.34	12.49	0.55	16.90

C18:1, C18:2, C18:3 are the dominant fatty acids for olive oil, sunflower oil and linseed oil, respectively (Zamora and Hidalgo, 2004). All models have 28.05-60.30 g

oleic acid/100g oil which is considerably high compared with other fatty acids. It was due to the fact that olive oil was added to all of them. The model, mixture of linseed and olive oil (A, D), were found to be rich in linolenic acid (33.56 and 32.64 g/100g). Linoleic acid (38.02 and 36.78 g/100g oil) was the main fatty acid for sunflower and olive oil blend (B, E). In models containing only olive oil, C18:1 amounts (60.30 and 54.34 g/100g oil) were much more higher compared to the other models.

4.3 Primary Oxidation Products

4.3.1 Peroxide value

The peroxide value is an indicator of oxidative stability and primary oxidation products in oils (Rustard, 2009). Peroxide values determined in each oil model were illustrated in Table A.1.

Hydroperoxide formation may be designate as 'high' when peroxide value is above 1 meq O_2/kg oil. The trend of peroxide value in autoxidation is; increase to a maximum value followed by a decrease in advanced stages (Frankel, 2005c). Peroxide value within 56 days have not showed remarkable changes in sunflower and olive oil blends. Due to the stable hydroperoxide formation, increase in peroxide value was expected afterwards. This result indicates that linseed, sunflower and olive oil blends may be considered as at earlier stages of autoxidation during 56 days.

Unlike constant trend of sunflower and olive oil models, linseed oil models showed an increase spesifically at day 56 (Figure 4.2). This is mainly caused by the linolenic acid content of linseed oil compared with other oil models. Lack of chlorophyll pigment in linseed oil blended with stripped olive oil model resulted in much more faster hydroperoxide formation than linseed oil including chlorophyll pigments. Despite the avaliability of chlorophyll pigments in linseed oil blended with virgin olive oil model, peroxide value reached 1 meq O_2/kg oil. In this case, effect of linolenic acid overcame the antioxidants.

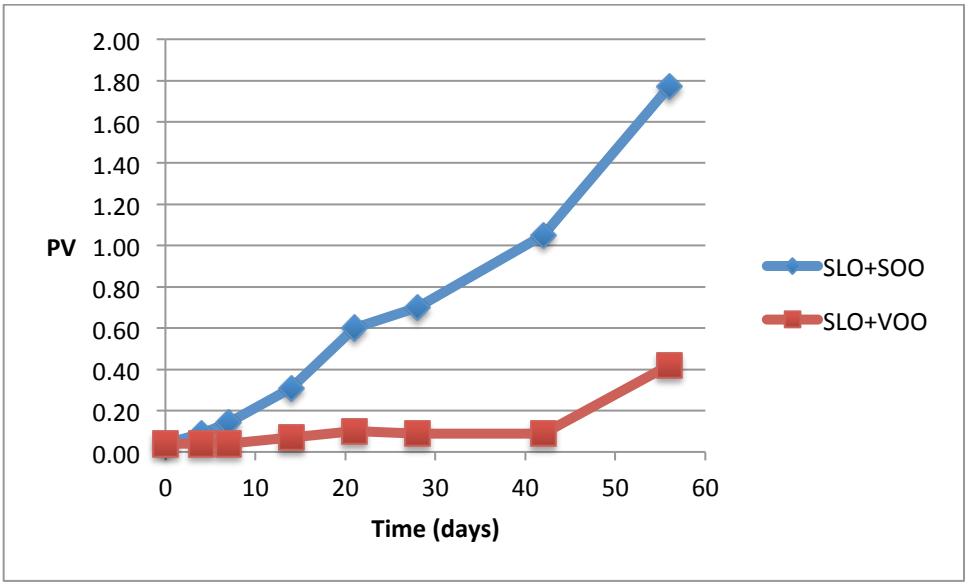

Figure 4.2 : Peroxide values of linseed oil models.

4.3.2 Conjugated diene and conjugated trienes formation

Conjugated dienes and trienes are formed by the reaction with pentadieneyl radical and oxygen, briefly (Warner, 1995). Conjugated diene and triene amounts of each oil model were presented in Table A.1.

Conjugated diene formation rate and amount in linseed oil blends was significantly higher compared with other oil types. However, trend in sunflower oil blend and olive oil models were stable and lower in amount (Figure 4.3 and Figure 4.4). Theoretically, oleic acid cannot form conjugated dienes when mechanism of formation was considered (White, 1995). Linoleic acid forms less amount of conjugated diene compared with linolenic acid. Thus, results obtained might be explained by formation mechanism of conjugated dienes.

Small amount of conjugated triene formation was observed in all of the models. Trends were stable except stripped linseed oil blended with stripped linseed oil which showed increasing trend. The reason could be the lower rate of autoxidation reactions.

Fluctuations occurred in sunflower oil blended with virgin olive oil, however, conjugated triene amount at the beginning and at the end of incubation was almost unchanged. UV range used for conjugated triene measurement was very close to conjugated diene, therefore, overestimation or fluctuations could be observed (Gordon, 200).

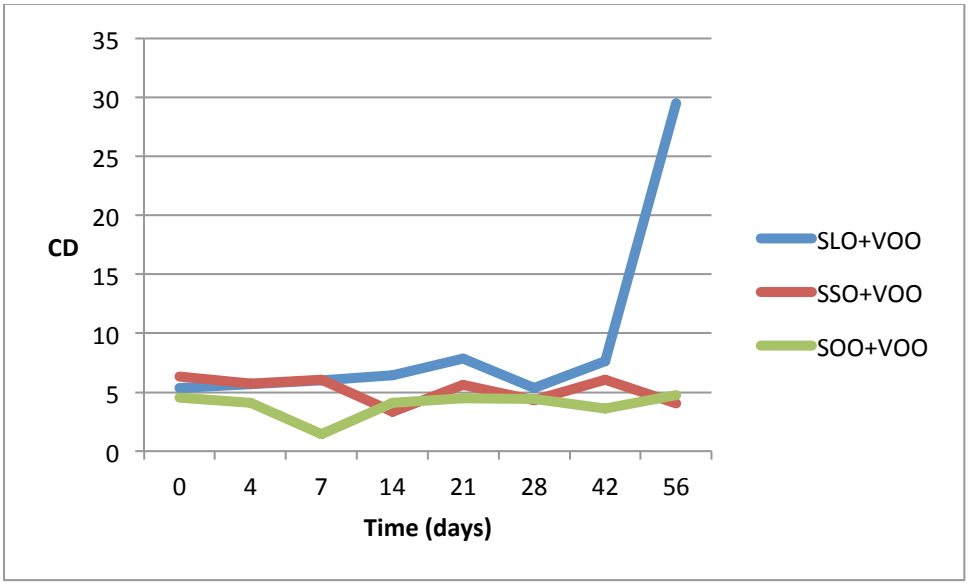

Figure 4.3 : Conjugated diene formation of non-control models.

Conjugated diene amounts were much more higher compared with conjugated trienes. Limitations in conjugated trienes amounts could be related with the formation mechanism. Conjugated trienes formed as the same mechanism with conjugated dienes but only if there is three double bonds in conjugation (Warner, 1995).

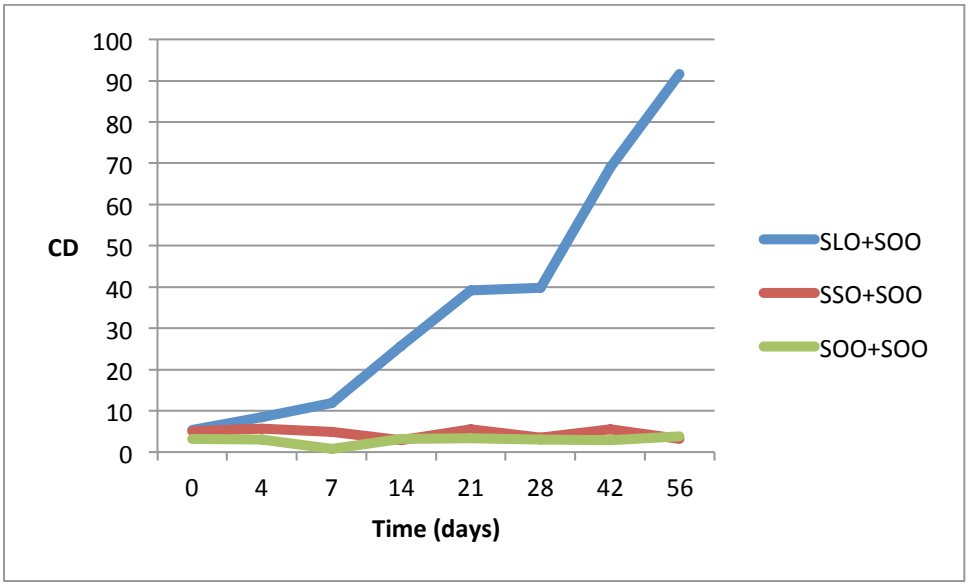

Figure 4.4 : Conjugated diene formation of control models.

4.4 Secondary Oxidation Products

4.4.1 p-Anisidine value

p-anisidine value is the indicator of high molecular weight saturated and unsaturated carbonyl compounds which are defined as secondary oxidation products (Frankel, 2005c). p-AV of each oil model were presented in Table A.1.

Linseed oil and sunflower oil blends with virgin olive oil have both formed the highest amount of p-anisidine value at the end of 56 days incubation. Linolenic acid was reported to have higher p-anisidine values compared with linoleic and oleic acid (Frankel, 2005c).

The trend was decreasing in models containing linoleic acid and oleic acid. Linseed oil blends showed different trends depending on chlorophyll pigment availability. Stripped linseed oil blended with virgin olive oil model showed an increased at the end of incubation whereas stripped linseed oil blended with stripped olive oil remained constant. Fluctuations were monitored both in linseed oil blends and sunflower oil blendS. Fluctuations in amounts as well as trends could be caused by the iso-octane used during experiments.

4.4.2 Quantification of epoxy fatty acids

The comparison of mean values of 12 epoxy fatty acids (EFAs) formed are displayed in Table A.2.

At first sight, stripped models of sunflower and olive oil blends have not formed detectable amounts of epoxy fatty acids at the end of 56 days. Limit of quantification for oils were defined as 5.5 μg g−1 of sample (Mubiru et al., 2014). Lack of chlorophyll pigments in these models was expected to increase the sensitivity to autoxidation. However, by stripping process not only antioxidants but also all other minor components like metals which have an important effect on the rate of autoxidation was removed.

Formation of epoxy fatty acids in linseed oil blends were higher when compared with sunflower and olive oil models containing chlorophyll pigments. Propagation and chain branching reactions are accelerated by the increase of unsaturation degree (Schaich, 2006). Linolenic acid (C18:3) had the highest unsaturation degree among

linoleic (C18:2) and oleic acid (C18:1). Linolenic acid oxidation is indicated as 25 times faster then oleic acid oxidation (Şimşek, 2008).

As shown in Figure 4.5, total EFAs formed in linseed oil blended with stripped olive oil model (533.0 μg/g oil) was approximately 5 times higher than linseed oil blended with virgin olive oil model (122.9 μg/g oil). Due to the removal of all minor components and high content of linolenic acid in the model including stripped olive oil, lipid oxidation was faster. Although the amount of epoxy fatty acids are higher in linseed oil without chlorophyll pigment, initiation of formation took much more time. This might be explained again by the absence of transtion metals. Once EFAs were formed rate of oxidation is faster but metals showed to be important for the initation.

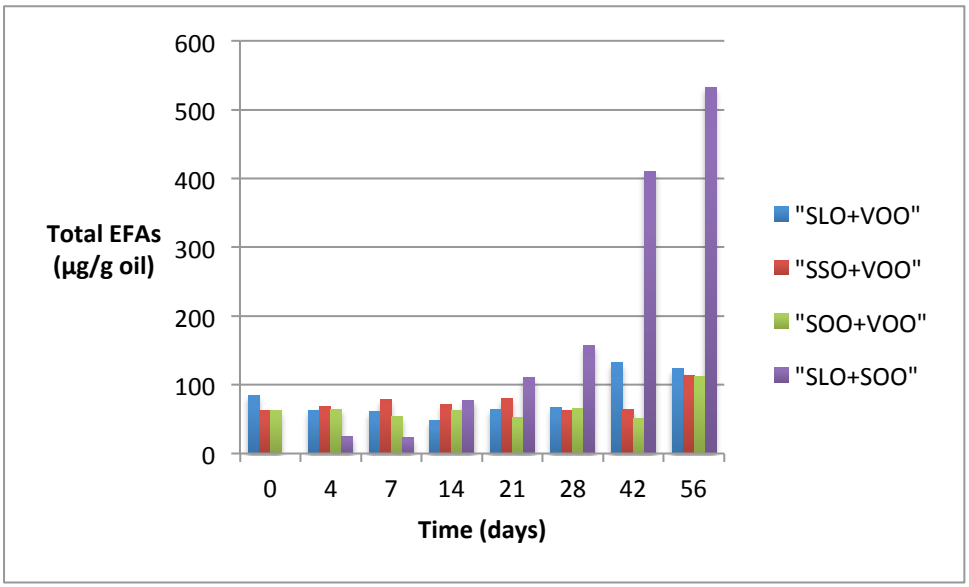

Figure 4.5 : Total epoxy fatty acid content of all models during 56 days of incubation.

Except the models which had not showed significant amount of formation, the other models had showed increasing trend of total epoxy fatty acid amount especially after 28 days.

High amounts of epoxy stearate (ES) formation was associated with olive oil containing C18:1. Sunflower oil blends, rich in C18:2, formed epoxy oleate (EO). In linseed oil blends epoxy linoleate (EOL) formation was observed with respect to C18:3. 12.5-14% of C18:2 content enabled epoxy oleate formation also in linseed oil blends and non-stripped pure olive oil (Table A.3). These results were reasonable compared with literature (Mubiru et al., 2014).

Cis and *trans* isomers of the same epoxy compounds showed different trends. Although higher amount of *cis* isomers were formed (Figure 4.6), linseed oil blended with stripped olive oil have formed more *trans* isomers (Figure 4.7).

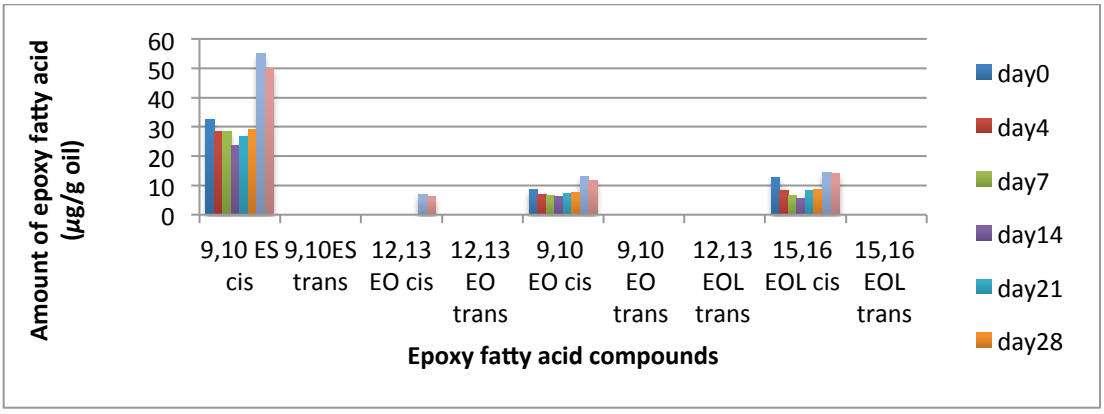

Figure 4.6 : *Cis* and *trans* epoxy fatty acid formation in stripped linseed oil blended with virgin olive oil.

Only information in literature about *cis* and *trans* isomers is: *cis* configuration is more sensitive to autoxidation than *trans* isomers. Therefore, hydroperoxides in *cis* position do not shift to *trans* isomers but undergo cyclization reaction to form epoxy fatty acids in *cis* configuration (Frankel, 2005b; Schaich, 2012). However, it is not clear why linseed oil blended with stripped olive oil formed *trans* epoxy fatty acids more.

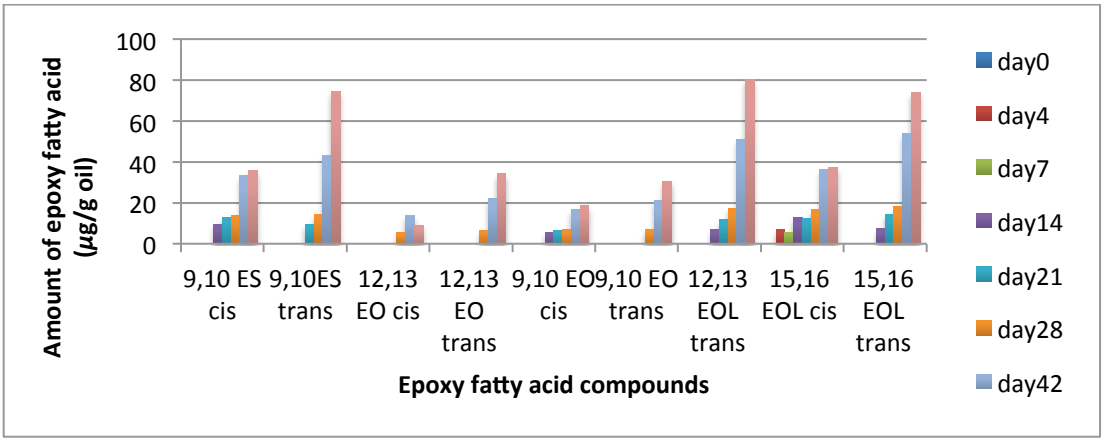

Figure 4.7 : *Cis* and *trans* epoxy fatty acid formation in stripped linseed oil blended with stripped olive oil.

4.5 Correlations

Pearson's correlation was applied to identify the relations between peroxide value, conjugated diene, conjugated trienes, p-anisidine value and total epoxy fatty acids

(Table 4.2). In sunflower oil and olive oil models blended with stripped olive oil, epoxy fatty acids were not detected. Therefore, the correlations between EFAs and other oxidation parameters were not operated.

Table 4.2 : Correlations between PV, CD, CT and total EFAs.

Correlations		
Model		EFA
A (stripped linseed oil and virgin olive oil blend)	PV	0.760*[1]
	CD	0.646
	CT	0.684
	p-anisidine	-0.652
B (stripped sunflower oil and virgin olive oil blend)	PV	0.473
	CD	-0.330
	CT	0.483
	p-anisidine	0.004
C (stripped olive oil and virgin olive oil blend)	PV	0.536
	CD	0.964*
	CT	0.726*
	p-anisidine	-0.199
D (stripped linseed oil and stripped olive oil blend)	PV	0.959**[2]
	CD	0.953**
	CT	0.872*
	p-anisidine	-0.638

[1]Correlation is significant at 0.05 confidence level (2-tailed).
[2] Correlation is significant at 0.01 confidence level (2-tailed).

Peroxides are unstable and rapidly transformed into secondary oxidation products (Hahm and Min, 1995). Only linseed oil blends showed strong correlation between peroxide value and epoxy fatty acids. Peroxide values for other models were under 1 meq O_2/kg oil therefore, not considered as high amounts. This could be the reason for the other models which have not showed correlation with EFA.

Apart from Pearson correlation, by direct comparision of the formation amounts, epoxy fatty acids are considerably high. Similar results were also reported by Mubiru

et al., (2013). This result indicates, even small amount of hydroperoxide formation could lead to epoxy fatty acid formation.

Another parameter for primary oxidation products determination is conjugated dienes and trienes. All models excluding stripped sunflower oil blended with virgin olive oil showed positive correlation between conjugated dienes and total epoxy fatty acid content. Olive oil model containing chlorphyll pigment and linseed oil model without chlorophyll model had showed strong positive correlation with conjugated trienes. However, it was mentioned in literature that oleic acid cannot form conjugated dienes and trienes which was linseed oil blends in our study (White, 1995). The reason could be the overestimation of conjugated dienes and trienes as their UV range is very close in the method used for measurement (Gordon, 2000).

No correlation found between epoxy fatty acids and p-anisidine value. p-anisidine value indicates the carbonyl groups which are secondary oxidation products (Rustard, 2010). Homolytic β scission of C-C bonds results in formation of volatile compounds whereas, epoxy fatty acids are produced by the 1,2 addition mechanism to the nearest double bond (Kolakowska, 2010; Mubiru et al., 2014). Due to the different formation mechanism, it is possible that epoxy fatty acids and aldehydes have no correlation as represented in our results.

Correlations between peroxide value and conjugated dienes were also performed as they have similar structure and results were gathered in Table 4.3. Only models containing linseed oil showed positive correlation. Pure olive oil blends were not expected to form conjugated dienes as described above. No correlations were observed in sunflower oil blends, probably due to the low amounts of peroxide and conjugated diene formation.

Table 4.3 : Correlations between PV and CD.

Correlations		
Model		CD
A *(SLO and VOO blend)*	PV	0.989^2
B *(SSO and VOO blend)*		-0.322
C *(SOO and VOO blend)*		0.520
D *(SLO and SOO blend)*		0.989^2
E *(SSO and SOO blend)*		-0.510
F *(SOO and SOO blend)*		0.369

5. CONCLUSION

The objective of the present study was to determine epoxy fatty acids and compare their formation with respect to fatty acid composition and availability of chlorophyll pigments during oxidation reactions.

As a result, storage time, fatty acid composition and chloropyll pigment existance had showed a great impact on epoxy fatty acid formation. By stripping process, not only chlorophyll pigments was removed but also other minor components. Among them, metal presence might be the most effective factor. An interesting result was that EFA formation might be an important reaction of autoxidation due to their existence in high amounts even hydroperoxide amount was low. Additionaly, the main factor effecting difference formation pathways of *cis* and *trans* epoxy fatty acids seems to be unclear. Thus, there are some missing point needs to be well-understand. In conclusion, understanding EFA formation mechanism should be the first step taken.

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APPENDICES

APPENDIX A: Results
APPENDIX B: Chromatograms

APPENDIX A

Table A.1: Peroxide value, p-anisidine, conjugated diene, conjugated triene and chlorophyll content of oil models.

	Time (days)	PV (meq O_2 kg^{-1} oil)	p-AV (g^{-1} oil)	CD (μmol kg^{-1} oil)	CT (μmol kg^{-1} oil)	Chlorophyll content (mg kg^{-1} oil)
A *(stripped linseed oil and virgin olive oil blend)*	0	0.04	3.50	5.38	0.30	6.80
	4	0.04	9.96	5.70	0.34	0.19
	7	0.04	16.11	6.00	0.28	6.79
	14	0.07	23.84	6.44	0.26	6.66
	21	0.10	19.10	7.83	0.44	6.73
	28	0.09	6.57	5.36	0.27	3.38
	42	0.09	6.64	7.65	0.47	6.55
	56	0.42	6.40	29.5	0.72	6.56
B *(stripped sunflower oil and virgin olive oil blend)*	0	0.02	7.76	6.33	2.48	6.85
	4	0.02	9.05	5.71	2.24	6.90
	7	0.02	2.89	6.08	2.02	5.91
	14	0.02	5.86	3.33	0.17	3.16
	21	0.02	9.11	5.62	2.10	6.70
	28	0.03	3.47	4.31	2.02	6.70
	42	0.02	5.86	6.04	2.34	6.71
	56	0.03	6.08	4.04	2.49	6.63

Table A.1 (continue) : Peroxide value, p-anisidine, conjugated diene, conjugated triene and chlorophyll content of oil models.

Oil model	Time (days)	PV (meq O_2 kg^{-1} oil)	p-AV (g^{-1} oil)	CD (μmol kg^{-1} oil)	CT (μmol kg^{-1} oil)	Chlorophyllcontent (mg kg^{-1} oil)
C *(stripped olive oil and virgin olive oil blend)*	0	0.02	6.26	4.52	0.02	6.66
	4	0.02	2.97	4.12	0.02	6.73
	7	0.02	2.48	1.43	0.01	2.99
	14	0.02	3.25	4.09	0.01	6.68
	21	0.02	2.56	4.46	0.02	6.92
	28	0.02	2.65	4.44	0.03	6.61
	42	0.02	2.51	3.62	0.02	7.29
	56	0.02	2.79	4.73	0.04	6.63
D *(stripped linseed oil and stripped olive oil blend)*	0	0.04	2.14	5.30	0.56	0.25
	4	0.09	13.78	8.50	0.37	0.07
	7	0.14	15.47	11.85	0.46	0.22
	14	0.31	7.93	25.74	3.99	0.31
	21	0.60	13.72	39.12	2.01	0.04
	28	0.70	9.69	39.89	2.36	0.32
	42	1.05	13.29	68.97	3.88	0.02
	56	1.77	2.06	91.68	7.87	0.40

Table A.1 (continue) : Peroxide value, p-anisidine, conjugated diene, conjugated triene and chlorophyll content of oil models.

Oil model	Time (days)	PV (meq O2 kg^{-1} oil)	p-AV (g^{-1}oil)	CD (μmol kg^{-1} oil)	CT (μmol kg^{-1} oil)	Chlorophyll content (mg kg^{-1} oil)
E	0	0.004	8.65	5.08	2.04	0.21
(stripped sunflower oil	4	0.001	7.04	5.62	2.11	0.20
and stripped olive oil	7	0.02	4.56	4.97	1.98	0.00
blend)	14	0.01	3.82	2.89	1.79	0.00
	21	ND	5.74	5.54	2.14	0.00
	28	0.06	5.48	3.47	1.98	0.00
	42	0.01	0.08	5.59	2.09	0.00
	56	0.02	0.60	3.12	2.34	0.00
F	0	0.001	4.27	3.14	0.11	0.00
(stripped olive oil and	4	0.002	0.85	2.98	0.13	0.00
stripped olive oil blend)	7	0.004	0.78	0.77	0.01	0.00
	14	0.01	0.89	3.24	0.01	0.00
	21	0.01	0.77	3.41	0.14	0.00
	28	0.01	0.54	3.06	0.18	0.00
	42	0.01	1.09	2.92	0.20	0.00
	56	0.01	0.77	3.81	0.30	0.00

Table A.2: Amount of epoxy fatty acid compounds in oil model.

Oil models	Time (day)	Epoxy fatty acids (μg/g oil)										
		9,10 ES *trans*	9,10ES *cis*	12,13 EO *trans*	9,10EO *trans*	12,13 EO *cis*	9,10 EO *cis*	12,13 EOL *trans*	15,16 EOL *trans*	12,13 cis&9,10 EOL *trans*	15,16 EOL *cis*	9,10 EOL *cis*
A *(stripped linseed oil and virgin olive oil blend)*	0	ND	32.53±0.79^{a}	ND	<LOD	ND	8.51±0.10^{a}	ND	ND	14.79 ±0.21^{a}	12.67±0.06^{a}	13.92±0.55^{a}
	4	ND	28.42 ±0.86^{b}	ND	<LOD	ND	7.00 ± 0.06^{a}	ND	ND	10.73 ±0.07^{b}	8.27±0.19^{b}	8.73 ±0.89^{b}
	7	ND	28.57±1.28^{b}	ND	<LOD	ND	6.63 ±0.51^{a}	ND	ND	10.04 ±0.50^{b}	6.64 ±0.40^{bc}	8.56±1.37^{b}
	14	ND	23.66 ±1.40^{c}	ND	<LOD	ND	6.37 ±1.44^{a}	ND	ND	12.28±0.77^{ab}	5.63 ± 0.44^{c}	<LOQ
	21	ND	26.78±1.11^{bc}	ND	<LOD	ND	7.35 ±0.34^{a}	ND	ND	12.01 ±0.61^{ab}	8.42 ±0.52^{b}	9.35±0.31^{b}
	28	ND	28.96±0.65^{bc}	ND	<LOD	ND	7.69±0.41^{a}	ND	ND	11.66±0.89^{b}	8.71 ±0.35^{b}	10.26±0.38^{b}
	42	6.68±0.24^{a}	54.91±1.70^{d}	ND	<LOD	7.04 ±0.77^{a}	12.97±1.48^{b}	ND	ND	20.38±2.01^{c}	14.32 ±0.19^{a}	15.57±0.32^{ac}
	56	6.52±0.82^{a}	49.87±1.16^{e}	ND	<LOD	6.31 ±0.41^{a}	11.66±1.49^{b}	ND	ND	19.15±0.22^{c}	14.16±1.94^{a}	16.19±3.05^{c}
B *(stripped sunflower oil and virgin olive oil blend*	0	ND	33.70 ±3.11^{a}	ND	ND	11.96±1.75^{a}	17.31 ±1.91^{ac}	ND	ND	<LOQ	ND	ND
	4	ND	36.55±0.85^{a}	ND	ND	14.04 ±0.37^{a}	18.02 ±0.91^{ac}	ND	ND	<LOQ	ND	ND
	7	ND	41.02 ±0.54^{a}	ND	ND	16.64 ±0.32^{ab}	20.37±0.87^{ac}	ND	ND	<LOQ	ND	ND
	14	ND	37.50 ± 3.06^{a}	ND	ND	14.94 ±2.33^{a}	18.55 ±2.38^{a}	ND	ND	<LOQ	ND	ND
	21	ND	41.61 ±2.52^{a}	ND	ND	17.05 ±0.95^{b}	21.14 ±1.51^{c}	ND	ND	<LOQ	ND	ND
	28	ND	34.14 ±2.99^{a}	ND	ND	12.94 ±1.60^{a}	15.60 ±1.86^{a}	ND	ND	<LOQ	ND	ND
	42	ND	34.84±2.07^{a}	ND	ND	13.00±2.05^{a}	15.77±1.97^{a}	ND	ND	<LOQ	ND	ND
	56	<LOQ	65.88±3.14^{b}	ND	ND	20.31±0.65^{b}	27.32±2.45^{b}	ND	ND	<LOQ	ND	ND
C *(stripped olive oil and virgin olive oil blend)*	0	ND	46.95±0.29^{ab}	ND	ND	5.97±0.28^{a}	8.86±0.43^{a}	ND	ND	<LOQ	ND	ND
	4	ND	49.17±2.46^{ab}	ND	ND	5.95±0.40^{a}	9.07±0.61^{a}	ND	ND	<LOQ	ND	ND
	7	ND	47.60 ±1.26^{ab}	ND	ND	5.87±0.20^{a}	8.72±0.31^{a}	ND	ND	<LOQ	ND	ND
	14	ND	48.48±0.18^{ab}	ND	ND	5.64 ±0.09^{a}	8.56±0.2^{a}	ND	ND	<LOQ	ND	ND
	21	ND	43.99±2.45^{ab}	ND	ND	<LOQ	8.24 ±0.66^{a}	ND	ND	<LOQ	ND	ND
	28	ND	50.15±2.80^{b}	ND	ND	6.25±0.37^{a}	8.80 ±0.41^{a}	ND	ND	<LOQ	ND	ND
	42	ND	42.87±1.90^{a}	ND	ND	<LOQ	8.18±0.37^{a}	ND	ND	<LOQ	ND	ND
	56	<LOQ	81.24±4.35^{c}	ND	ND	10.83±2.37^{b}	20.07±0.21^{b}	ND	ND	<LOQ	ND	ND

Table A.2 (continue) : Amount of epoxy fatty acid compounds in oil model.

Oil model	Time day	Epoxy fatty acids (μg/g oil)										
		9,10 ES *trans*	9,10ES *cis*	12,13 EO *trans*	9,10EO *trans*	12,13 EO *cis*	9,10 EO *cis*	12,13 EOL *trans*	15,16 EOL *trans*	12,13 cis&9,10 EOL *trans*	15,16 EOL *cis*	9,10 EOL *cis*
D	0	ND	<LOQ	ND	ND	ND	ND	ND	ND	<LOQ	ND	<LOQ
(stripped	4	ND	<LOQ	ND	ND	ND	ND	ND	ND	8.99±1.00^{a}	6.93 ±0.50^{a}	9.55 ±0.60^{a}
linseed oil	7	ND	<LOQ	ND	ND	ND	ND	<LOQ	<LOQ	9.59±1.17^{a}	5.72 ±0.97^{a}	7.27±1.59^{a}
and	14	ND	9.36±0.80^{a}	ND	ND	<LOQ	5.72 ±0.41^{a}	7.17±0.45^{a}	7.31 ±0.42^{a}	17.82 ±1.37^{ab}	12.88 ±1.82^{ab}	16.70 ±1.49^{ab}
stripped	21	9.38±1.28^{a}	12.92 ±0.89^{a}	<LOQ	<LOQ	<LOQ	6.58±0.83^{a}	11.74 ±1.60^{a}	14.44 ± 1.19^{a}	27.20 ± 3.09^{ab}	12.54 ± 1.49^{ab}	15.57±2.18^{ab}
olive oil	28	14.46±0.71^{b}	13.76±0.65^{a}	6.67±0.27^{a}	6.82 ±0.35^{a}	5.52 ±0.37^{a}	6.89±0.34^{a}	17.40 ±0.24^{a}	18.37±0.56^{a}	30.46±1.12^{b}	16.83 ±1.08^{b}	19.37±1.63^{b}
blend)	42	43.23 ±1.21^{c}	33.31 ±3.29^{b}	22.00±1.65^{b}	21.07±0.65^{b}	13.61 ±1.14^{b}	16.88±1.0^{b}	51.19±1.59^{b}	54.11 ±1.60^{b}	79.60 ±2.75^{c}	36.22 ±2.57^{c}	39.51 ±2.54^{b}
	56	74.59±4.39^{d}	35.58±3.70^{b}	34.19±0.60^{c}	30.50±0.26^{c}	8.87±0.96^{a}	18.51±0.08^{b}	80.42 ±7.67^{c}	74.16 ±4.44^{c}	98.61 ±6.17^{c}	37.40 ±4.00^{c}	40.17±3.03^{b}
E	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
(stripped	4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
sunflower	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
oil and	14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
stripped	21	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
olive oil	28	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
blend)	42	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	56	ND	<LOQ	ND	ND	ND	ND	ND	ND	ND	ND	ND
F	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
(stripped	4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
olive oil	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
and	14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
stripped	21	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
olive oil	28	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
blend)	42	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	56	ND	<LOQ	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table A.3: Total amount of epoxy fatty acids separated by isomer group.

Oil models	Time (day)	Epoxy fatty acids (μg/g oil)			
		Total ES	Total EO	Total EOL	Total EFAs
A *(stripped linseed oil and virgin olive oil blend)*	0	32.53	8.51	41.38	82.42
	4	28.42	7.00	27.73	63.15
	7	28.57	6.63	25.24	60.44
	14	23.66	6.37	17.91	47.94
	21	26.78	7.35	29.87	63.91
	28	28.96	7.69	30.63	67.28
	42	61.59	20.01	50.27	131.87
	56	55.45	17.97	49.50	122.92
B *(stripped sunflower oil and virgin olive oil blend)*	0	33.70	29.27	<LOQ	62.97
	4	36.55	32.06	<LOQ	68.61
	7	41.02	37.01	<LOQ	78.03
	14	37.50	33.49	<LOQ	70.99
	21	41.61	38.19	<LOQ	79.8
	28	34.14	28.54	<LOQ	62.68
	42	34.84	28.77	<LOQ	63.61
	56	65.88	47.63	<LOQ	113.51
C *(stripped olive oil and virgin olive oil blend)*	0	46.95	14.83	<LOQ	61.78
	4	49.17	15.02	<LOQ	64.19
	7	47.60	5.87	<LOQ	53.47
	14	48.48	14.20	<LOQ	62.68
	21	43.99	8.24	<LOQ	52.23
	28	50.15	15.05	<LOQ	65.20
	42	42.87	8.18	<LOQ	51.05
	56	81.24	30.90	<LOQ	112.1

Table A.3 (continue) : Total amount of epoxy fatty acids separated by isomer group.

Oil modes	Time (day)	Epoxy fatty acids (μg/g oil)			
		Total ES	Total EO	Total EOL	Total EFAs
D *(stripped linseed oil and stripped olive oil blend)*	0	<LOQ	ND	<LOQ	<LOQ
	4	<LOQ	ND	25.40	25.40
	7	<LOQ	ND	22.58	22.58
	14	9.4	5.72	61.88	77.00
	21	22.3	6.58	81.49	110.37
	28	28.22	25.9	102.43	156.55
	42	76.54	73.56	260.63	410.73
	56	110.17	92.07	330.76	533.00
E *(stripped sunflower oil and stripped olive oil blend*	0	ND	ND	ND	ND
	4	ND	ND	ND	ND
	7	ND	ND	ND	ND
	14	ND	ND	ND	ND
	21	ND	ND	ND	ND
	28	ND	ND	ND	ND
	42	ND	ND	ND	ND
	56	<LOQ	ND	ND	<LOQ
F *(stripped olive oil and stripped olive oil blend)*	0	ND	ND	ND	ND
	4	ND	ND	ND	ND
	7	ND	ND	ND	ND
	14	ND	ND	ND	ND
	21	ND	ND	ND	ND
	28	ND	ND	ND	ND
	42	ND	ND	ND	ND
	56	ND	ND	ND	ND

APPENDIX B

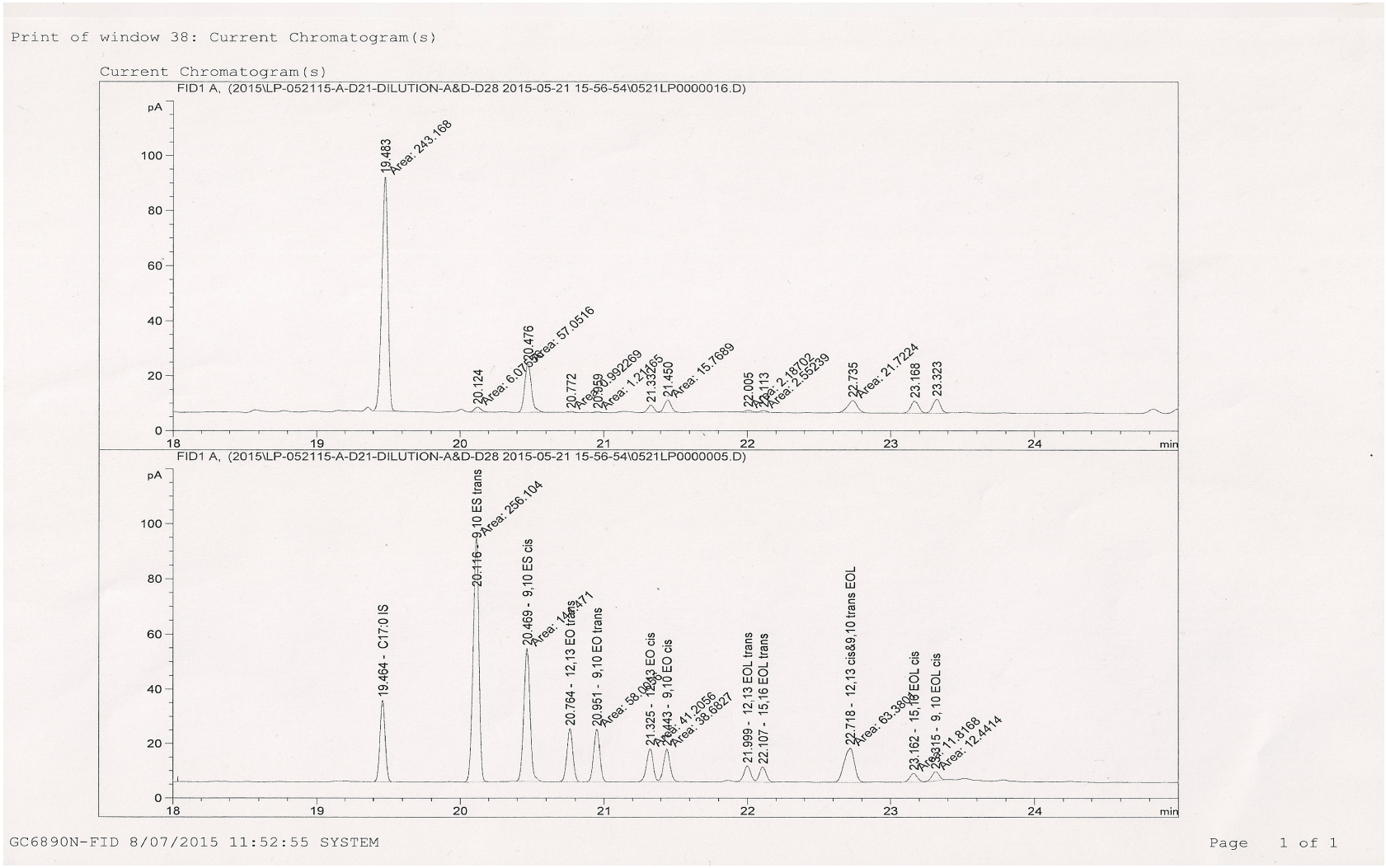

Figure B.1 : Epoxy fatty acid chromotagram for the sample (top) and epoxy standard (bottom).

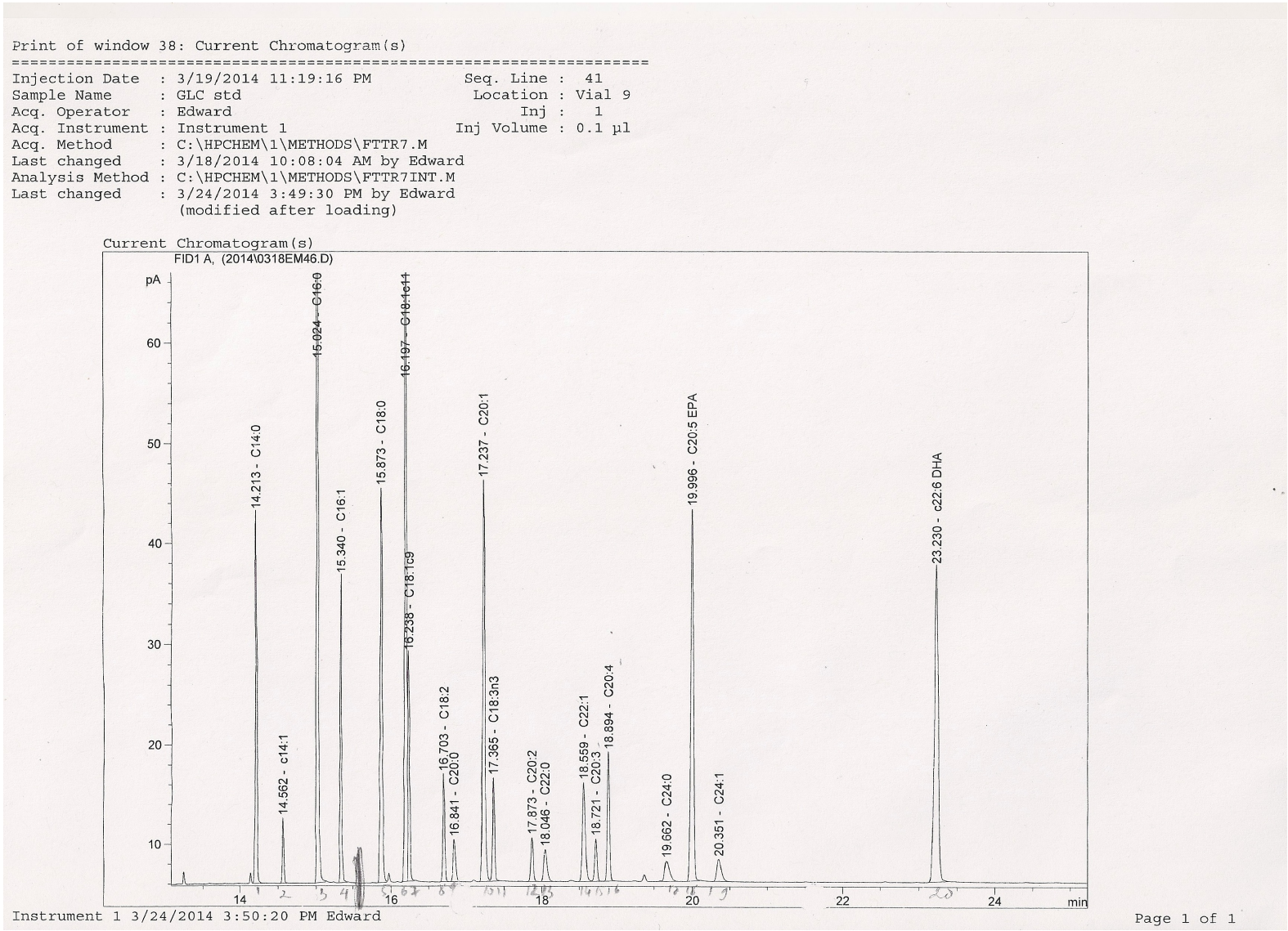

Figure B.2 : Referance chromotagram for fatty acid composition analysis.

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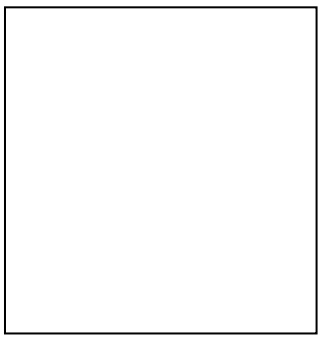

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