Formation of epoxy fatty acids during autoxidation of oils at elevated temperature

Ziggy Buyle

Promotor: Prof. dr. ir. Bruno De Meulenaer
Tutor: MSc. Edward Mubiru

Master thesis submitted to obtain the degree of Master of Science in Bioscience: Food Industry
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Ghent, 8th of June 2014
Preface

The practical work of this thesis was carried out at the department of Food Safety and Food Quality at Ghent University, located in Ghent, Belgium.

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Last but not least, special thanks to my friends and family for the continuous support and help.
Summary

Lipid oxidation is a free-radical chain reaction which leads to oxidative rancidity. During lipid oxidation many oxidation products are formed. Some of these oxidation products can be harmful to human health. That is why it is important to understand the formation mechanism of these oxidation products and to know how fast these compounds are being formed. This work focuses on epoxy fatty acids, which are secondary oxidation products.

To monitor the oxidation process and the formation of epoxy fatty acids, stripped and non-stripped oil models containing primarily oleic, linoleic or linolenic fatty acids were stored at an elevated temperature of 70 °C for 0 to 5 days. After 0, 1, 3 and 5 days sampling was done and the peroxide value, the conjugated dienes, the conjugated trienes and the amount of epoxy fatty acids were determined.

The stripped oil models showed a very fast oxidation process. The models containing mostly unsaturated fatty acids were oxidizing at a higher rate than the oil models with less unsaturated fatty acids. The models containing a significant amount of linolenic acid oxidized faster and formed the highest amount of epoxy fatty acids. In only five days, up to 5 mg/g oil of epoxy fatty acids were formed, which is very high. The non-stripped oil models showed the same oxidation kinetics but were oxidizing much slower. Only the oil models with linolenic acid are showing a significant increase of epoxy fatty acids in such a short time.
Table of contents

Preface .................................................................................................................................... III

Summary ................................................................................................................................. IV

1 Introduction ..................................................................................................................... 3

2 Literature overview ......................................................................................................... 4

   2.1 Lipid oxidation .......................................................................................................... 4

   2.1.1 Mechanisms of lipid oxidation .............................................................................. 5

   2.1.1.1 Initiation ........................................................................................................... 5

   2.1.1.2 Propagation ....................................................................................................... 5

   2.1.1.3 Termination ..................................................................................................... 6

   2.1.1.4 Photoxidation ................................................................................................. 8

   2.1.1.5 Thermoxidation .............................................................................................. 8

   2.1.2 Kinetics of lipid oxidation .................................................................................... 9

   2.1.2.1 Induction period ........................................................................................... 9

   2.1.2.2 Peroxide formation and decomposition ........................................................ 9

   2.1.3 Negative effects of lipid oxidation ...................................................................... 11

   2.1.3.1 Effect on food quality .................................................................................. 11

   2.1.3.2 Effect on health ............................................................................................ 11

   2.1.4 Epoxy fatty acids ............................................................................................... 12

   2.2 Measurement of lipid oxidation level ..................................................................... 15

   2.2.1 Peroxide value ...................................................................................................... 15

   2.2.1.1 Iodometric titration ....................................................................................... 15

   2.2.1.2 Ferric thiocyanate method .......................................................................... 16

   2.2.2 Conjugated dienes and trienes ......................................................................... 16

   2.2.3 Malondialdehyde value ..................................................................................... 17

3 Material and Methods ................................................................................................... 18

   3.1 Chemicals and reagents ........................................................................................... 18

   3.2 Samples .................................................................................................................... 18

   3.2.1 Preparation of the oil models ............................................................................. 19

   3.3 Sample preparation .................................................................................................. 20

   3.3.1 Stripping of the oils ........................................................................................... 20

   3.3.1.1 First step ....................................................................................................... 20

   3.3.1.2 Second step ................................................................................................. 20

   3.4 Treatment and storage of the samples .................................................................... 21

   3.5 Base-catalyzed-transmethylation .......................................................................... 22

   3.6 Solid phase extraction (SPE) .................................................................................. 22

   3.6.1 Activation of the silica gel ................................................................................. 22

   3.6.2 Preparation of the column ................................................................................. 22

   3.6.3 Separation of the polar fraction .......................................................................... 23

   3.7 Gas chromatography and flame ionization detection ............................................. 24
3.8 Peroxide value ............................................................................................................. 25
  3.8.1 External calibration curve and equations .......................................................... 25
3.9 Conjugated dienes and conjugated trienes ............................................................... 27
3.10 Determination of fatty acid profile using acid-catalysed esterification and transesterification ................................................................................................................. 27
3.11 Determination of chlorophyll pigments .................................................................. 28
3.12 Data processing ........................................................................................................ 28

4 Results and discussion ................................................................................................... 29
  4.1 Fatty acid composition of the models .................................................................... 29
  4.2 Chlorophyll level ..................................................................................................... 30
  4.3 Stability of the oils .................................................................................................. 31
  4.4 Formation of primary oxidation products .............................................................. 33
  4.5 Formation of epoxy fatty acids ............................................................................. 38
  4.6 Correlations .......................................................................................................... 44

5 Conclusion ..................................................................................................................... 47

6 Appendices .................................................................................................................... 50
  Appendix 1: Flowchart thesis ..................................................................................... 51
  Appendix 2: IDF method for peroxide determination .................................................... 52
  Appendix 3: Chromatogram of one of the samples ...................................................... 55
1 Introduction

Lipid oxidation is not only responsible for sensory degradation, it can also be a risk to human health. It is shown that oxidation products are involved in diseases like atherosclerosis and cancer and that they can impair certain metabolic functions (Dobarganes & Marquez-Ruiz, 2003). Recent studies (Mubiru et al., 2013, 2014) optimized the method for detecting and quantifying one of the secondary oxidation products, epoxy fatty acids. This work is a continuation on these studies and focuses on the formation of epoxy fatty acids in oils while oxidizing at an elevated temperature. The thesis revolves on autoxidation of C18:1, C18:2 and C18:3 and is part of a bigger study where photoxidation and thermoxidation are also looked into.

The aim of this work was to determine the stability of the oils by determining the peroxide value, to follow the formation of other primary oxidation products, that is conjugated dienes and conjugated trienes and to monitor the formation of epoxy fatty acids by base-catalyzed-transmethylation and detection and quantification with GC-FID. Finally correlations between peroxide value, conjugated dienes, conjugated trienes and amount of epoxy fatty acids were found.

This work starts with a review of the literature, which gives some background information on the mechanisms and the kinetics of lipid oxidation. It also shows some insights into the measurement of the oxidation level in oils and it talks about the negative effects of lipid oxidation. Next, the material and methods used are explained, including standards and the design of the study. Thereafter the results of the experiments are given and discussed and finally a conclusion is presented.
2 Literature overview

2.1 Lipid oxidation

Oxidation, a free-radical chain reaction which leads to oxidative rancidity and hydrolysis, the splitting of triacylglycerol into fatty acids by reacting with water, are the two basic reactions of lipid deterioration. Oxidation is more responsible for the deterioration than hydrolysis (List et al., 2005). Oils with high concentrations of polyunsaturated fatty acids (PUFAs) are more susceptible to lipid oxidation. They can autoxidize due to molecular oxygen in the oil reacting with the fatty acids of the triglycerides, they can photoxidize due to light exposure, they can thermoxidize due to exposure to heat and they can also oxidize enzymatically due to enzymatic reactions (Frankel, 2005). Photoxidation can only occur when oils are exposed to direct sunlight or to fluorescent light in presence of a photosensitiser. Enzymatic oxidation is of little concern in commercial oil samples, as the enzymes in the oil are inactivated during the refinement process (List et al., 2005).

The rate and degree of oxidation depends on several factors: fatty acid saturation, amount of molecular oxygen present in the sample, water content and the amount of anti- and pro-oxidants, etc. Antioxidants and pro-oxidants influence the induction period. This is the period where very little oxidation occurs. Pro-oxidants, such as iron and copper, reduce the induction period significantly, whereas antioxidants, such as \( \alpha \)-tocopherol, extend the induction period (Steele, 2004). A typical oxidation curve with an induction period is shown in figure 1.

![Figure 1: Typical oxidation curve with induction period (IP). (Adapted from Steele, 2004)](image-url)
2.1.1 Mechanisms of lipid oxidation

A chemical reaction that occurs at room temperature between molecular oxygen and an organic compound is usually referred to as autoxidation. It is important to note that this reaction is autocatalytic or self-sustaining. Therefore, the rate of oxidation is slow at the start and increases with time. There are three main steps in the autoxidation: initiation, propagation and termination (Porter et al., 1995).

2.1.1.1 Initiation

The first step of the lipid oxidation is the formation of a lipid radical, R•. There are three ways from where this lipid radical can be formed. It can be formed due to a thermal or photochemical homolytic cleavage of a RH bond or by the abstraction of a hydrogen atom from a RH bond by a free radical. This free radical can be a hydroperoxide which was formed in the plant oil prior to and during the extraction process. The homolytic cleavage of the RH bond is seen as the main initiation reaction in edible oils (Porter et al., 1995; Steele, 2004).

2.1.1.2 Propagation

The lipid radical, or alkyl radical, formed in the initiation step reacts with molecular oxygen. There are three orbital states in which molecular oxygen can exist, namely two singlet states and a triplet state (Ho et al., 1995), as shown in figure 2.

![Diagram showing partial 2p orbitals of triplet oxygen and two singlet oxygens](image)

Figure 2: The partially filled 2p orbitals of respectively triplet oxygen and two singlet oxygens. (Adapted from Ho et al., 1995)
The singlet states are electronically excited and are therefore highly reactive. It can react directly with an unsaturated fatty acid (Alberti & Orfanopoulos, 2010). The triplet state is less reactive and can react with the previously formed lipid radical first to form a peroxyl radical, ROO•. This peroxyl radical is highly reactive and will react with a new fatty acid to form a hydroperoxide, ROOH, and another lipid radical. By formation of a new lipid radical, the oxidation process continues. Because of these chain reactions this step is called the propagation. The rate at which this occurs is dependent on the energy needed to break the RH bond of the fatty acid. The dissociation energy of an allylic hydrogen is 10 kcal/mol higher than the dissociation energy of a bisallylic hydrogen. That is why PUFAs are much more susceptible to oxidation than other fatty acids (Porter et al., 1995; Steele, 2004).

2.1.1.3 Termination

When two alkyl radicals react with each other, they form a non-reactive dimer, RR. In case of excess oxygen in the sample, most of the alkyl radicals will react with oxygen to form peroxyl radicals. When there is consequently an excess of peroxyl radicals, the peroxyl radicals will participate in the termination as well. They can react with other peroxyl radicals or with alkyl radicals to form peroxides, ROOR.

Peroxide can decompose to form alkoxy radicals, RO•, which in turn can decompose to form volatile compounds, such as hydrocarbons, alcohols and aldehydes, and non-volatile compounds, such as non-volatile alcohols, ketones and epoxides. These compounds are referred to as secondary oxidation products. The volatile compounds, and more specifically the aldehydes are mostly responsible for the aroma changes in oxidized oils (Steele, 2004). Figure 3 shows a summary of the mechanism of lipid oxidation.
Figure 3: Generalized scheme of autoxidation of unsaturated lipids. (Adapted from Shahidi, 1997)
2.1.1.4 Photoxidation

Light can turn triplet oxygen into singlet oxygen, with aid of photosensitizers, such as riboflavin, chlorophyll, myoglobin, porphyrins, and synthetic colorants (Lee, 2002). Singlet oxygen can start the oxidation reaction due to its high reactivity. Photoxidation depends on the formation of singlet oxygen and it cannot be quenched by free radical antioxidants. It can be inhibited though by singlet oxygen quenchers like carotenoids. Photoxidation is of less concern, unless under direct sunlight or visible light, because light with wavelength less than 220 nm cannot be absorbed by lipids (List et al., 2005).

2.1.1.5 Thermoxidation

Thermoxidation is the oxidation process at high temperatures. At 180 °C and above, oxidation products are being formed which consumers believe to smell and taste good. This can be explained by the difference of solubility of oxygen between low and high temperatures. At low temperatures, solubility of oxygen is high, hence primarily hydroperoxides are formed. At high temperatures, solubility of oxygen is low, although the oxidation process is increased drastically. It is shown that hydroperoxides are almost absent at 150 °C, and high amounts of triacylglycerol monomers and polymers are formed. The formation of compounds at high temperatures is complex, as not only oxidation reactions but also thermal reactions are occurring (Dobarganes & Marquez-Ruiz, 2003).

Lipids oxidized at low temperatures show maximum 4-5% oxidized triacylglycerols before being classified as rancid. Frying fats on the other hand display often more than 25% of oxidized compounds, which is the upper limit permitted in most countries (Dobarganes & Marquez-Ruiz, 2003). However, it is believed that many fast food places use fat with even higher amounts of oxidized compounds (Saguy & Dana, 2003).


2.1.2  Kinetics of lipid oxidation

2.1.2.1  Induction period

During the induction period or initial phase, the oxidation process proceeds slowly. The length of this phase can be increased by lowering the temperature, by adding antioxidants, by decreasing the oxygen concentration or by decreasing the activity of pro-oxidants.

In theory the oxidation process increases exponentially with the increase of temperature. Practically speaking, this is not completely correct because of the oxygen solubility decreasing with increasing temperature. Antioxidants prolong the initial phase by catching the free radicals in the oil and by forming them into less reactive compounds. By decreasing the activity of pro-oxidants, the induction phase will be extended. Pro-oxidants like iron and copper are very effective in reducing the initial phase, even in concentrations of one part per million or less. They also catalyze the decomposition of hydroperoxides (Steele, 2004).

2.1.2.2  Peroxide formation and decomposition

Initially hydroperoxides are formed faster than they are destroyed, which means their level increases over time. At a certain critical peroxide level the oxidation enters the second phase. In this phase the oxidation rate increases exponentially. From this point on the oil will begin to smell and taste rancid (List et al., 2005). The peroxide levels will keep increasing over time, until the decomposition rate of the hydroperoxides gets higher than the formation rate, as shown in figures 4 and 5. The time required to reach the maximum levels depends on the saturation level of the fatty acids. Highly unsaturated lipids will reach this point sooner (Frankel, 2005). This means that in the early stages of the oxidation process the primary oxidation products will dominate and in the later stages the secondary oxidation products will dominate. The final stages of oxidation, polymerization and degradation, come accompanied with a big increase of viscosity, as shown in figure 5 (List et al., 2005).
Figure 4: Development of primary and secondary oxidation products during lipid oxidation. (Adapted from Frankel, 2005)

Figure 5: Peroxide formation and decomposition as function of time. (Adapted from List et al., 2005)
2.1.3 \textit{Negative effects of lipid oxidation}

2.1.3.1 \textit{Effect on food quality}

The main concern of lipid oxidation is rancidification. This process covers the decomposition of lipids, such as fats and oils. It is characterized by off flavors and undesirable odors. In some cases, such as during the frying process, oxidation creates desirable compounds. At present, the emphasis is being given to the use of unsaturated fatty acids in food due to the fact that PUFAs are considered healthy. Consequently, the food sector has problems with rancidity, given that PUFAs are more susceptible to oxidation. Besides the produced flavors and odors, oxidation changes the nutritional quality as well as the fatty acid composition.

To control oxidation, it is advised to minimize the amount of metal in the oil, and to optimize the amount of antioxidants, such as tocopherol. Unfortunately, antioxidants often are destroyed or removed during the refining process (Frankel, 1996). Therefore, antioxidants are often added to the final product (Wsowicz et al., 2004).

2.1.3.2 \textit{Effect on health}

At present, it is difficult to determine the impact of oxidized lipids on our health. The main reason for this is the fact that it is hard to get trustworthy information on the daily intake of these fats. This is partly blamed on the change of lifestyle in the last decades. The ratio of visible to invisible fats has decreased due to the increasing consumption of convenient foods and fast food. This makes it hard to predict the composition of consumed fats. Secondly, it is difficult to estimate the degree of oxidation of digested fats and thirdly, the oxidation process produces a cocktail of compounds of which the toxicity is not always fully clear yet. Therefore it becomes hard to predict if the amount of oxidized fats in diets is sufficient to express physiological effects (Dobarganes & Marquez-Ruiz, 2003).

Lipids oxidized at low to moderate temperature (below 20 °C) are not believed to be a problem for human health, as these products show sensory degradation and would be discarded before consumption. It is still important to keep in mind that small amounts of oxidation products are ingested through fats which do not show degradation. On the other hand, lipids that are oxidized at high temperatures (above 180 °C) contain several oxidation products that humans seem to appreciate. Therefore these products are being eaten and are a potential risk to our health. That is why oxidation processes at high temperature are believed to be the primary source of oxidation products in our diets (Dobarganes & Marquez-Ruiz, 2003).
In addition, it is important to notice that human gastric fluid is a perfect medium for further oxidation of the lipids. This suggests an underestimation of the dietary intake of oxidation products (Kanner & Lapidot, 2001). High intake of oxidation products can attribute to atherosclerosis, cancer and impairment of metabolic functions, but more data are needed on the intake of these compounds to see if the intake is sufficient to produce these effects. Literature suggests that a moderate intake of oxidized fats is safe. However, ingestion of highly oxidized fats, such as overused frying fats, can be harmful in the long term (Dobarganes & Marquez-Ruiz, 2003).

2.1.4 *Epoxy fatty acids*

Epoxides are molecules with a ring structure of three atoms containing an ether group, as can be seen in figure 6b. Epoxides can be used as biomarkers of lipid oxidation since they are secondary oxidation products. They are believed to be formed by two different mechanisms. The first mechanism consists of the addition of a peroxy radical to a non-conjugated double bond. The double bound will undergo a 1,3- cyclization which forms an epoxide and eliminates an alkoxy radical (figure 6a). The second mechanism is a rearrangement or a cyclization, involving a 1,2- addition to an adjacent double bond and formation of an epoxyallylic radical (figure 6b) (Mubiru et al., 2014).

![Figure 6: Mechanism of epoxy fatty acid formation. (adapted from Schaich, 2005)](image-url)
Recent studies have shown that especially the C18 epoxy fatty acids appear in high amounts in oxidized oils and foods (Mubiru et al., 2013, 2014). This may have consequences on human health, as epoxides have been found to be leukotoxic and some isomers are implicated in acute respiratory distress (Greene et al., 2000; Hayakawa et al., 1990).

There are 12 epoxy fatty acids from the dominant C18 fatty acids, that is oleic, linoleic and linolenic acid. Two from oleic acid (c18:1) namely methyl trans-9,10-epoxystearate and methyl cis-9,10-epoxystearate. Four epoxy fatty acids from linoleic acid (c18:2) namely methyl trans-12,13-epoxyoleate, methyl cis-12,13-epoxyoleate, methyl trans-9,10-epoxyoleate and methyl cis-9,10-epoxyoleate and six from linolenic acid (c18:3) namely methyl trans-12,13-epoxy-9,15-octadecadienoate, methyl cis-12,13-epoxy-9,15-octadecadienoate, methyl trans-15,16-epoxy-9,12-octadecadienoate, methyl cis-15,16-epoxy-9,12-octadecadienoate, methyl trans-9,10-epoxy-12,15-octadecadienoate and methyl cis-9,10-epoxy-12,15-octadecadienoate (Mubiru et al., 2014). Their structures can be seen in figure 7 on the next page.
Figure 7: Structure of formed epoxy fatty acids.
2.2 Measurement of lipid oxidation level

2.2.1 Peroxide value

Free radicals, abstracted from fatty acids, react with molecular oxygen to form peroxide radicals (primary oxidation products). These peroxide radicals react with unsaturated fatty acids, forming other free radicals and hydroperoxide. Hydroperoxides are very unstable and will form secondary oxidation products. The peroxide value is therefore correlated with the degree of oxidation of fats.

2.2.1.1 Iodometric titration

The AOCS Official Method 965.33 is commonly used to determine the peroxide value of oils and fats. This method is based on the reaction of peroxides in the oil sample with iodide ion present in a saturated potassium iodide solution. The reaction will form iodine which is an indicator of the initial quantity of peroxides present in the sample. The amount of iodine can be measured by titration with sodium thiosulphate with starch as end point indicator. The peroxide value is expressed in milliequivalents of peroxide per kilogram oil. The reaction mechanism is illustrated below.

\[
\text{ROOH} + 2\text{H}^+ + 2\text{I}^- \rightarrow \text{I}_2 + \text{ROH} + \text{H}_2\text{O} \\
\text{I}_2 + 2\text{S}_2\text{O}_3^{2-} \rightarrow \text{S}_4\text{O}_6^{2-} + 2\text{I}^-
\]

Despite being the official method for determining the peroxide levels in oils, this method has some limitations. First of all, the procedure requires large amounts of oil, it is time consuming and has difficulties in determining the titration end point (Dobarganes & Velasco, 2002). Secondly, iodine can react with unsaturated bonds, which results in a lower peroxide value and thirdly, oxygen in the sample can liberate iodine from the potassium iodide and consequently raise the peroxide value (Min et al., 1985).

These limitations along with a relatively high detection limit, thus low sensitivity, has led to the development of different methods for determining the peroxide value, such as the ferric thiocyanate method. However, despite all the above limitations, it is still a popular method for determination of peroxide value in oxidized oils.
2.2.1.2 Ferric thiocyanate method

The ferric thiocyanate method is based on the fact that peroxides oxidize ferrous ions to ferric ions. These ferric ions are then complexed with thiocyanate in a red iron complex, which can be measured with a spectrophotometer at 500 nm (Lips et al., 1943). This method is ten times more sensitive than the iodometric titration. There still can be an error due to the presence of oxygen in the sample, but this can be avoided by bubbling the sample with nitrogen.

As with the iodometric titration method, the peroxide value is expressed in milliequivalents of peroxide per kilogram oil. It has to be taken into account that the peroxide value found in the last method has to be divided by two, to be compared with the values obtained from the iodometric titration method (Lea, 1952).

2.2.2 Conjugated dienes and trienes

One of the first things happening in the oxidation process of PUFAs is a positional shift of a double bond. This shift happens when one hydrogen atom breaks apart from a methylene group between two double bonds. The formed pentadienyl radical can shift to form two possible conjugated dienes. The conjugated dienes can react with oxygen to form conjugated hydroperoxides. When there are three double bonds in conjugation, they are called conjugated trienes. Therefore there is a correlation between the amount of displaced bonds and the degree of oxidation (Warner & Eskin, 1995).

The quantity of conjugated dienes and trienes can be measured at a wavelength of 232-234 nm or a wavelength of 268 nm respectively. The method is very simple as the sample only has to be diluted with iso-octane before being analysed with a spectrophotometer. Another advantage of this method is the fact that the sample size is small (0,1 g) and that it does not depend on chemical reactions (Frankel, 2005).

There also are some limitations to the method, the amount of conjugated dienes is dependent on the fatty acid composition. Samples containing high amounts of PUFA will have more conjugated dienes and trienes, whereas for example oleic acid theoretically cannot have conjugated dienes and trienes. Therefore the comparison of different oils is not possible (Warner & Eskin, 1995). A second limitation is that secondary oxidation products have the same UV detection range as the dienes and trienes. Which means an overestimation of the amount of dienes and trienes can be obtained (Steele, 2004). Nevertheless, the determination of conjugated dienes and trienes is still a good relative measurement of the degree of oxidation, if the fatty acid composition of the sample is known.
2.2.3  **Malondialdehyde value**

Malondialdehyde (MDA) is a secondary oxidation product of polyunsaturated fatty acids. The malondialdehyde value can be used as a biomarker of oxidative stress. The most frequently used test to determine the MDA value is the thiobarbituric acid reactive substances (TBARS) method. TBA reacts with MDA to form a complex which can be quantified spectrophotometrically at 532 nm. The reaction mechanism is illustrated in figure 8 (Janero, 1990).

![Reaction mechanism TBARS](image)

Figure 8: Reaction mechanism TBARS. Adapted from Janero, 1990.

However, this traditional method of determination presents relatively low recovery levels (under 71%) and its accuracy and specificity for MDA is not optimal, as compounds other than MDA can interfere with the complex formation. Hence it is better to use a second method of determination, namely high performance liquid chromatographic (HPLC) (Mendes et al., 2009; Papastergiadis et al., 2012).
3 Material and Methods

3.1 Chemicals and reagents

Silica gel 60 (0.063 – 0.100 mm) and boron trifluoride-methanol solution were bought from Merck Chemicals (Overijse, Belgium). Hexane (95%+), isoctane, sodium chloride (99.8% purity), sodium sulfate, sea sand (acid washed and calcinated), diethylether, barium chloride, hydrochloric acid (37%) and ammonium thiocyanate were purchased from Chem-Lab NV (Zedelgem, Belgium). Aluminium oxide (Brockmann I activated), sodium methoxide (25% w/v) and iron sulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Petroleum ether, hexane (95%+), methanol, sulfuric acid (95%+) and chloroform were bought from Fisher Scientific (Tournai, Belgium). Finally methyl-tert-butylerether was obtained from Acros Organics (Geel, Belgium). Methyl cis-10,11-epoxyheptadecanoate (C17:0) was used as internal standard. It was obtained as previously described (Mubiru et al., 2014).

3.2 Samples

All oils were bought freshly from a supermarket in Belgium. Six models of stripped oils with their control were formulated depending on the fatty acid composition in a 2:1 ratio (oil: virgin olive oil; v:v) as follows: two models with stripped olive oil, two models with stripped sunflower oil and two models with stripped linseed oil. The remaining three control models were mixed with stripped refined olive oil, also in a 2:1 ratio as described above.

Another six models of non-stripped oils were made with the same setup as the stripped models, only the stripped oils replaced by non-stripped oils. These six models are further referred to as the non-stripped samples.

Virgin olive oil was added in order to compare the results of this work with the results of further experiments on photoxidation. Virgin olive oil is rich in chlorophyll pigments, which are needed for the photoxidation of fatty acids. Because chlorophyll acts as an antioxidant as well, a control group for each model was made where the virgin olive oil was replaced with refined olive oil. Table 1 clarifies the composition of the models.
Table 1: Composition of the models.

### Stripped samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Composition</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Stripped refined olive oil + Virgin olive oil</td>
<td>SOO + VOO</td>
</tr>
<tr>
<td>B</td>
<td>Stripped sunflower oil + Virgin olive oil</td>
<td>SSFO + VOO</td>
</tr>
<tr>
<td>C</td>
<td>Stripped linseed oil + Virgin olive oil</td>
<td>SLO + VOO</td>
</tr>
<tr>
<td>D</td>
<td>Stripped refined olive oil</td>
<td>SOO</td>
</tr>
<tr>
<td>E</td>
<td>Stripped sunflower oil + Stripped refined olive oil</td>
<td>SSFO + SOO</td>
</tr>
<tr>
<td>F</td>
<td>Stripped linseed oil + Stripped refined olive oil</td>
<td>SLO + SOO</td>
</tr>
</tbody>
</table>

### Non-stripped samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Composition</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Refined olive oil + Virgin olive oil</td>
<td>OO + VOO</td>
</tr>
<tr>
<td>B</td>
<td>Sunflower oil + Virgin olive oil</td>
<td>SFO + VOO</td>
</tr>
<tr>
<td>C</td>
<td>Linseed oil + Virgin olive oil</td>
<td>LO + VOO</td>
</tr>
<tr>
<td>D</td>
<td>Refined olive oil</td>
<td>OO</td>
</tr>
<tr>
<td>E</td>
<td>Sunflower oil + Refined olive oil</td>
<td>SFO + OO</td>
</tr>
<tr>
<td>F</td>
<td>Linseed oil + Refined olive oil</td>
<td>LO + OO</td>
</tr>
</tbody>
</table>

### 3.2.1 Preparation of the oil models

For each model, oil was measured with a graduated cylinder in a 2:1 ratio, as previously mentioned. The oils were blended together using an Ultra-Turrax blender (Janke & Kunkel, IKA-Werk, Staufeb, Germany) for 1 min at 14000 rpm.
3.3 Sample preparation

3.3.1 Stripping of the oils

3.3.1.1 First step

A glass column with valve was filled with hexane and 25 g of silica gel was added to pack the column. To prevent air trapping the valve was opened, to create a small vortex, while adding the silica. Next, 50 g of sample was dissolved in 50 ml of hexane and was loaded onto the column. The column was wrapped in aluminum foil to prevent light-induced oxidation during the stripping process. The sample was eluted with 50 ml of hexane in three steps and collected in a round bottom flask. The oil was recovered by evaporating the hexane at room temperature using a rotary evaporator (Heidolph, Germany).

3.3.1.2 Second step

A glass column with valve was packed with petroleum ether and 100 g of aluminium oxide. The obtained oil of the first step was then dissolved in petroleum ether (1:1, v/v) and loaded onto the aluminum wrapped column. The oil was then eluted with 100 ml of hexane and dried at room temperature with the rotary evaporator.
3.4 Treatment and storage of the samples

Accelerated oxidation was performed in an oven at 70 °C ± 5 °C for up to 5 days. Sampling was done at 0, 1, 3 and 5 days. Each sample was done in triplicate. After the oxidation process and in between experiments the samples were stored at -20 °C. Figure 9 shows the sampling scheme for an oil model.

Figure 9: Sampling scheme for an oil model (PV = peroxide value; CD = conjugated dienes; CT = conjugated trienes; EFAs= epoxy fatty acids).
3.5  Base-catalyzed-transmethylation

The transmethylation method used for the epoxy fatty acids was the base-catalyzed-transmethylation with sodium methoxide at room temperature as already described by other studies (Mubiru et al., 2013, Dobarganes & Velasco, 2002). First of all, 50 µg of internal standard (C17:0) was accurately pipetted into a 25 ml glass centrifuge tube and was dried under nitrogen using a nitrogen generator (Domnick Hunter, Parker, Cleveland, USA). 200 mg of sample was weighted into the glass centrifuge tube and 5 ml of methyl-tert-butylether (tBME) was added. Then 2.5 ml of 0.2 M sodium methoxide solution in methanol was added to the sample and the tube was vortexed for 1 min. After exactly 2 minutes standing at room temperature the reaction was stopped by adding 0.17 ml of 0.5 M sulfuric acid and vortexing the mixture for a few seconds. This was needed for neutralization purposes and to prevent saponification. Eventually 5 ml of water was added and the mixture was vortexed for 30 seconds. After the phases separated, the organic layer was collected and the extraction was repeated with 5 ml of tBME. The organic layer was dried under nitrogen and the resulting fatty acid methyl esters (FAMEs) were dissolved into 2 ml of n-hexane-diethyl ether (98/2, v:v).

3.6  Solid phase extraction (SPE)

3.6.1  Activation of the silica gel

The procedure used has been described by Mubiru et al, 2013. Briefly, the silica gel was activated in a muffle furnace at 450 °C for 12 hours and cooled in a desiccator. The moisture content was then modified to 10% and the silica was equilibrated on a shaker for 1 hr.

3.6.2  Preparation of the column

One gram of the activated silica gel was weighed into the SPE cartridge (6 mL, 6.5 cm × 1.3 cm, Waters, Zellik, Belgium) and 5 ml of the elution solvent, n-hexane-diethyl ether (98/2, v:v), was added. To avoid trapping air the silica was mixed with a pasteur pipette and a layer of sea sand (1 cm) was put on top to protect the column.
3.6.3 Separation of the polar fraction

The obtained FAMEs were loaded onto the prepared SPE column and the non-polar fraction was then eluted with 15 ml of n-hexane-diethyl ether (98/2, v:v). Subsequently the polar fraction, which contains the epoxy, was eluted with 15 ml of n-hexane-diethyl ether (90/10, v:v) and collected in a 15 ml glass centrifuge tube. The solvent was then removed by drying under nitrogen and the resulting epoxy was dissolved in 300 µL of isoctane ready for GC analysis. Figure 10 shows an example of the SPE set up.

Figure 10: Solid phase extraction set up.
3.7 Gas chromatography and flame ionization detection

The epoxy fatty acids (EFAs) were analyzed by gas chromatography and flame ionization detection (GC-FID) using an Agilent 6890N series gas chromatograph (figure 11) (Agilent Technologies, USA). The samples were dissolved in iso-octane, and 0.1 µl was injected directly into the column using a cold on column injector (COC); separation was performed in a CP-Sil 88 for FAME (60 m × 0.25 mm i.d.) capillary column coated with a 0.2 µm film. A deactivated fused silica precolumn 3 m × 0.25 mm i.d. (Agilent Technologies, USA) was fitted to protect the column. The oven temperature program was set as follows: 50 °C hold for 4 min, then ramp to 225 °C at 12 °C min⁻¹, and hold for 25 min. The flame ionization detector temperature was set at 300 °C. The detector flow rates for hydrogen, air and helium (makeup) were 40, 400 and 20 ml min⁻¹ respectively. The column flow rate of helium as a carrier gas was 1 ml min⁻¹. Identification of individual EFAs was carried out by comparison of retention times with those of an epoxy standard. The procedure, as well as the used epoxy standard and the used response factors, were according to Mubiru et al.,( 2013).

Figure 11: Agilent 6890N series gas chromatograph.
3.8 Peroxide value

The peroxide value was determined according to the ferric thiocyanate method (IDF, 1974). The iron (II) chloride solution was made by dissolving 0.5 g iron (II) sulfate into 50 ml of water, dissolving 0.4 g barium chloride in 50 ml water and by pouring them slowly together with constant stirring and in dimmed light. The ammonium thiocyanate solution was made by dissolving approximately 30 g ammonium thiocyanate in 100 ml of water. Finally, the solvent was made by mixing 70 volume parts of chloroform with 30 volume parts of methanol. The solvent was then mixed with 0.5% ammonium thiocyanate and 0.5% iron (II) chloride to create the reaction solvent.

To measure the peroxide value 0.01 g of sample was weighed into a glass centrifuge tube and 10 ml of reaction solvent was added. After exactly 5 minutes the absorbance at 500 nm was measured with a Varian Cary 50 Bio UV-visible spectrophotometer (Agilent Technologies, USA). The absorbance was corrected with the pure reaction solvent as blank and the peroxide value was calculated using an external reference calibration curve.

3.8.1 External calibration curve and equations

To make the stock solution, 1 ml was taken from a 1 mg/ml iron (III) solution and diluted to 100 ml with the solvent. Next, six samples containing 5 to 30 µg iron (III) were made for analysis by the IDF method for calibration (table 2).

<table>
<thead>
<tr>
<th>Calibration samples composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe (III) (µg)</td>
</tr>
<tr>
<td>Stock solution (ml)</td>
</tr>
<tr>
<td>Solvent (ml)</td>
</tr>
</tbody>
</table>

The spectrophotometer was set at 500 nm and was set blank with the solvent. Next, 50 µl of the ammonium thiocyanate solution was added to the calibration samples and after exactly 5 minutes the absorbance was measured. The measured absorbance was then plotted against the added amounts of iron (III). This plot is displayed in figure 12.
The peroxide value of the models was then calculated as milligram equivalents oxygen per kilogram oil (mEq O\textsubscript{2}/kg) using this formula:

$$ PV = \frac{Corrected\ absorbance \times m}{55.84 \times W \times 2} $$

Where:

Corrected absorbance = absorbance sample – absorbance blank
m = slope of calibration curve
W = mass in gram of the samples taken
55.84 = atomic weight of Iron

Figure 12: Calibration curve for the peroxide value.
3.9  **Conjugated dienes and conjugated trienes**

The conjugated dienes and conjugated trienes were determined by weighing 10 mg and 50 mg of sample respectively into a glass centrifuge tube. Then samples were dissolved into 10 ml isooctane and the absorbance was measured at respectively 233 nm and 268 nm with pure isooctane as a blank.

Because the 233 and 268 nm wavelengths are part of the ultraviolet spectrum, quarts cuvettes were used. The calculations were made using the Lambert-Beer law:

\[
A = \varepsilon \times l \times C
\]

Where:

- \( A \) = corrected absorbance
- \( \varepsilon \) = extinction coefficient
- \( l \) = width cuvette
- \( C \) = concentration

3.10  **Determination of fatty acid profile using acid-catalysed esterification and transesterification**

This method was adapted from the AOCS official method Ce 2-66 (AOCS, 2009) and was used to determine the fatty acid composition of the used oils. Approximately 50 mg of oil was weighed into a glass centrifuge tube and 2 ml of a 0.5 N methanolic NaOH solution was added to saponify the triacylglycerols. The tube was placed in a boiling water bath for 7 min and was eventually cooled with water. For the esterification 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. After adding 3 ml of isooctane, 5 ml of saturated sodium chloride was added and vortexed for 30 seconds. After letting the phases separate the organic layer was collected with a pasteur pipette and the extraction was repeated with 3 ml of isooctane. The resulting FAMEs were then analyzed on the GC-FID using the same method as described in section 3.8.
3.11 Determination of chlorophyll pigments

The chlorophyll level was determined spectrophotometrically by measuring the absorbance at 670 nm. The result was corrected for the background absorption, and the content was calculated with use of the absorptivity of pheophytin A, which is the main chlorophyll pigment in vegetable oils.

The samples were measured at 630, 670 and 710 nm in a 10 mm thick spectrophotometer cell against air. The amount of chlorophyll pigments was then calculated using following formula:

\[
C = \frac{345,3 \times (A_{670} - 0,5 \times A_{630} - 0,5 \times A_{710})}{L}
\]

Where:

- \( C \) = content of chlorophyll pigments in mg of pheophytin A in 1 kg of oil
- \( A \) = absorbance
- \( L \) = thickness of the spectrophotometer cell

3.12 Data processing

To combine all the collected data, to make calculations and to make the charts displayed in this thesis, Microsoft Excel was used. For the correlations a Pearson’s correlation was used using IBM SPSS.
4 Results and discussion

4.1 Fatty acid composition of the models

The fatty acid composition of the oil models was analyzed. As shown in Table 3 the C18 fatty acids were the most common in the models. Model A, which contains olive oil, shows 71% oleic acid (C18:1), 10% linoleic acid (C18:2), but trace levels of linolenic acid (C18:3). Model B, which contains sunflower oil, had 46% oleic acid, 40% linoleic acid and trace levels of linolenic acid. Model C, which is primarily linseed oil, shows 40% oleic acid, 12% linoleic acid and 34% linolenic acid. Model D, E and F showed almost the same composition as model A, B and C respectively since they were the controls of the first three oil models. The small deviations could have been caused by the substitution of virgin olive oil with refined olive oil in model D, E and F.

Model A and D had approximately 14% of palmitic acid (C16:0). However, this is of less concern, as it is a saturated fatty acid and therefore is not capable of forming epoxy fatty acids.

Table 3: Fatty acid composition of stripped and non-stripped oils (g / 100 g fatty acid).

<table>
<thead>
<tr>
<th>Model</th>
<th>C14:0</th>
<th>C16:0</th>
<th>C16:1</th>
<th>C18:0</th>
<th>C18:1c11</th>
<th>C18:2</th>
<th>C20:0</th>
<th>C20:1</th>
<th>C18:3n3</th>
<th>C22:0</th>
<th>C22:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.3</td>
<td>13.7</td>
<td>1.3</td>
<td>2.7</td>
<td>71.0</td>
<td>9.6</td>
<td>0.5</td>
<td>0.3</td>
<td>0.6</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>B</td>
<td>0.4</td>
<td>8.3</td>
<td>0.3</td>
<td>3.0</td>
<td>46.4</td>
<td>39.7</td>
<td>0.9</td>
<td>0.2</td>
<td>0.4</td>
<td>0.4</td>
<td>0.0</td>
</tr>
<tr>
<td>C</td>
<td>0.3</td>
<td>7.7</td>
<td>0.3</td>
<td>4.2</td>
<td>39.9</td>
<td>12.3</td>
<td>0.4</td>
<td>0.3</td>
<td>34.3</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>D</td>
<td>0.3</td>
<td>14.5</td>
<td>1.5</td>
<td>2.7</td>
<td>68.4</td>
<td>11.0</td>
<td>0.5</td>
<td>0.3</td>
<td>0.6</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>E</td>
<td>0.3</td>
<td>9.0</td>
<td>0.6</td>
<td>3.1</td>
<td>43.4</td>
<td>41.5</td>
<td>0.9</td>
<td>0.2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>F</td>
<td>0.3</td>
<td>8.6</td>
<td>0.6</td>
<td>4.3</td>
<td>37.3</td>
<td>13.7</td>
<td>0.4</td>
<td>0.3</td>
<td>34.2</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>
4.2 Chlorophyll level

Chlorophyll pigments are known photosensitizers and can affect the oxidation process. It was necessary to determine the chlorophyll level since this work is part of another study which also wanted to insights into the formation of epoxy fatty acids during photoxidation. By measuring the chlorophyll level of the oil models, comparison between the two works is possible.

Table 4: Chlorophyll levels (mg/kg oil) in the models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Stripped (mg/kg)</th>
<th>Non-stripped (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10.8</td>
<td>12.2</td>
</tr>
<tr>
<td>B</td>
<td>10.6</td>
<td>10.9</td>
</tr>
<tr>
<td>C</td>
<td>10.8</td>
<td>11.3</td>
</tr>
<tr>
<td>D</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td>E</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>F</td>
<td>0.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>

As shown in table 4, the stripped models with virgin olive oil have a chlorophyll concentration of approximately 11 mg/kg oil. The stripped models without the virgin olive oil have no chlorophyll pigments. This means that all chlorophyll was removed during the stripping process. The results of the non-stripped models show that refined oils have less chlorophyll pigments than virgin olive oil. The non-stripped samples without the virgin olive oil have a chlorophyll level between 1 and 2 mg/kg oil. The non-stripped models with virgin olive oil show a slightly increased chlorophyll concentration compared with the stripped models. They range between 10.9 and 12.2 mg chlorophyll pigments per kg oil.
4.3 Stability of the oils

The stability of the oil models was determined using the peroxide value. The method used was the ferric thiocyanate spectrophotometric method. The different models were left to oxidize at 70°C for 0, 1, 3, 5 or 7 days. The peroxide value given for each model is the mean value based on triplicate analyses and the uncertainty is given as a standard deviation. The results are shown in table 5 and 6 (p. 41-42). To simplify the data figure 13 and 14 are given. Please note that displayed trends are not always correct between two dots. The dots are merely connected to make it easier on the eye.

During the first 24 hours there was little to no oxidation (figure 13). After this induction period all models started accumulating peroxides (primary oxidation products). The oxidation process of model D, E and F seemed to be much faster than the oxidation process of model A, B and C. This may be caused by the presence of chlorophyll pigments and other pigments, which are present in the virgin olive oil of these models. These pigments may have an antioxidative activity which stabilizes the oils better than the ones where only refined olive oil was added. At day 3, the concentration of primary oxidation products seemed to reach its peak in the models without virgin olive oil and the peroxide value started to decrease, which means that the peroxides were reacting away to form secondary oxidation products. All models peaked on day 3, however model A and B showed a much lower maximum peroxide value. The peak amount of peroxides in all models can be higher than recorded. This would be caused by a peak between measured days.

Figure 13: Peroxide value (mEq O₂/kg oil) of stripped oil models during 0, 1, 3, 5 and 7 days of storage at 70°C.
In comparison with the stripped samples there was no significant difference between the models with virgin olive oil and the ones with refined olive oil (figure 14). This can be explained by the presence of antioxidants in all non-stripped models. The degree of saturation of oils was clearly more important in these models. The models with olive oil (A and B) were still in the induction period, or at least did not oxidize a lot yet. The models with sunflower oil and linseed oil started to oxidize, but did not reach their maximum peroxide value. The models with linseed oil (C and F) displayed the highest peroxide value. The peroxide value of the non-stripped models was far lower than the peroxide value of the stripped models (0-60 mEq O$_2$/kg oil in comparison with 0-700 mEq O$_2$/kg oil). While the amount of formed peroxides was different in each model, they all follow the normal peroxide value kinetics.

Figure 14: Peroxide value (mEq O$_2$ kg$^{-1}$ oil) of non-stripped oil models during 0, 1, 3 and 5 days of storage at 70°C.

These hypotheses seem to be the most logical, but actual trends can differ as continuous monitoring of the peroxide value during the oxidation process was not possible. The measurements of the epoxy fatty acids, the conjugated dienes and the conjugated trienes were done on the same day for each model, so comparisons and correlations are possible.
4.4 Formation of primary oxidation products

The formation of primary oxidation products can be monitored by measuring the conjugated dienes and conjugated trienes, as previously mentioned in the literature. These methods have the same goal as the peroxide value and function as a supplement. It will also be used for correlation purposes with the epoxy fatty acids. The results shown in table 5 (p. 41), table 6 (p. 42) and figure 15 to 18 are the means of each model based on triplicate analyses. The uncertainty is given by the standard deviation.

To simplify the data figures 15 to 18 are given. Please note that displayed trends are not always correct between two dots. The dots are merely connected to make it easier to visualize the trend.

During day 1, model A, B, C and D were not forming conjugated dienes, while model E and F were (figure 15). These were the only models which consisted primarily of fatty acids with two or more double bonds and which did not have virgin olive oil. Day 3 showed a peak for every model, although model A, B and C stayed roughly at the same level for almost the whole experiment. After the maximum, the concentration of conjugated dienes decreased, as secondary oxidation products were being formed. After five days, all models, with the exception of model A, started to rise again. This may be attributed to the limitation of this method. The formed secondary oxidation products may interfere with the conjugated dienes and cause an overestimation. The concentration of conjugated dienes laid between 3 and 120 µmol/g oil, not considering the results of day 7. Model E showed the highest concentration overall. This can be explained by the fact that models B and E had the highest concentration of linoleic acid (C18:2) and were most capable to form conjugated dienes. Model B did not show a high concentration of conjugated dienes, probably due to the presence of virgin olive oil.
As with the peroxide value, the presence of virgin olive oil did not seem to influence the oxidation process a lot for the non-stripped models. The fatty acid composition caused the difference in formed conjugated dienes (figure 16). Models B and E already had a higher concentration of conjugated dienes than the other models on day 0. Models A and D remained constant throughout the experiment due to their low concentration of linoleic acid. The other models started rising after day 1 and continued to do so for the whole experiment. Models C and F had roughly the same concentration of linoleic acid as models A and D, but had a high concentration of linolenic acid, which is also capable of forming conjugated dienes. The concentration of conjugated dienes ranged between 6 and 26 µmol/g oil. This was much lower than the concentration of the stripped models. This can probably be attributed to the antioxidants in the non-stripped oils, which stabilized them.

Figure 15: Conjugated dienes (µmol/g oil) of stripped oil models during 0, 1, 3, 5 and 7 days of storage at 70°C.
Figure 16: Conjugated dienes (µmol/g oil) of non-stripped oil models during 0, 1, 3 and 5 days of storage at 70°C.

Figure 17 displays the results of the conjugated trienes experiment on the stripped models. Only model F showed a large increase in conjugated trienes. This was to be expected as this model had no antioxidants and contained a lot of linolenic acid (C18:3). Model C also contained the linolenic acid but did not show a big increase in concentration of conjugated trienes. Once again this can probably be explained by the presence of antioxidants. Model E showed a slight increase as well, but did not have C18:3. This seemed to occur due to formation of secondary oxidation products which caused an overestimation of conjugated trienes. That is also why the concentration of all models increased after day 5. The concentration of the conjugated trienes ranged from 0 to 13 µmol/g oil, without including the results of day 7.
Results of the conjugated trienes experiment of the non-stripped models are given in figure 18. The concentrations were very low ranging from 0.5 to 2.5 µmol/g oil. The concentrations of conjugated trienes on day 0 were probably caused by interference, as even model A, which did not contain linolenic acid, had a higher concentration than model C and model F. The trends seemed to be logical, as models A, B, D and E did not increase in concentration due to a lack of C18:3 while models C and F did increase over time.
As with the peroxide value these four hypotheses seem to be the most logical, but actual trends can differ as continuous monitoring of the concentration of conjugated dienes and trienes during the oxidation process was not possible.

Figure 18: Conjugated trienes (µmol/g oil) of non-stripped oil models during 0, 1, 3, and 5 days of storage at 70°C.
4.5 Formation of epoxy fatty acids

The formation of epoxy fatty acids is displayed in table 5 (stripped) and table 6 (non-stripped) for each model (p. 41-42). The 12 possible epoxy fatty acids are shown, as well as the total amount of epoxy fatty acids in the samples. The variation of the amount of total EFAs formed with time is shown in figures 19 and 20. As with the conjugated dienes, the conjugated trienes and the peroxide value, the measurements were done on day 0, day 1, day 3 and day 5. The represented values are means of triplicate analyses.

The starting amount of epoxy fatty acids was higher in the non-stripped models than in the stripped models. For the non-stripped models it varied between approximately 280 and 1820 µg/g oil, while for the stripped models it varied between 8 and 135 µg/g oil. This seemed logical as the stripping process removed all epoxy fatty acids present in the oils. Models B and E of the non-stripped oils already displayed a high amount of epoxy fatty acids on day 0. This was due to the sunflower oil, which is known to have high amounts of epoxy fatty acids even before the oil is significantly oxidized.

Although the stripped oils had less epoxy fatty acids at the start of the experiment, it was clear that they were far less stable than the non-stripped oil models. While the total amount of epoxy fatty acids of the non-stripped models almost stayed constant during the experiment, the total amount of EFAs of the stripped models skyrocketed. Especially the total amount of epoxy fatty acids of the stripped models without the virgin olive oil rised very fast. Models C and F, which both contained high amounts of C18:3, were forming epoxy fatty acids the quickest and caused even a rise in the non-stripped models. C18:3 was expected to oxidize faster than C18:2 and C18:1, as it was the most unsaturated and therefore the most unstable fatty acid. At day 5 the total amount of epoxy fatty acids varied between 150 and 5000 µg/g oil for the stripped models (figure 19), and between 300 and 1950 µg/g oil for the non-stripped models (figure 20).
Figure 19: Formation of epoxy fatty acids (µg/g oil) in stripped oil models after 0, 1, 3 and 5 days of storage at 70°C.

Figure 20: Formation of epoxy fatty acids (µg/g oil) in non-stripped oil models after 0, 1, 3 and 5 days of storage at 70°C.
When observing the data displayed in tables 5, 6 and 7 (p. 41-43), it is clear that the models with primarily C18:1, that is model A and model D, were forming mostly methyl trans-9,10-epoxystearate and methyl cis-9,10-epoxystearate. There were also traces of methyl trans-12,13-epoxyoleate, methyl cis-12,13-epoxyoleate, methyl trans-9,10-epoxyoleate and methyl cis-9,10-epoxyoleate. This was to be expected as these models also contained some C18:2. The 6 isomers of epoxylinoleate were not present, due to the lack of significant amounts of C18:3 fatty acids in these models. Models B and E, which contained primarily C18:1 and C18:2 fatty acids, were showing high amounts of both the 2 epoxystearate isomers and the 4 epoxyoleate isomers. The 6 isomers of epoxylinoleate were not present, due to the same reason as above. The models with C18:3 fatty acids, model C and model F, had all sorts of epoxy fatty acids, as they also contained C18:1 and C18:2. These models also had the highest total amount of epoxy fatty acids due to having the most unsaturated bonds and therefore being the most susceptible to oxidation. Looking at the non-stripped samples, models B and E had the highest total amount of epoxy fatty acids. This was already explained by the high amounts of epoxides in sunflower oil and by the fact that the oils were not stripped. The non-stripped models showed a fairly high basic amount of methyl trans-12,13-epoxy-9,15-octadecadienoate and methyl trans-9,10-epoxy-12,15-octadecadienoate. This amount was not increasing in the models without C18:3.
Table 5: Epoxy fatty acids (µg g\(^{-1}\) of oil), PV (mEq O\(_2\) kg\(^{-1}\) of oil), conjugated dienes and conjugated trienes (µmol g\(^{-1}\) oil) of the stripped oil models.

<table>
<thead>
<tr>
<th>Oil model</th>
<th>Time (days)</th>
<th>9,10ES tr</th>
<th>9,10ES cis</th>
<th>12,13EO tr</th>
<th>12,13EO cis</th>
<th>9,10EO tr</th>
<th>9,10EO cis</th>
<th>12,13 EOL tr</th>
<th>12,13 cis &amp; 9,10 EOL tr</th>
<th>15,16 EOL cis</th>
<th>9,10 EOL cis</th>
<th>Total epoxy</th>
<th>Peroxide value(^a)</th>
<th>Conjugated dienes(^a)</th>
<th>Conjugated trienes(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>3.7</td>
<td>81.8</td>
<td>0.0</td>
<td>0.0</td>
<td>5.4</td>
<td>8.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>99.3</td>
<td>6.6</td>
<td>3.7</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.9</td>
<td>75.8</td>
<td>0.0</td>
<td>0.0</td>
<td>5.6</td>
<td>8.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>94.8</td>
<td>8.0</td>
<td>4.4</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11.6</td>
<td>104.0</td>
<td>1.8</td>
<td>4.1</td>
<td>8.5</td>
<td>13.5</td>
<td>0.0</td>
<td>0.0</td>
<td>7.5</td>
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<td>51.0</td>
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</tr>
<tr>
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<td>5</td>
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<td>104.3</td>
<td>2.0</td>
<td>0.0</td>
<td>8.1</td>
<td>11.4</td>
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Abbreviations: ES, epoxystearate; EO, epoxyoleate; EOL, epoxylinoleate

\(^a\) Values are means of triplicate analyses.
Table 6: Epoxy fatty acids (µg g⁻¹ of oil), PV (mEq O₂ kg⁻¹ of oil), conjugated dienes and conjugated trienes (µmol g⁻¹ oil) of the non-stripped oil models.

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<td>42.2</td>
<td>995.1</td>
<td>54.6</td>
<td>23.1</td>
</tr>
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</table>

Abbreviations: ES, epoxystearate; EO, epoxyoleate; EOL, epoxylinoleate

a Values are means of triplicate analyses.
Table 7: Total amounts of epoxy fatty acids (µg g⁻¹ of oil) split by isomer group.

<table>
<thead>
<tr>
<th>Oil model</th>
<th>Time (days)</th>
<th>Stripped models</th>
<th>Non-stripped models</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Total epoxystearate</td>
<td>Total epoxyoleate</td>
<td>Total epoxylinoleate</td>
</tr>
<tr>
<td>A</td>
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<td>13.8</td>
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<td>80.7</td>
<td>14.1</td>
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<tr>
<td></td>
<td>3</td>
<td>115.6</td>
<td>27.9</td>
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<td>49.2</td>
</tr>
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<td></td>
<td>1</td>
<td>79</td>
<td>49.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>162.8</td>
<td>123.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>149.3</td>
<td>136.9</td>
</tr>
<tr>
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<td>17.1</td>
</tr>
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<td>1</td>
<td>78.5</td>
<td>20.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>197.1</td>
<td>79.5</td>
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<td></td>
<td>5</td>
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<td>161.7</td>
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<td>137.9</td>
<td>186.7</td>
</tr>
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<td>3</td>
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<td>1096.9</td>
<td>1666.3</td>
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<tr>
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<td>1</td>
<td>122.2</td>
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<td></td>
<td>5</td>
<td>1432.4</td>
<td>880.8</td>
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4.6 Correlations

One of the aims of this study was to try to correlate the peroxide value, the conjugated dienes and the conjugated trienes with the amount of epoxy fatty acids formed while oxidizing the models at 70°C. Although it is hard to correlate primary oxidation products with a single secondary oxidation product since many products form, the results obtained are shown in table 8 (p. 46). As the peroxide value and the conjugated dienes and trienes rised to a peak and went down afterwards, it was hard to correlate them with the amount of formed epoxy fatty acids as this amount only went up.

There was a significant positive correlation of approximately 0.9 to 1 between the peroxide value and the conjugated dienes. This was expected as these two methods measured both the primary oxidation products. The models with primarly C18:1 fatty acids, that is models A and D, would not be expected to show a significant correlation, as they would not be able to form conjugated dienes. However, they still had some C18:2 that can oxidize when not quenched by pigments from the virgin olive oil. This was probably the reason for the correlation in model D. The non-stripped model A showed a correlation as well. This was probably because the peroxide value and the conjugated dienes both stayed constant and the oxidation rate was low during the experiment.

When considering the non-stripped models, there was only a significant correlation in models C and F between the peroxide value and the conjugated trienes. Both had a positive correlation around 0,95. It is quite normal these models in particular showed a relationship because they were the only models containing C18:3, hence they were the only models that could form significant amounts of conjugated trienes. Of the stripped models, model C, model D and model E showed a positive correlation of 0,95 to 1. Model F would be expected to have a significant correlation instead of model D and E, but models D and E probably only had a significant correlation due to the limitations in the method of analysis of conjugated trienes. The formation of secondary oxidation products probably interfered with the readings and caused the conjugated trienes to be overestimated. Table 5 shows clearly the amount of epoxy fatty acids increasing at the moment the supposedly conjugated trienes increase. This was probably also the reason why model F did not have a significant correlation. The oxidation process was going too fast.
The peroxide value was positively correlated with the total amount of epoxy fatty acids for models C, E and F of the non-stripped oils. These were the models where oxidation was faster. Perhaps the other models would show a positive correlation as well, if the experiment was continued for a longer time, now they did not form secondary oxidation products yet. For the stripped oils the correlations were not easy to investigate, because the oxidation process happened so fast the peroxide value was already decreasing at day 3, while the total amount of EFAs increased rapidly. This was why the correlations were probably an underestimation. Models A and C were still showing a positive correlation of approximately 0.95 because their peroxide value was not decreasing yet.

There was no significant correlation between the conjugated dienes or trienes and the total amount of epoxy fatty acids for the models with primarily C18:1. This was to be expected as C18:1 cannot form conjugated dienes and trienes, but can form epoxystearate. There was also no significant correlation between the conjugated dienes and the total amount of EFAs for the other stripped models, this was probably caused by the decrease in conjugated dienes after the peak, thus by a faster oxidation process. Models C, E and F of the non-stripped models showed a positive correlation between the conjugated dienes and the total amount of EFAs. The conjugated trienes and the total amount EFAs were positively correlated for models B, C and F of the non-stripped models and for models C, E and F of the stripped models. Model B of the non-stripped models showed a correlation due to staying relatively constant in both conjugated trienes and total EFAs, while model E and maybe also model C and F of the stripped models showed a correlation due to previously mentioned limitation of the conjugated trienes method and the following overestimation.
Table 8: Correlations between peroxide value (PV), conjugated dienes (CD), conjugated trienes (CT) and total epoxy fatty acids (EFAs) using the Pearson correlation coefficient.

<table>
<thead>
<tr>
<th>Model</th>
<th>PV</th>
<th>CD</th>
<th>CT</th>
<th>EFA</th>
<th>Model</th>
<th>PV</th>
<th>CD</th>
<th>CT</th>
<th>EFA</th>
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<td></td>
<td>Dns (n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PV</td>
<td>1</td>
<td>0.972*</td>
<td>-0.336</td>
<td>0.337</td>
<td>CD</td>
<td>0.921*</td>
<td>1</td>
<td>0.567</td>
<td>-0.232</td>
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<tr>
<td>CD</td>
<td>0.972*</td>
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<td>-0.303</td>
<td>0.324</td>
<td>CT</td>
<td>-0.336</td>
<td>-0.303</td>
<td>1</td>
<td>0.768</td>
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<tr>
<td>CT</td>
<td>-0.336</td>
<td>-0.303</td>
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<td>0.768</td>
<td>EFA</td>
<td>0.337</td>
<td>0.324</td>
<td>0.768</td>
<td>1</td>
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<tr>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>As (n=4)</td>
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<td></td>
<td></td>
<td>Ds (n=4)</td>
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<td></td>
<td>Ens (n=4)</td>
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<tr>
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<td>CD</td>
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<tr>
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<tr>
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<td>Es (n=4)</td>
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<td>0.861</td>
<td>0.512</td>
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<tr>
<td>EFA</td>
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<td>0.861</td>
<td>0.512</td>
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<tr>
<td>Cns (n=4)</td>
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<td>Fs (n=4)</td>
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<td></td>
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<tr>
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<td>1.000**</td>
<td>0.968*</td>
<td>0.955*</td>
<td>CD</td>
<td>0.995**</td>
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<tr>
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<td>0.964*</td>
<td>CT</td>
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<td>0.972*</td>
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<td>Fs (n=4)</td>
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<tr>
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<td>0.943*</td>
<td></td>
<td></td>
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</table>

* Correlation is significant at the 0.05 level (1-tailed).  ns = non-stripped
** Correlation is significant at the 0.01 level (1-tailed).  s = stripped
5 Conclusion

The purpose of this work was to monitor the epoxy fatty acids during autoxidation at an elevated temperature. To frame these results, the peroxide value and the conjugated dienes and trienes were determined as well.

The stripped oil models showed a very fast oxidation process. This can be seen in every experiment. The peroxide value showed a big increase while the peroxides were accumulating and showed a decline when the secondary oxidation products were being formed. The conjugated dienes showed a peak as well and after the decline the secondary oxidation products were forming so rapidly that there was an increase again due to the limitation of the method. Finally in only five days, up to 5 mg/g oil of epoxy fatty acids were being formed, which is very high. That was why the non-stripped oil experiment was put in place as a control group.

The non-stripped oil models showed the same oxidation kinetics but were oxidizing much slower. After five days, the primary oxidation products were still forming and not many epoxy fatty acids were being formed.

The difference between the oil models was clear as well. The models containing highly unsaturated fatty acids were oxidizing at a higher rate than the oil models without. The models containing a significant amount of C18:3 oxidized the quickest and formed the most epoxy fatty acids, as they could form all 12 epoxy fatty acid isomers. Finally, the virgin olive oil added to some models seemed to slow the oxidation process significantly.

In a next study, the non-stripped oil models should be left oxidizing for a longer period of time to complete the oxidation process. This way, a bigger resemblance should be found between the non-stripped oil models and the stripped oil models.
References


6 Appendices
Appendix 1: Flowchart thesis

September '13 - December '13
Literature and preparation of experiment

- Meeting promotor, tutor and colleagues
- Getting used to the lab and the topic of the thesis
- Reading literature and writing a literature review
- Practicing and testing methods and equipment
- Setting up and testing the design of the experiment
- Stripping of the oils

February '14 – April ‘14
Experiment

- Preparing the mixtures and starting the accelerated oxidation
- Measuring the peroxide value
- Measuring the conjugated dienes and trienes
- Measuring and determining the epoxides
- Measuring the chlorophyll pigments in the mixtures
- Determining the fatty acid composition in the mixtures
- Discarding the TBARS method
- Presenting progress and results to promoter and colleagues

April ‘14 – June ‘14
Processing data and writing thesis

- Looking for trends and correlations
- Writing thesis
-Preparing Defense
Appendix 2: IDF method for peroxide determination

Method based on:


And

http://www.fao.org/DOCREP/003/X6537E/X6537E05.htm

1 International Standard FIL-IDF 74: 1974 – Anhydrous milk fat – Determination of the peroxide value

1.1 Definition

The peroxide value means the number of milligram-equivalents of oxygen per kilogram of fat, determined by the procedure described.

1.2 Principle of the method

Solution of a weighed amount of the sample in a mixture of chloroform and methanol and addition of some iron (II) chloride and ammonium thiocyanate. After a fixed reaction time, photometric determination of the amount of red iron (III) complex.

1.3 Reagents

All reagents shall be of analytical reagent quality. Water used shall be distilled water or water of at least equivalent purity.

a. Mixture of 70 volume parts of chloroform (trichloromethane) and 30 volume parts of anhydrous methanol.

b. Iron (II) chloride solution

c. Ammonium thiocyanate solution

Solution b. shall be prepared in undirected, dimmed light. Dissolve approximately 0,4 g barium chloride (BaCl₂.2H₂O) in about 50 ml of water.

Dissolve approximately 0,5 g iron (II) sulfate (FeSO₄.7H₂O) in about 50 ml of water.

Slowly pour the barium chloride solution, with constant stirring, into the iron (II) sulfate solution and add about 2 ml of approximately 10 N hydrochloric acid.

Allow the precipitate of barium sulfate to settle or centrifuge the mixture until the upper liquid layer is clear. Decant the clear solution into a brown bottle. Do not store the solution for more than 1 week.
Note: The iron (II) chloride solution can also be prepared by dissolving approximately 0.35 g iron (II) chloride (FeCl₂·4H₂O) in about 100 ml of water and adding 2 ml of approximately 10 N hydrochloric acid.

For solution c., dissolve approximately 30 g ammonium thiocyanate (NH₄SCN) in water and dilute to 100 ml. If the solution is not colorless, remove the color by extracting the solution several times with small amounts (e.g. 5 ml portions) of iso-amyl alcohol.

1.4 Procedure

Precautions

In order to eliminate lipid oxidation, the following precautions shall be followed:

Exposure of the sample of fat to light shall be avoided.

Take care that the procedure described below is completed within 10 minutes.

Carry out the test in dimmed light, subdued as much as is practicable.

Determination

*Standard solution of iron (III) chloride (10 µg/ml)*

Dissolve 0.5 g iron powder in about 50 ml of 10 N hydrochloric acid and add 1 to 2 ml of about 30% (m/m) hydrogen peroxide solution.

Remove the excess of hydrogen peroxide by boiling for 5 minutes. Cool to room temperature and dilute with water to 500 ml. Transfer, by means of a pipette 1 ml of this solution to a measuring flask and dilute with the mixture of chloroform and methanol (7:3) to 100 ml. Standard Fe (III) samples containing 1 to 40 µg iron (III) are prepared from above stock solution for analysis for calibration. For example you can keep as follows:

<table>
<thead>
<tr>
<th>Calibration samples composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe (III) (µg)</td>
</tr>
<tr>
<td>Stock solution (ml)</td>
</tr>
<tr>
<td>Solvent (ml)</td>
</tr>
</tbody>
</table>

*Reference curve*

Add from a graduated pipette 50 µl of ammonium thiocyanate solution to the calibration samples and vortex for some seconds. Set spectrometer at 500 nm and set blank with solvent. Wait for exactly 5 minutes and measure the absorbance.

Plot the extinctions of the series against the added amounts of iron (III), expressed as µg Fe (III). Construct the best fitting straight line through the points and calculate the slope of the graph. The line should be perfectly straight.
**Analysis of the sample**

Set spectrometer at 500 nm and set blank with solvent.

**a) Reagent blank absorbance (E_{reagent blank})**

Take 10 ml of solvent, add 50 µl ammonium thiocyanate solution and mix for some seconds, then add 50 µl Fe (II) solution and vortex for 2 to 4 seconds and take absorbance after exactly 5 minutes.

**b) Sample Absorbance (E_{sample})**

Take sample weight from 0.01 to 0.3 g according to degree of oxidation. Add 10 ml of the solvent very accurately and vortex for some seconds. Add 50 µl ammonium thiocyanate solution and mix for some seconds, then add 50 µl Fe (II) solution and vortex for 2 to 4 seconds and take absorbance after exactly 5 minutes.

**1.5 Expression of results**

**Method of calculation**

The peroxide value of the models is then calculated as milligram equivalents oxygen per kilogram oil (mEq O₂/kg) using this formula:

\[
P_V = \frac{Corrected \ absorbance \times m}{55,84 \times W \times 2}
\]

Where:

Corrected absorbance = E_{sample} – E_{reagent blank}

m = slope of calibration curve

W = mass in gram of the samples taken

55,84 = atomic weight of Iron

**Repeatability**

The difference between the results of two determinations, carried out simultaneously or in rapid succession by the same analyst, using the same apparatus, should not exceed 0.05 units of peroxide value.
Appendix 3: Chromatogram of one of the samples