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SUMMARY

Vegetable oils consist of triglycerides between 95-99%. They also contain soluble vitamins (A, D, E, and K), phytosterols, natural pigments and phospholipids. The increasing content of unsaturated fatty acids the possibility of oxidation increases as well.

The heat influences the oxidation of the oils which leads to the formation primary oxidation products and secondary oxidation products. Hydroperoxides are the primary oxidation products of lipid oxidation. These products are generally unstable and decompose into a variety of secondary oxidation products, including the carbonyls compounds which were compounds focusing in this dissertation.

Carbonyl compounds are more stable than hydroperoxides and the measurement is a good index of oxidative changes in lipids. The derivatization of carbonyls with 2,4-dinitrophenylhydrazine and HPLC measurement of the aldehydes is correlated with rancidity. The oxidation experiments were done in a Ranzimat at 120 °C with an air flow of 20 l/h to have reproducible and comparable conditions.

The formation of carbonyls was measured in coffee oil, triolein, palm oil, rapeseed oil, and sunflower oil. The aldehydes formed during roasting of coffee were hydroxypentanal and heptatrienal. In triolein – a model substance –hexanal, heptanal, octanal, nonanal, and decanal were identified. In rapeseed oil and sunflower oil hexanal, octanal, nonanal, decenal, and 2-undecanal were identified. In rapeseed oil the maximum was observed after 7.5 h. Sunflower oil oxidised 1 hour earlier.

In addition, two antioxidants commonly present in edible oils were investigated. These showed a slight reduction of the aldehyde formation in the experiments.

ZUSAMMENFASSUNG

Pflanzenöle bestehen aus Triglyceriden zwischen 95-99%. Sie enthalten auch Vitamine (A, D, E und K), Phytosterole, Pigmente sowie Phospholipide. Der zunehmende Grad an ungesättigter Fettsäure erhöht die Oxidationsmöglichkeit.

Erhöhte Temperaturen können die Oxidation in den Ölen beeinflussen, die zur Bildung von primärem und sekundären Oxidationsprodukten führen. Hydroperoxide sind im Allgemeinen instabil und zersetzen sich zu einer Vielzahl von sekundären Oxidationsprodukten, einschließlich den Carbonylverbindungen, die in dieser Arbeit untersucht wurden.

Carbonylverbindungen sind stabiler als Hydroperoxide und ihre Messung ist ein guter Index der oxidativen Veränderung des Öls. Die Carbonyle werden mit 2,4-Dinitrophenylhydrazin derivatisiert und mittels HPLC gemessen.

Die Bildung von Carbonylen als sekundäre Oxidation wurde in Kaffeeöl, Triolein und einigen Speiseölen wie Palmöl, Rapsöl und Sonnenblumenöl untersucht. Im extrahiertem Öl von geröstetem Kaffee fanden sich Hydroxypentanal und Heptatrienal. In Triolein wurden Hexanal, Heptanal, Octanal, Nonanal und Decanal gefunden. In Raps- und Sonnenblumenöl wurden Hexanal, Oktanal, Nonanal, Decenal und 2-Unekanal gefunden. Rapsöl zeigt die größte Menge an Carbonylen nach 7,5 h, während in Sonnenblumenöl die maximale maximale Konzentration an Carbonylen nach 6,5 Stunden zu finden war.

Darüber hinaus wurden zwei Antioxidantien (α-Tocopherol und β-Carotin) zugesetzt, um die Auswirkung auf die Bildung von Carbonylen zu untersuchen. Beide zeigen einen geringen reduzierenden Effekt.

SECTION I

ANALYSIS FATTY ACIDS AND SECONDARY OXIDATION PRODUCTS IN ROASTING COFFEE WITH HPLC-LC/MS

I. INTRODUCTION

The coffee production coffee is consumed by more than 800 million people who enjoy its taste, flavour, and health benefits. The taste of coffee related to the quality of coffee and the lipids contain in coffee can contribute the quality of coffee. Lipid presents in coffee approximately 10-20% of the total chemical compounds that are found in *Coffea arabica* L. and *Coffea canephora*, which are two varieties of coffee approximately 70% of the world production (Illy & Viani, 2005).

The main fatty acids present in all the samples are linoleic acid, with an average percentage of 44.1%, and palmitic acid with an average percentage of 34%. Minor acids are myristic acid, palmitoleic acid, eicosenoic acid, and behenic acid whose contents are lower than 1.0% of each and the percentage of linolenic acid and arachidic acid are both between 1.5% and 3% (Oliveira, et al., 2006)

Green coffee has no intensive taste or aroma on its own. The desirable aroma develops during the roasting process. The obvious changes which occur during the roasting process are the change in color, flavor which increase the consumer acceptance of the product. In addition, roasting induces severe transformation on coffee's chemical composition. There are three main reactions taking place simultaneously which are the Maillard reaction, lipid oxidation, and sugar decomposition.

The Maillard reaction describes as non-enzymatic reaction between amines and carbonyls (Maillard, 1912). It is known because of heated, dried and stored foods. Thus, the reaction has not

only an impact on the color, flavour, and nutritive value but also on the formation of stabilizing and mutagenic compounds.

In green coffee the carbohydrates and amino acids are the main components that contribute to the formation of the typical aroma during roasting. The standard procedures for the roasting coffee range from 240 °C for 6 min to 270 °C for 3 min. Normally, industrial coffee roasting is done at the higher temperatures. This condition not only take place to forming the aroma and colour but also undesirable substances like acrylamide or furans (Bagdonaite et al, 2008).

The most important lipids in coffee are the diterpenes kahweol and cafestol related to one another by their fragrance and importance to consumer health. The fatty acid composition of green coffee depends of several factors, such as plant species/variety and climatic conditions during growth (Alves, et. al, 2003; Villareal, 2009).

The roasting experiments described here were carried out in a laboratory roaster (Probat type 1-Z) at 140 °C for 11 minutes and compared to green coffee. Thermal decomposition of lipids includes the formation of hydroperoxides and the formation of carbonyls (Wsowicz, et al, 2004 ; Frankel, 2005). In this study, the main fatty acids of arabica coffee (which are linoleic acid (C18:2), palmitic acid (C16:0), oleic acid (C18:2), and stearic acid (C18:0) were analysed by trans-esterification of the triacylglycerols with methanol. The BF_3-methanol method was used to derivatise the fatty acids. The derivatized fatty acids have been determined by gradient elution reversed phase high performance liquid chromatography (RP-HPLC) with evaporate light scattering detection (ELSD) and HPLC-UV.

II. LITERATURE REVIEW

2.1 Availability of coffee in world

Coffee is one of the top commodities worldwide. Around 70 countries produce coffee, with the overwhelming majority of the supply coming from the developing countries which were Brazil (2,594,100 tons), Vietnam (1,650,000 tons), Colombia (810,000 ton), Indonesia (739,020,000 ton), Ethiopia (483,287,000 ton) in 2017 (Walton, 2015 ; Szenthe, 2017).

2.2 Coffee varieties

There are two species of coffee which are *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta). Arabica coffee is of higher quality than robusta coffee. Arabica coffee has a high quality of aroma so it used for production of roasted coffee while robusta coffee is often used for the production of instant coffee (Budryn, et al., 2012).

Sucrose dominates the carbohydrates with significantly higher concentrations in arabica beans (mean = 73 mg/g) compared to robusta beans (mean = 45 mg/g). As a result the aroma of arabica is more intense than robusta (Murkovic & Derler, 2006). Robusta coffee tends to contain more caffeine contents than arabica coffee. In addition, arabica coffee has a lower content of chlorogenic acids (CGA), a higher content of lipids, resulting in a highly aromatic fragrance and a rounded flavour (Carrera et al., 1988).

2.3. Fatty acids in coffee

The main fatty acids present in all the samples are linoleic acid with an average percentage of 44.1% and palmitic acid, with an average percentage of 33.3%. Minor acids are myristic, palmitoleic, eicosenoic and behenic whose contents are lower than 1.0%, whereas the percentage of linoleic and arachidic acids are between 0.6 and 3.5%. Robusta coffee has a higher contents of

oleic acid around 12.3% than Arabica with 8.3%. However, robusta coffee has a lower linolenic acid with 0.9% while arabica has 1.5% (Martin, et al., 2001).

2.4 Roasting in coffee beans

Roasting is a critical process in coffee production, during roasting the flavour develops. To produce high-quality coffee beans, the roasting degree is probably the single most important factor. The roasting degree can be measured by judging the beans' color by eye or a colorimeter, or by weight loss of water after roasting (Tab. 1)

Table 1. Roasting degree of coffee beans

Roasting Degree	Roasting Name	I. Value	Weight Loss (% of Green Coffee Bean)
Light roasting degree	Light roast	30.2	10.0-14.0
	Cinnamon roast	27.3	
	Medium roast	24.2	
Medium roasting degree	High roast	21.5	14.0-17.0
	City roast	18.5	
	Fully city roast	16.8	17.00-21.00
Dark roasting degree	French roast	15.5	
	Italian roast	14.2	>21.0
Source: Wei &Tanokura, 2015			

The standardized procedures for coffee roasting range 240 °C for 6 min to 270 °C for 3 min. Industrial coffee roasting is done at higher temperatures (Zyzak et al., 2003). Not only the aroma and colour are formed but also some nondesirable compounds such as polycyclic aromatic hydrocarbons (PAHs). PAHs can come from the contaminants of green coffee beans itself and from the roasting process. PAHs which are formed during roasting occur in the the μg kg^{-1} range (Houessou, et al., 2007, 2008).

The final result of roasted coffee varies according to the raw material, roasting degree, and other roasting variables such as roaster type and time, temperature, and air-flow in the roasting device. The relevant reaction during roasting are pyrolysis, caramelization, and the Maillard reaction (Chu & Farah, 2012). During coffee roasting, many substances are formed due to numerous chemical reactions occurring at high temperatures that can contribute to the taste and aroma of coffee having variable volatility (Albertini, et al., 1985)

III. MATERIALS AND METHODS

3.1. Reagents and solvents

Arabica coffee which was present from Ily Café (Trieste, Italy) . The solvent acetonitrile (ACN) (Chem Lab, NV, Belgium) was of gradient grade purity. Boron trifluoride in methanol from Fluka (Buchs, Switzerland). Sodium hydroxide was purchased from Sigma Aldrich. 2,4-Dinitrophenylhydrazine (2,4-DNPH) was purchased from Sigma-Aldrich (St. Louis, USA), hydrochloric acid (HCl 37%) was purchased from Merck (Darmstadt, Germany), all other solvents (e.g. methanol acetone) used were of HPLC grade and were purchased from Merck (Darmstadt, Germany), acetic acid was purchased from Roth (Karlsruhe, Germany), β-carotene and α-tocopherol were from Sigma Aldrich (St. Louis, USA).

3.2. Analysis of fatty acids in coffee oil

3.2.1. Transesterification with methanol.

Twenty mg of extracted coffee oil were hydrolyzed in a Pyrex glass tube with 6 ml of 0.5 M NaOH in methanol at 80 °C for 1 h. After cooling to room temperature, the FFAs were methylated with 6 ml of BF_3 in methanol at 80 °C for 15 min. Water was added to the solution, and then FAMEs were extracted with 10 ml of heptane.

3.2.2. Chromatography of aldehydes and their identification.

Study of the fatty acids which are found in arabica coffee was carried out by the ELSD and UV-HPLC (Agilent series 1100 (Waldbronn, Germany) equipped with a quaternary pump, vacuum degasser, autosampler, and variable wavelength detector (VWD). The column was an AccQ-Tag Column, 60 Å, 4 µm, 3.9 mm × 150 mm). The solvent is used 95% acetonitrile in water.

3.3 Analysis of aldehydes in coffee oil oxidation

3.3.1. Oxidation of coffee oil

40 g of green coffee were roasted in the laboratory roaster at 140 °C for 11 min with sampling 3, 6, 9 and 11 min (fully roasted). After cooling to room temperature, the roasted coffee beans were ground and extracted with with a soxhlet apparatus for 5 h with petroleum benzene.

3.3.2. Derivatization with 2,4-dinitrophenylhrazine (DNPH)

To 1 ml of the oxidized oil samples 4 ml of acetonitrile were added and mixed with 3 ml of the reagent 2,4-DNPH (3.48 mg/ml). The reaction mixture was kept in the dark for 1 h. After completion of the reaction 2 ml ethyl acetate were added for extraction and 1 g KCl for better phase separation. This mixture was thoroughly shaken for 30 s and centrifuged for phase separation. The organic layer was analysed without further treatment by HPLC.

3.3.3. Liquid chromatography-mass spectrometry condition for aldehydes identification.

The analyses of the DNPH derivatives of the carbonyls formed during oxidation were done by HPLC (Agilent 1100, Waldbronn, Germany) using a reversed phase column (Kinetex, EVO C18, 150 × 3 mm, Phenomenex, Aschaffenburg, Switzerland). For elution a gradient was used starting with methanol (45%), water (30%), and acetonitrile (25%) changing to methanol (6%), water (4%), and acetonitrile (90%) linearly within 15 min. The absorption of the eluent was measured at 400 nm for the presence of the DNPH derivatives.

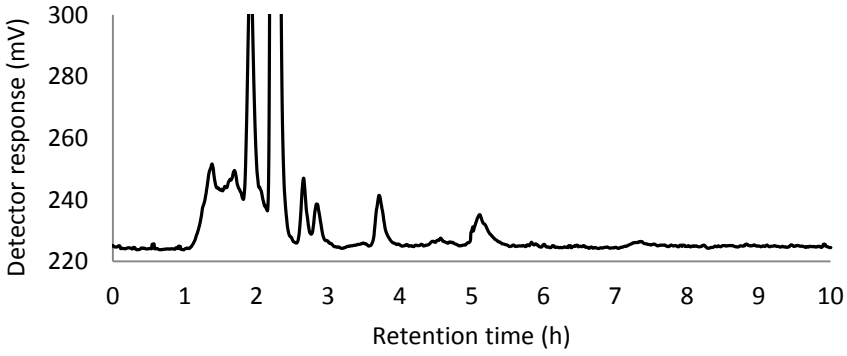
For mass selective detection a QTRAP 2000 (AB Sciex, Framingham, MA, USA) was used. Ionization was done using the APCI mode with a gas drying temperature of 250 °C, capillary voltage of 4000 V, and a fragmentor potential of 150 V.

IV. RESULTS AND DISCUSSION

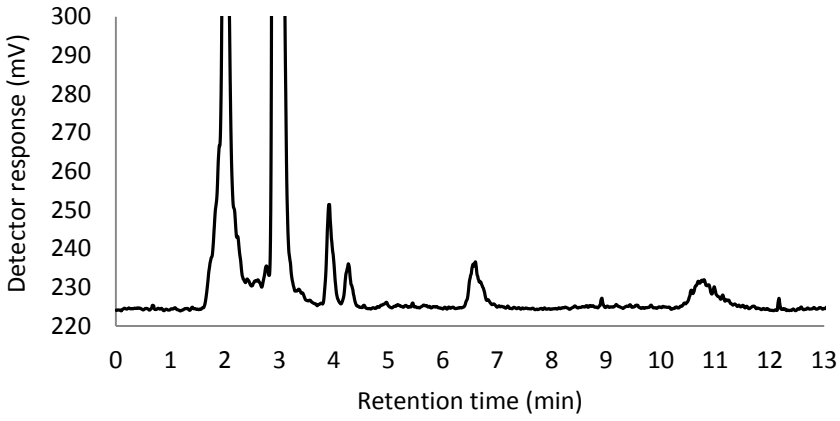
4.1. Fatty acid content in coffee oil

Several eluent combinations were used to separate the fatty acids from the coffee oil. The organic solvent itself or the combination with water tried to separate the TAG such as acetonitrile 100 %, acetonitrile and water = 95:5 and then acetonitrile:methanol:water = 47.5:47.5:5with ELSD-HPLC. The acetonitrile and water mixture (95:5) is the optimal eluent combinations for separation of the methylated fatty acids (Fig. 1 a-c) Saponification with BF_3-methanol was used in order to quantify the fatty acids in those samples.

(a)



(b)



(c)

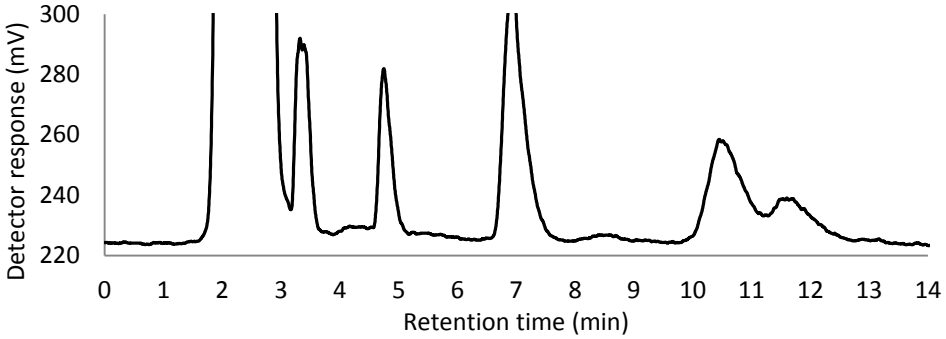


Figure 1. Fatty acid separation with different solvent combinations, (a) acetonitrile 100 %; (b) acetonitrile:water (95:5); (c) methanol:acetonitrile:water (475:475:50)

Furthermore, gradient elution RP-HPLC was used to determine the fatty acids in roasted arabica coffee. Saponification followed by methylation is a classical method for preparation of FAMEs from glycerolipids and sterol esters (Ses). A gradient of acetonitrile and water with 0.1 M formic acid is the method to separate of $FAME_s$ from the roasted coffee oil. Formic acid in the mobile phase is needed to improve the chromatographic peak shape and to provide a source of protons in reverse phase liquid chromatography–mass spectrometry (LC/MS) (Fig. 2).

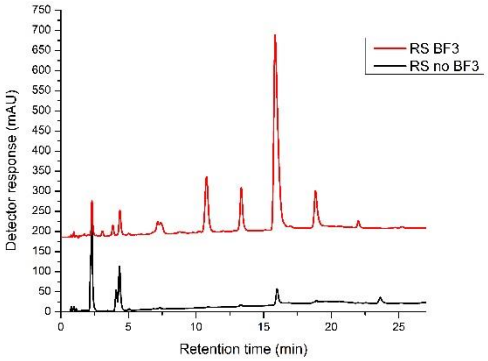
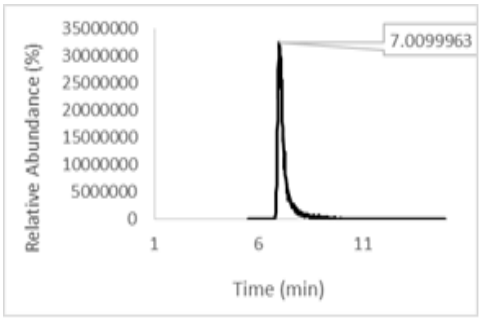
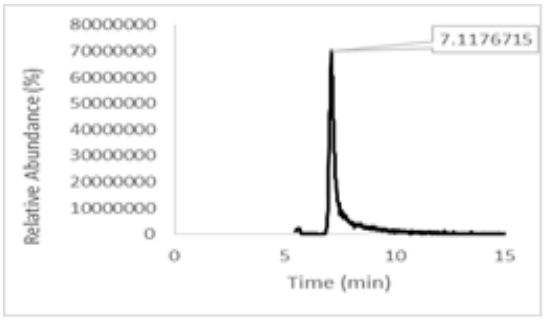


Figure 2. Fatty acids in coffee oil profile with/without BF_3

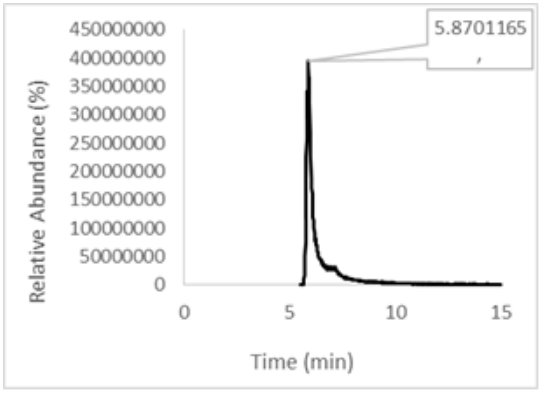
The main fatty acids content in coffee lipid extracts of arabica coffee have been determined with LC/MS. Four fatty acids were are considered: Palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and stearic acid (C18:0) (Fig. 3 a-d).



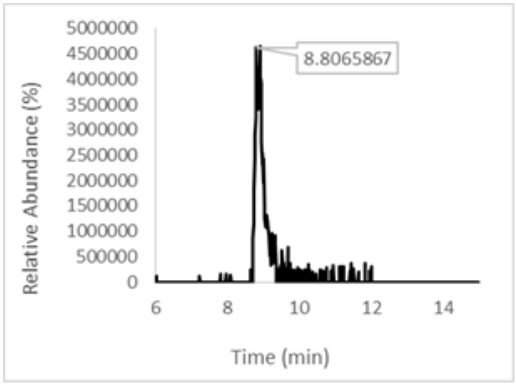
(a) palmitic acid



(b) oleic acid



(c) linoleic acid



(d) stearic acid

Figure 3. Identification fatty acids in coffee oil with LC/MS, (a) palmitic acid, (b) oleic acid, (c) linoleic acid, (d) stearic acid

Fatty acids are combined in more complex molecules such as acylglycerols, cholesterol esters, waxes and glycosphingolipids, they can be obtained free by *saponification* (inorganic or organic basic solution) or *acidic hydrolysis* and then *derivatized*. FAME may be also obtained directly by transesterification (alcoholysis or methanolysis) of the fatty acid containing lipids.

Alternative analysis of fatty acids which used the reversed-phase HPLC has been done. It is convenient for the analysis of non-volatile acylglycerols, which need to be derivatised before the analysis. This method is more common to determine the fatty acids i.e C_{14} to C_{22} of fatty acids. The extraction, hydrolysis and methylation of coffee oil in continuing steps required to find clearly separation of saponifiable and unsaponifiable material. Also, it is recommended for very small samples to avoid any loss of fatty acids during analysis.

4.2 Carbonyls formation during the coffee roasting

Arabica coffee was roasted in a Probat roaster 1 Z at ca. 140 °C for 11 minutes to obtain a roasted coffee comparable to standard quality (Fig. 4)

Non-volatile lipid secondary oxidation products of roasting coffee oils were analysed using MS coupled with a reversed phase-HPLC. Most of the LC methods available for other food matrices are based on the detection of stable carbonyl derivatives. Therefore, the carbonyl compound in triacylglycerol are volatile, it is preferable derivatise them into a non-volatile stable form. One of the most popular methods for the determination of individual secondary oxidation product in edible oils is the reaction of 2,4- dinitrophenyl hydrazine (Decker, 2010).

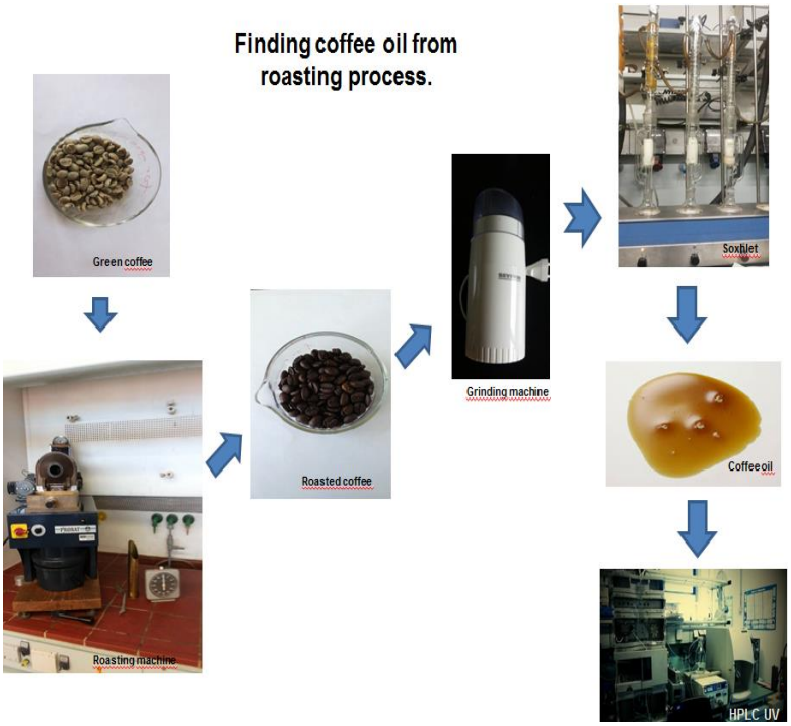


Figure 4. Roasted coffee process

Hydrazine, such as 2,4- dinitrophenyl hydrazine (DNPH), selectively react with aldehydes and ketones to form stable hydrazones. The aromatic hydrazine reacts with carbonyls under acidic condition, forming insoluble hydrazone derivatives. Total carbonyl content can be determined in oxidized lipids by the reaction with 2,4-dinitrophenylhydrazine, and the coloured hydrazone (2,4-DNPH) due to its high reactivity, selectivity, and stability. Some concentration of secondary oxidation products detected can be seen in Tab. 2.

Table 2. Secondary oxidation products in roasting coffee

Oxidation products (μmol/ml)	Roasting time (min)				
	0	3	6	9	11 (fully roasted)
A	1.58	1.31	7.88	6.15	57.45
B	1.67	1.53	8.25	6.58	42.17
C	1.48	1.40	4.42	3.58	224.80
D	1.34	1.40	3.18	4.01	36.80

During 3 minutes of roasting, the oxidation product is low. Starting from 6 minutes the formation of carbonyls increases. The highest number and concentration of the carbonyls can be observed at more than 9 minutes of roasting. The secondary oxidation products with low molecular weight including carbonyls can be identified by an Orbitrap LC-MS which has high sensitivity and an excellent mass resolution (Fig. 5).

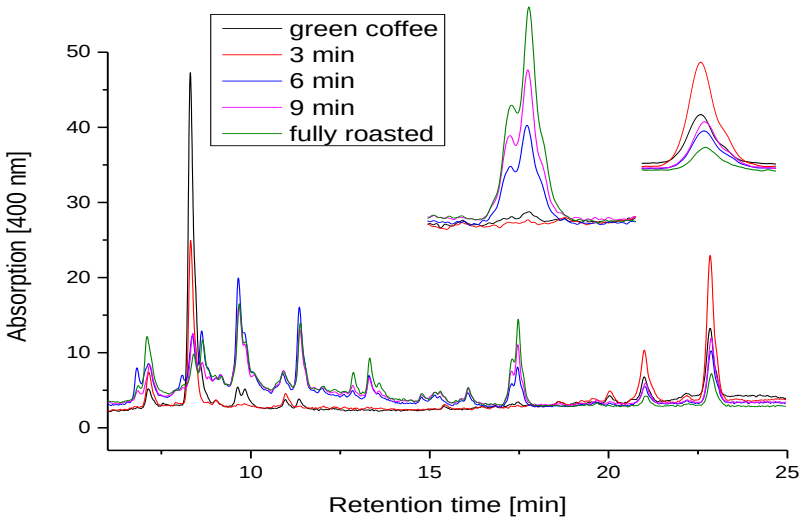


Figure 5. The profile of secondary oxidation products in coffee oil during roasting at 0, 3, 6, 9, 11 min (fully roasted).

The secondary oxidation products with low molecular weight including carbonyls can be identified by an Orbitrap LC-MS (Fig. 6)

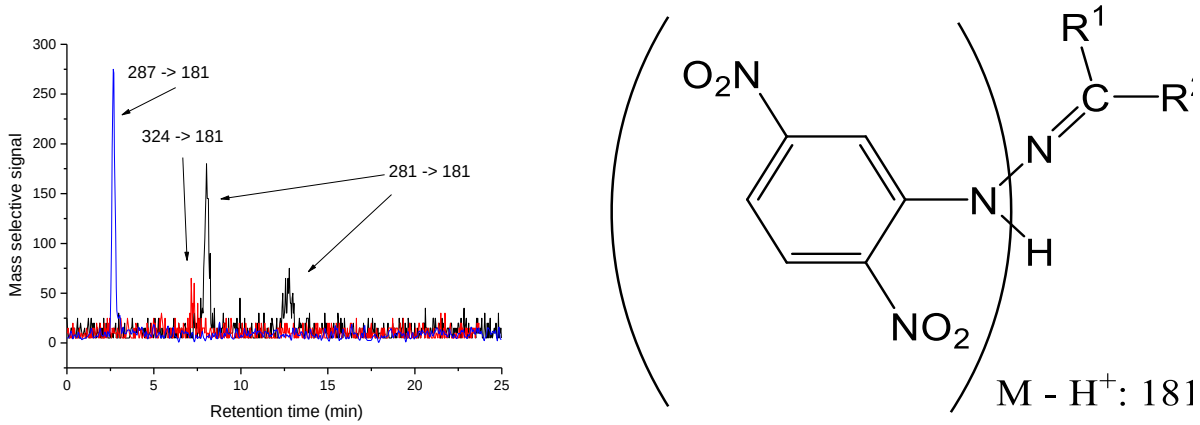


Figure 6 : LC/MS analysis of coffee oil

From the orbitrap's data of the fully roasted, we can identify two kinds of carbonyl compounds with the molecular mass of 101 and 107 (Tab.3).

Table 3. The molecular mass of the carbonyl compounds in rosted coffee

Full ms2 (-H+)	Molecule	Fragment	Proposed Structure
281	282	101	Hydroxypentanal
287	288	107	Heptatrienal

V. CONCLUSIONS

Alternative analysis of fatty acids which used the RP-HPLC has been done. The main fatty acids in arabica coffee have been analysed which are palmitic acid, stearic acid, oleic acid, and linoleic acid. The extraction, hydrolysis and methylation of coffee oil in continuing steps are required to obtain a good separation of the saponifiable and unsaponifiable material.

MS coupled with a reverse phase-HPLC was used for the determination of secondary oxidation products (carbonyls). They are usually occurring at low concentrations. The dinitrophenylhydrazone (DNPH) method produces stable hydrazone derivatives. Some carbonyl compounds that have been analysed which were hydroxypentanal and heptatrienal.

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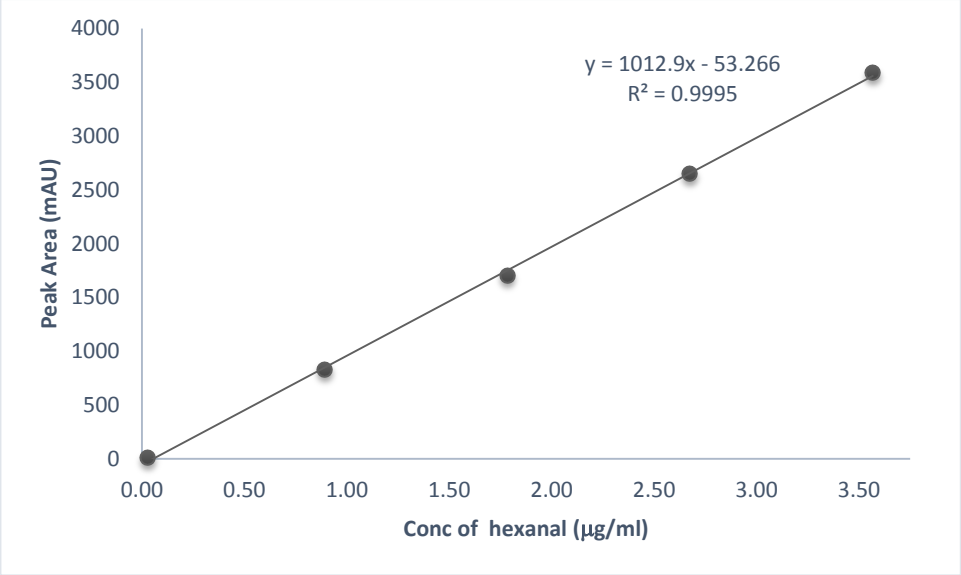
Apendix

Table 1. Standard curve of hexanal



SECTION 2

CHARACTERIZATION OF SECONDARY PRODUCT OXIDATION WITH 2,4 DNPH IN TRIOLEIN VIA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH ATMOSPHERIC PRESSURE CHEMICAL IONIZATION ORBITRAP MASS SPECTROMETRY

I. INTRODUCTION

Lipid oxidation in oil is associated with the unsaturation of the oil, especially with di-unsaturated and polyunsaturated acyl groups. The reaction leads to the formation of a series of the intermediate compounds of hydroperoxides. The decomposition of lipid hydroperoxide is always accompanied by the formation of a great variety of aldehydes (Farhoosh & Pazhouhanmehr, 2009).

Medium and short chain aldehydes becomes intensively studied because they have responsibility to the rancid off-flavour of the oil. There are two kinds of aldehydes depending on their β-cleavage reaction of lipid alkoxyl radicals. One group, where the aldehyde functions still remain at the lipid parent molecule and the second group which are the fragmentation products formed from the methyl terminus of the fatty acids.

More than one method should be used to determine the degree of oxidation by measuring different types of products, including initial products of lipid oxidation (e.g hydroperoxides) and their decomposition products (e.g. aldehydes). Decomposition products of oxidation can be measured by analysis of carbonyls compounds or volatiles by high performance liquid chromatography (Frankel, 2005)

Aldehydes and ketones present as a secondary oxidation product are derivatised with 2,4-dinitrophenylhydrazin (DNPH) under acidic conditions for at least 1h at room temperature. These

treatments aim to measure the absorption of the hydrazones as a means for estimating the rancidity. 2,4-Dinitrophenylhydrazine can be used to qualitatively detect the carbonyl functionality of **a ketone** or **aldehyde** functional group (Schulte, 2002)

The derivatization method with 2,4 DNPH has been used many years ago. This method depends on measuring the colour of intensity of the hydrazones as a means for estimating rancidity. However, it has a weakness which cannot differentiate between short chain aldehyde and the higher carbonyl compounds (Schulte, 2002)

Atmospheric pressure chemical ionization (APCI) is a relatively recent mass spectrophotmetric ionization technique which is a soft ionisation meaning that mainly molecular ions are formed. It has considerable potential for combined HPLC/MS analysis because it can improve quantification and positive identification of a number of carbonyls in oxidised oil. (Ochs, et al., 2015; Mottram, et al., 1997)

The aim of this study is to develop a routine method by using reversed-phase high performance liquid chromatography (RP-HPLC) coupled to mass spectrophotometry hyphenated with an atmospheric pressure chemical ionization (APCI) source with 2,4 DNPH derivatisation prior to chromatography to detect the carbonyl compounds in oxidized oils. This has the advantage that by interpretation of the optimized fragmentation separation pattern of carbonyls and relative abundance of the oxidative of fatty acids can be identified.

II. LITERATURE REVIEW

2.1. Reaction mechanisms from primary oxidation products to secondary oxidation products in edible oils.

Heat treatments such as cooking, frying and other preparation processes can cause the oxidation of lipids. The unsaturated fatty acids such as oleic acid, arachidonic acid, linoleic acid and linolenic acid can produce secondary oxidation products (aldehydes and ketons). Shibamoto has described the mechanism for the formation of secondary products from the oxidation of lipids.

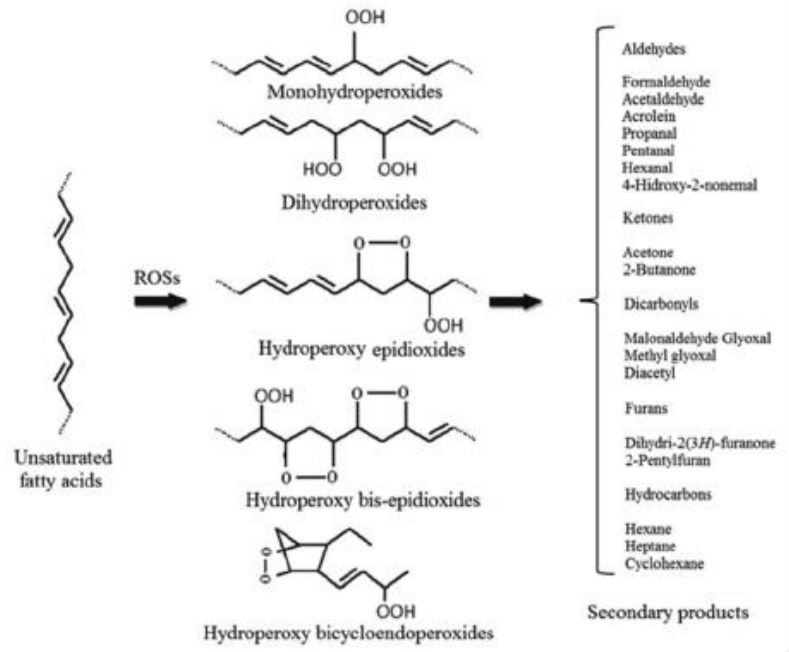


Figure 1. Mechanism reaction for the formation of secondary products from the oxidation of lipids (Shibamoto, 2006).

In the absence of metals, the primary oxidation products, lipid hydroperoxides are relatively stable at room temperature. However, in the presence of metals or high temperature they are readily

decomposed to alkoxy radicals and then they form aldehydes, ketones, acids, esters, alcohols, and short chain hydrocarbons. The most likely pathways of hydroperoxide decomposition is a homolytic cleavage between oxygen and the oxygen bond, in which alkoxy and hydroxyl radicals are produced (Choe & Min, 2006)

The energy required for removing hydrogen from fatty acids or acylglycerols depends on the hydrogen position in the respective molecule. The hydroperoxide positional isomers formed in the auto-oxidation of oleic, linoleic, and linolenic acids are shown in the Tab. 1.

Table 1. Hydroperoxide of fatty acids by auto-oxidation

Fatty acids	Hydroperoxides at	Relative amount (%)
Oleic acid	C8	26~28
	C9	22~25
	C10	22~24
	C11	26~28
Linoleic acid	C9	48~53
	C13	48~53
Linolenic acid	C9	26~35
	C12	8~13
	C13	10~13
	C16	28~35

Source: Frankel, 1985)

The most pivotal factor to be considered in evaluating the oxidative stability of oils or fats is the temperature, and especially unsaturation of the fatty acids. The mechanism of oxidation changes with temperature and different hydroperoxides of linoleate are produced. They work as precursors of volatile flavours which decompose at different temperatures. This happens because the rate of oxidation is exponentially related to temperature, the shelf life of food lipid decreases logarithmically with increasing temperature (Katragadda, et al., 2010).

2.2. Secondary oxidation products analysis.

During the oil degradation, aldehydes, ketone and alcohols, dienes and acids, commonly create, produce off flavour which reduced the quality of oil and for the future effect can cause the health problems (Fullana, Carbonell-Barrachina & Sidhu, 2004a, 2004b). 2,4-Dinitrophenylhydrazine can be used to selectively detect the carbonyl functionality of a ketone or aldehyde functional group. Derivatization with a pre-charged derivatising agent is often used by LC/MS researches to enhance the signal of charged ion.

The hydrazine group of DNPH reacts with the carbonyl group, forming a hydrazone. A positive test is signalled by a yellow, orange or red precipitate (known as a dinitrophenylhydrazone). If the carbonyl compound is aromatic, then the precipitate will be red; if aliphatic, then the precipitate will have a more yellow colour.

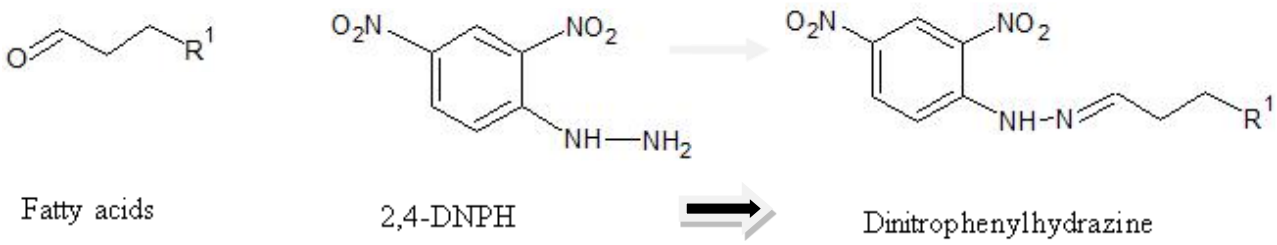


Figure 2. Formation of dinitrophenylhydrazone for identification of carbonyls

Hydrazine reagents have been established as highly efficient derivatising agents to identify aldehydes and ketones (Potter & Karst, 1996). The NH_2 of DNPH will react with carbonyls group to produce hydrazones (Fig. 2). The separation of the hydrazone of saturated, monounsaturated, and diunsaturated aldehydes was performed by reversed-phase high performance liquid chromatography (Reindl and Stan, 1982). Using the C18 column is recommended because of pre

charged of the derivatization reaction. It can improve to detect any salts which contain in the disinfection by products in drinking water because salts are unretained on the C 18 phase (Richardson, et al., 1999).

Unsaturated aldehydes, such as alkenals and alkadienals, show higher toxicity than alkanals (Meacher & Menzel, 1999 ; Tovar & Kaneda, 1977). Moreover, further literature indicates that some oils such as rapeseed oil, soybean oil, peanut oil, and lard, when exposed to high temperatures induce mutagenic and genetic toxicity (Chiang et al., 1997; Moreira et al., 1999; Qu et al., 1992).

Thermal decomposition of monohydroperoxides produces alkoxyl radicals undergoing homolytic β-scission to form aldehydes, alkylic and olefinic radicals. The formation of lower aldehydes proceeds by reaction of the alkyl radicals with oxygen to produce primary hydroperoxides. These can decompose further to aldehydes via an alkoxy radical, or cleave to produce formaldehyde and a lower alkyl radical containing one carbon less.

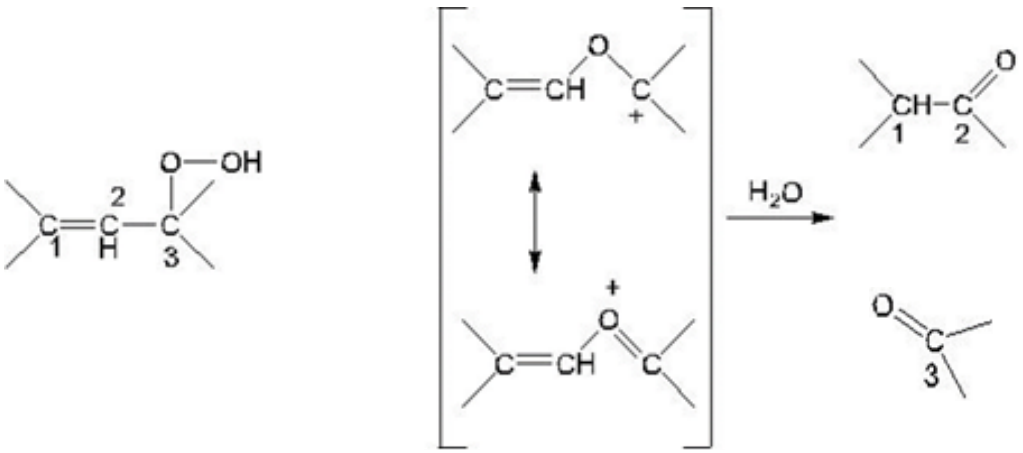
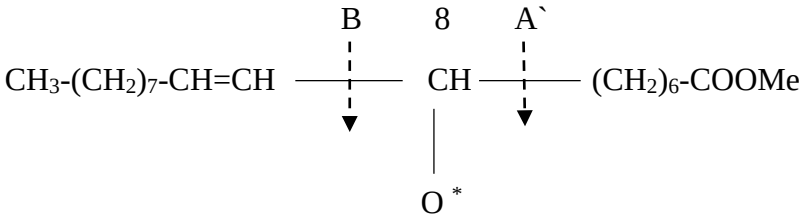


Figure 3. Hock cleavage mechanism for the fragmentation of allylic hydroperoxides into two carbonyl products

Other mechanism for the thermal decomposition of allylic hydroperoxides are recognized in free radical chemistry and photochemistry. The hock cleavage involves fragmentation of allyl hydroperoxides into two carbonyl products by oxygen insertation (Fig. 3).

4a.

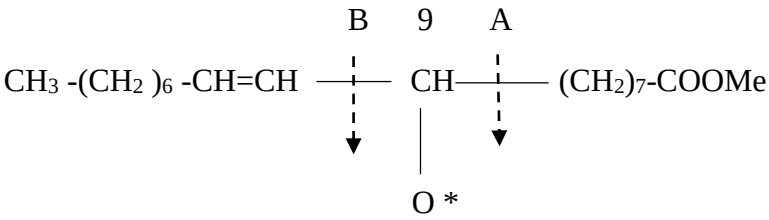


8OOH

Cleavage A: 2-undecenal (2%, 7%) + Me heptanoate (2%, 5%)

Cleavege B: decanal (4%, 2%) + Me 8-oxooctanoate (4%, 3%)

4b.

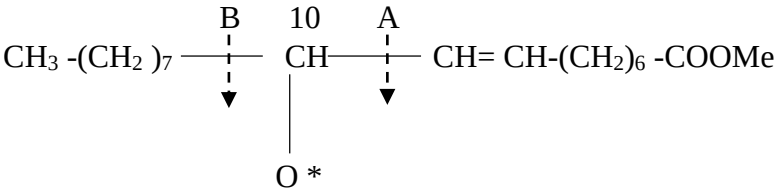


9OOH

Cleavage A: 2 decenal (5%, 12%) + Me octanoate (5%,10%)

Cleavage B: nonanal (15%,10%) + Me 9-oxononanoate (15%,11%)

4c.

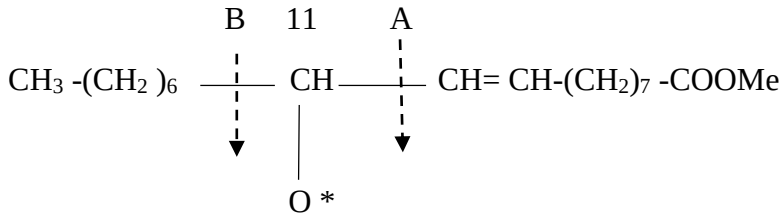


10OOH

Cleavage A: nonanal + Me 9-oxononanoate

Cleavage B: octane (3%, 10%) + Me 11-oxo-8-decenote (3%, 5%)

4d.



Cleavage A: octanal (11%, 4%) + Me 10-oxodecanoate (12%, 2%)

Cleavage B: heptane (4%, 5%) + Me-oxo-9-undecenoate (6%, 5%)

Figure 4 (a,b,c,d). Cleavage products from methyl oleate hydroperoxides. Relative percent values shown in parentheses are for autoxidation and photosensitized oxidation respectively

Source: Frankel, 2005

Oleate can be oxidized with a subsequent homolytic cleavage A and B. Alkoxyl intermediate group from 8-, 9-, 10- and 11-hydroperoxide produce different carbonyls. Decanal and 2-undecenal are produced from 8-hydroperoxide, nonanal from either 9-or 10- hydroperoxide, octane from 10 hydroperoxide, and octanal and heptane from 11-hydroperoxide (Fig. 4a-d). On the other hand, on the ester side oxo esters can be formed by cleavage A. From triolein monohydroperoxides are produced. In addition, conjugated oxo glycerides or aldehyde glycerides are formed. These are "core" aldehydes which have a high molecular weight and are non-volatile. The presence of these compounds is related to a reduced quality of the oil (Frankel, 2005).

III. MATERIALS AND METHODS

3.1. Reagents and solvents

2,4-Dinitrophenylhydrazine (2,4-DNPH) was purchased from Sigma-Aldrich (St. Louis, USA), hydrochloric acid (HCl 37 %) was purchased from Merck (Darmstadt, Germany), all solvents (e.g. methanol, ACN, acetone) used were of HPLC grade and were purchased from Merck (Darmstadt, Germany), acetic acid was purchased from Roth (Karlsruhe, Germany), triolein was purchased from FLUKA (Buchs, Switzerland), β-carotene and α-tocopherol were from Sigma Aldrich (St. Louis, USA).

3.2. Ranzimat oxidation method

The triolein oil samples were subjected to oxidation in a Ranzimat (679, Metrohm, Herisau, Switzerland). Eleven grams of sample were used for the oxidation experiments. The temperature was set to 120 °C and the air flow to 20 l/h. Triolein was treated for up to 10 h with the given sampling interval starting from 0, 2, 4, 5, 6, 6.5, 7, 7.5, 8, 9, and 10 hours for oil sample analysis. The oxidized samples were cooled immediately after the oxidation on ice and then stored under nitrogen at below -18 °C.

3.3. Derivatisation with 2,4-dinitrophenylhrazine (DNPH)

To 1 ml of the oxidized oil samples 4 ml of acetonitrile were added and mixed with 3 ml of the reagent 2,4-DNPH (3.48 mg/ml). The reaction mixture was kept in the dark for 1 h. After completion of the reaction 2 ml ethyl acetate were added for extraction and 1 g KCl for better phase separation. This mixture was thoroughly shaken for 30 s and centrifuged for phase separation. The organic layer was analysed without further treatment by HPLC.

3.4. Liquid chromatography-mass spectrometry

The analyses of the DNPH derivatives of the carbonyls formed during oxidation were done by HPLC (Agilent 1100, Waldbronn, Germany) using a reversed phase column (Kinetex, EVO C18, 150 × 3 mm, Phenomenex, Aschaffenburg, Switzerland). For elution a gradient was used starting with methanol (45%), water (30%), and acetonitrile (25%) changing to methanol (6%), water (4%), and acetonitrile (90%) linearly within 15 min. The absorption of the eluent was measured at 400 nm for the presence of the DNPH derivatives.

For mass selective detection a QTRAP 2000 (AB Sciex, Framingham, MA, USA) was used. Ionization was done using the APCI mode with a gas drying temperature of 250 °C, capillary voltage of 4000 V, and a fragmentor potential of 150 V.

3.5 Analyses of α-tocopherol and β-carotene

For the analyses of α-tocopherol and β-carotene 25 mg of the oil (triolein, triolein with β-carotene and triolein with α-tocopherol) were extracted with 1 ml of methanol in 2 ml reactions vials (Eppendorf, Wien, Austria). The samples were shaken for 2 min vigorously and centrifuged. Under these conditions both, α-tocopherol and β-carotene were extracted quantitatively. The methanolic extract was used directly for HPLC analysis on a reversed phase column (Kinetex, EVO C18, 150 × 3 mm, Phenomenex, Aschaffenburg, Switzerland) using a flow of 0.6 ml/min. α-Tocopherol was separated isocratically using 5% water in methanol detecting it at 292 nm. β-Carotene was chromatographed with 100% acetonitrile with detection at 450 nm.

IV. RESULTS AND DISCUSSION

Hydroperoxides are unstable to heat and will easily decompose to the more stable secondary oxidation products under such conditions. The decomposition of hydroperoxides was probably faster than the formation. Moreover, a considerable increase in the content of the more stable alkenals appeared in this oxidation reaction. The five products with highest alkenal content after heating were sunflower oil, soybean oil, and one corn oil, containing about 69%, 61%, and 62% PUFA, respectively. These results are consistent with the assumption that MUFA are more resistant to oxidation and heat than PUFA (Blomhoff, 2011).

Thermal decomposition of monohydroperoxides produces alkoxyl radicals undergoing homolytic β-scission to form aldehydes, alkyl and olefinic radicals. The formation of lower aldehydes proceeds by reaction of the alkyl radicals with oxygen to produce primary hydroperoxides. These decompose further to produce aldehydes via an alkoxy radical or cleave to produce formaldehyde and a lower alkyl radical containing one carbon less.

4.1. The carbonyls profile in the triolein

The formation of carbonyls began after 2 hours of oxidation at 120 °C while bubbling air through the oil. A strong increase of carbonyl formation was observed between 2 and 4 hours with a maximum after 6 hours and a slight decrease or constant concentrations afterwards. A series of linear aldehydes (from 6 carbons to 10 carbons) could be identified positively by LC-MS. In the first 2 h no carbonyl compounds could be analysed. There were appeared 5 significant peaks started after 4 h of oxidation and could be found until the end of the experiment (Fig. 5).

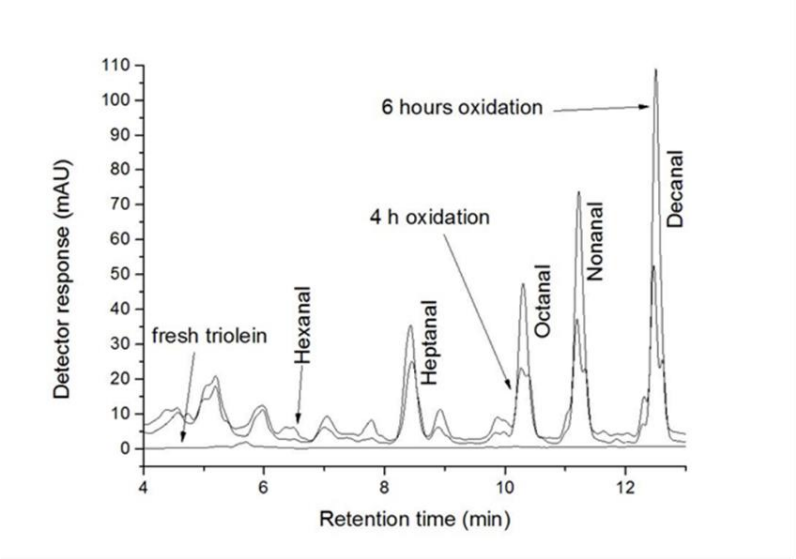


Figure 5. The chromatogram peaks from 2,4 and 6 h triolein's oxidation

During the oxidation, the series of carbonyl compounds was formed with the highest concentration at 6 h. The maximum concentration is 27 mM which is based on a calibration with hexanal. After 6 h the total peak area starts decreasing (Fig. 6)

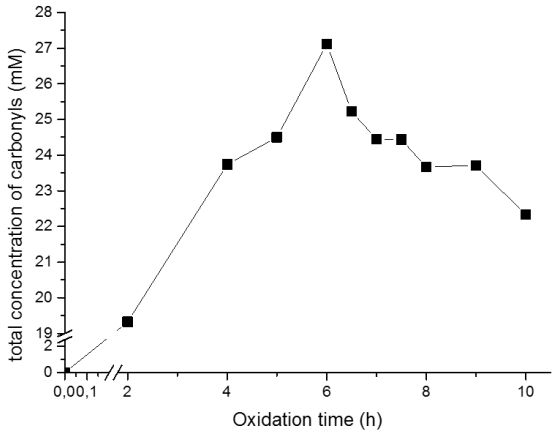


Figure 6. Formation of the total amount of carbonyls during oxidation of triolein at 120 °C with constant air sparging.

4.2. Identification of carbonyl compounds in the oxidised triolein

Some of the peaks in the chromatogram which developed, we observed a decrease in the later stage of the oxidation experiment. The five selected peaks which are A (6.04 min), B (8.58 min), C (10.29 min), D (11.21 min), and E (12.50 min) in minutes showed maximum at 6 h with a decrease starting after 7 h (Fig. 7 ; Fig. 8)

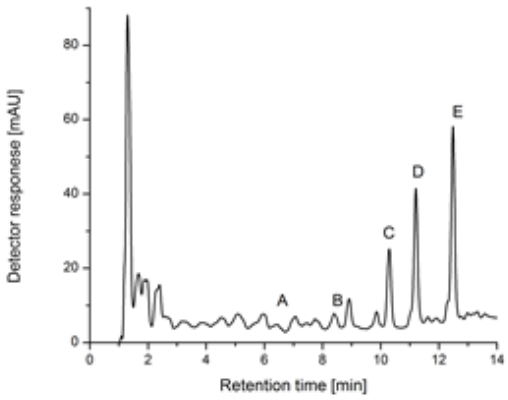


Figure 7. Chromatogram of derivatised carbonyls after 10 h oxidation

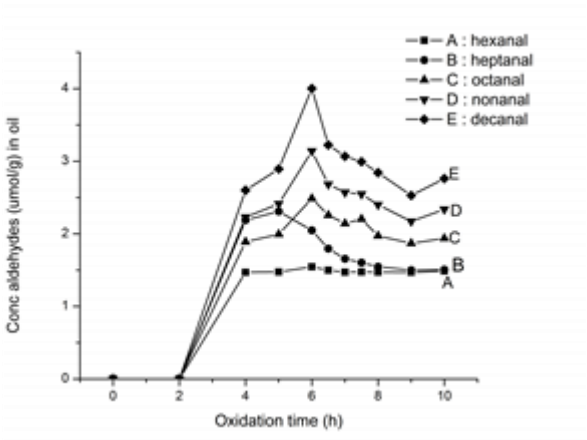
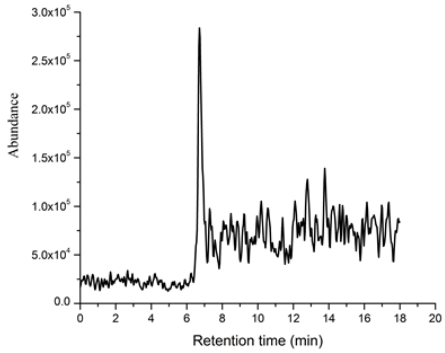


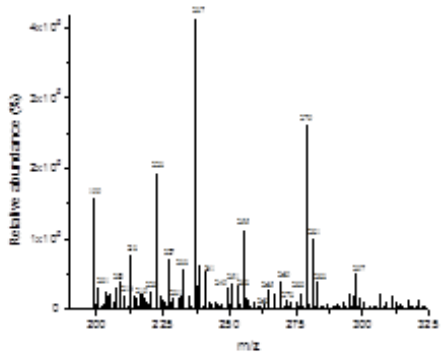
Figure 8. Formation of carbonyls compounds during oxidation, detection as DNPH derivatives, oxidation at 120 °C in Ranzimat with air flow of 20 L/h

Autoxidized oleate showed a homolytic cleavage A and B from were decanal and 2-undecenal on either side of the alkoxyl intermediate group from the 8-hydroperoxide, nonanal from either the 9- or 10-hydroperoxide, octane from the 10-hydroperoxide, and octanal and heptane from the 11-hydroperoxide. A mixture of 8-, 9-, 10-, and 11-oxo esters is produced by cleavage A on the ester side of the hydroperoxide. With the monohydroperoxides of triglycerides containing oleate, the corresponding oxo glycerides or aldehyde-glyceride are formed; these high molecular weight non-volatile compounds, referred also as “core” aldehydes, are useful predictors of quality edible oils (Frankel, 2005)

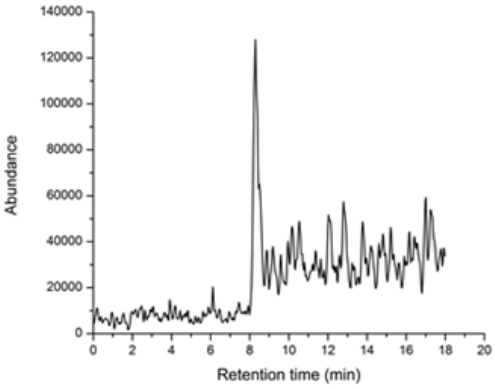
The main volatile decomposition products formed from oleate, linoleate, and linolenate are those expected from the cleavage of the alkoxyl readicals formed from the hydroperoxides of autoxidized and photosensitized oxidized fatty acid esters (Fig. 9)

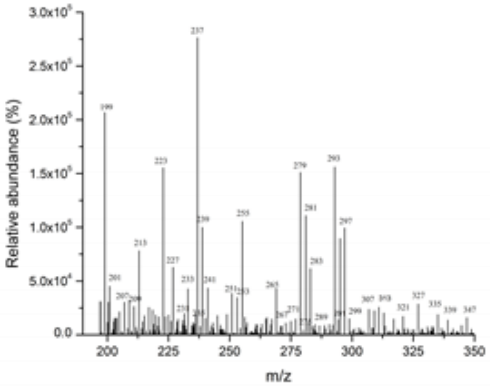
a.



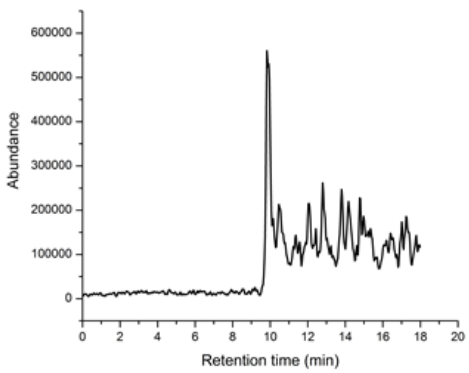


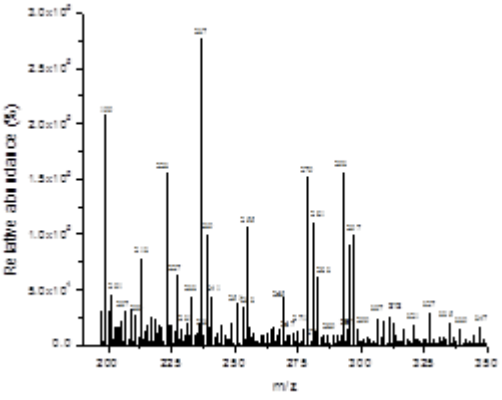
b.



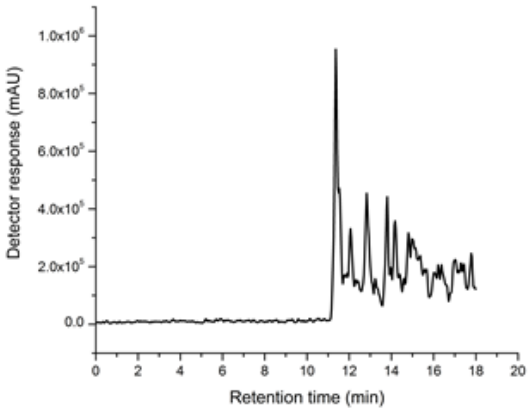


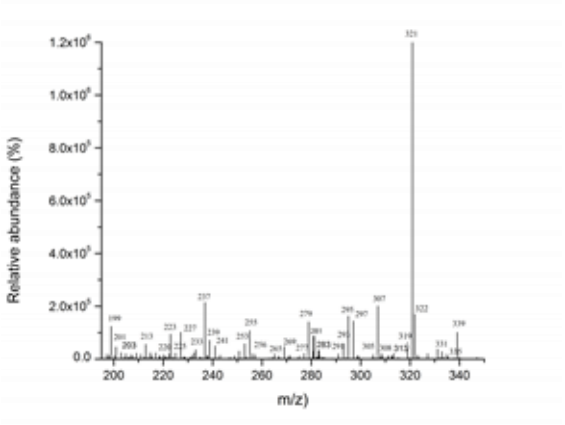
c.



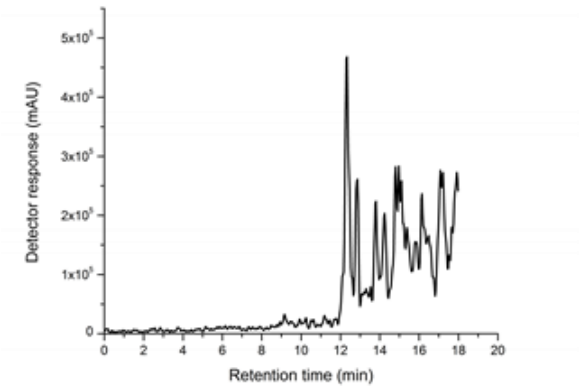


d.


Detector response (mAU)
Retention time (min)


Relative abundance (%)
m/z)

e.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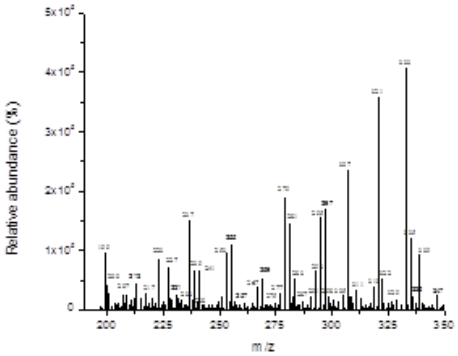
Relative abundance (%)
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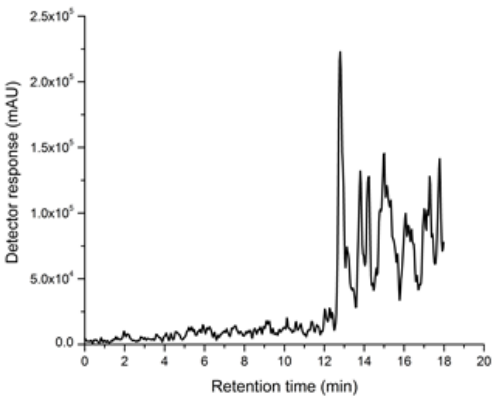
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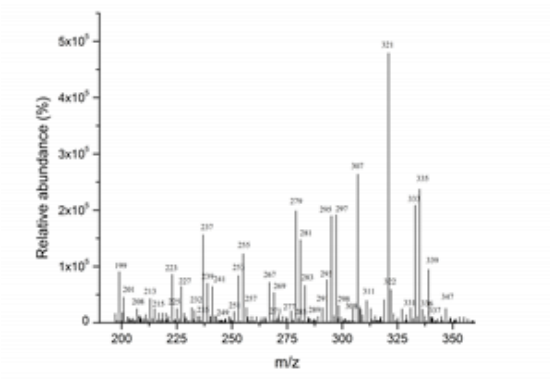
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g.

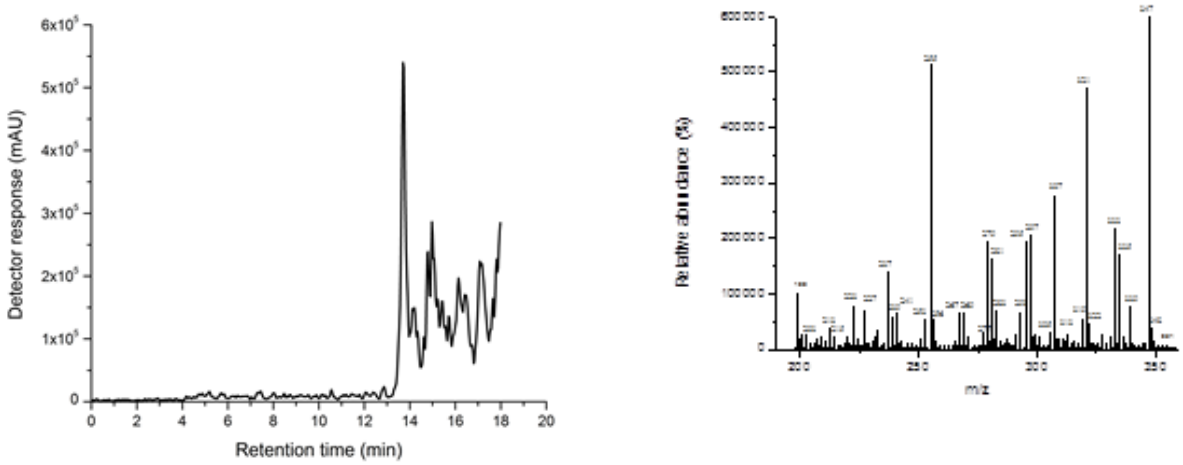

Figure 9. UV-HPLC and APCI/MS spectra of secondary lipid oxidation products. (a) hexanal (m/z 279), (b) heptanal (*m/z* 293), (c) octanal (*m/z* 307), (d) nonanal (*m/z* 321), (e) decanal (*m/z* 333), (f) decanal (*m/z* 335), (g) undecanal (*m/z* 347)

Hydrazones from aldehydes and ketones hydrazones can be differentiated by their different fragmentation pathways. Ketones show low or no formation of the fragment of *m/z* 163 and an ion of *m/z* 152 of high relative abundance, as shown in the MS/MS spectra abundant fragment cyclohexanone of acetone and cyclohexanone. Ketones also showed a highly (*m/z* 247). This ion was also found in the mass spectra of aldehydes, but with a relative low abundance (Ochs, et al., 2015).

Based on the calibration with hexanal all other dominant substances were in the similar concentration range with maximum concentrations of 1.6 µmol/ml of hexanal, 2.3 µmol/ml of heptanal, 2.5 µmol/ml of octanal, 3.2 µmol/ml of nonanal, 4.0 µmol/ml of decanal after 6 hours (Fig.10). These identified aldehydes were volatile and undergo further degradation reactions which may explain the reduced concentration.

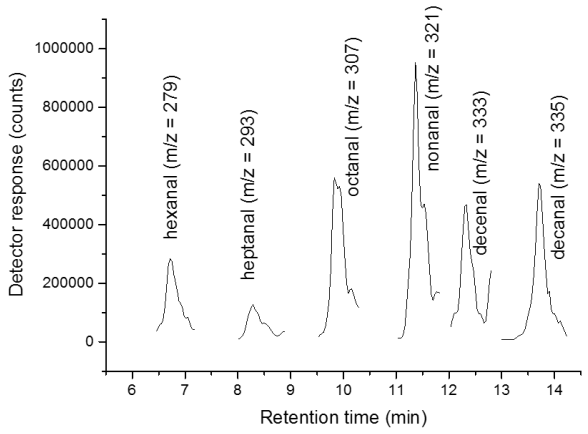

Figure 10. LC/MS analysis of triolein 10h

The higher the content of oleic acid the more nonanal is produced, the higher the linoleic acid the more hexanal is produced, and the higher the linolenic acid the more propanal is produced. In addition, hexanal being the main product from oleic, linoleic, and arachidonic acid, the high heptadienal content resulting from the oxidation of linolenic acid. More substantial features from oleic acid are the formation of octanal and nonanal, however the absence of heptanal because no linoleic acid present in the oil. The others aldehyde which were produced from oleic acid are 2-nonenal and 2,4-decadienal as well as the production of 2,4-nonadienal from arachidonic (Stan, 1982).

Undesirable degradation volatile compound in the heated triolein were fruity and plastic, with other negative odours of acrid and grassy. Some volatile aldehyde were increasing concentration during heating were hexanal (grassy), octanal (fruity), (*E*)-2-decenal (plastic), nonanal (fruity), and (*E*)-2-undecenal (plastic) (Neff, 2000).

4.3 Influence of lipid soluble antioxidants on carbonyl formation

In a second series of experiments, two lipid soluble antioxidants were added, namely α-tocopherol and β-carotene. The earlier experiments showed that the best stabilization was obtained by

addition of 300 ppm of ascorbyl palmitate to soybean oil (SO). In addition, a combination of ascorbyl palmitate (300 ppm) and α-tocopherol (1000 ppm) was able to limit hydroperoxide and hexanal formation in SO at 35 °C for 12 weeks (Sarkar, 2015).

The fortified oils were oxidized within 2 hours. The fast degradation was attributed to the lack of the protective compounds. In analogous experiments using corn oil, the β-carotene present was still measurable after 10 hours or more (Murkovic et al., 1997; Zeb & Murkovic, 2013). During the course of the oxidation in this study, the levels of the formed carbonyls were lower in the samples with added antioxidants (Fig. 11).

In addition, the pattern the chromatograms did not change significantly indicating the same carbonyls are formed but at lower amounts. The sample containing β-carotene showed a lower of carbonyls formation (Fig. 12)

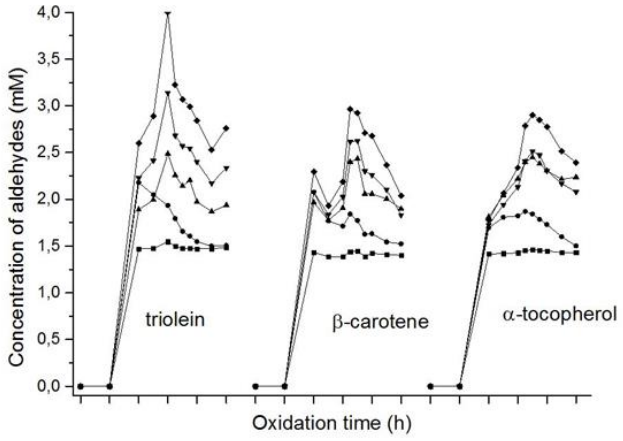

Figure 11. Formation kinetics of single compounds during oxidation of triolein in presence of lipid soluble antioxidants at 120 °C with constant air sparging for 10 h (-●-hexanal; -■-heptanal; -▲-octanal; -▼-nonanal; -♦-decanal).

For this, β-carotene and α-tocopherol were dissolved in acetone and added to the triolein. The concentrations of the antioxidants were 300 µg/g ± 0.5 µg. The fortified triolein was stirred for 15 min and then flushed with nitrogen for 30 min to remove the acetone before sealing airtight in glass bottles. For the oxidation in the Ranzimat 20 g of triolein were used. The concentrations which were applied were in the same range as it can be expected in natural oils. The α-tocopherol concentration was 300 µg/g and the level of β-carotene was 500 µg/g. The concentrations were selected according to the concentrations normally occurring in red palm oil which are in the range of 600 – 1000 µg/g for vitamin E and 400 – 3500 µg/g for α- and β-carotene (Oii, 1999)

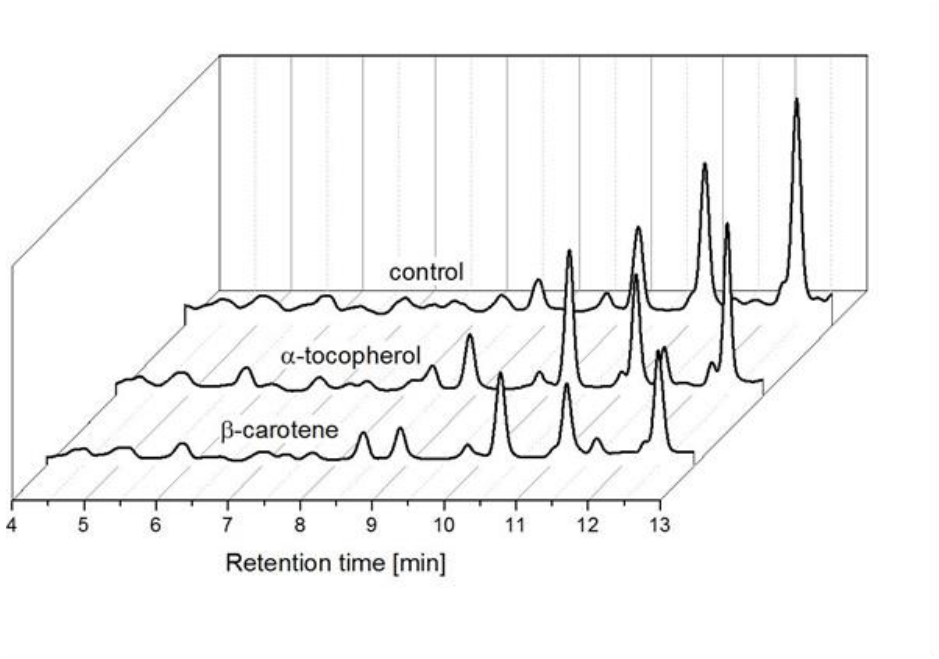

Figure 12. Comparison of the carbonyl formation in presence of lipid soluble antioxidants (α-tocopherol, β-carotene) after 10 h of oxidation at 120 °C. The carbonyls are measured as DNPH derivatives detected at 400 nm.

As no other protecting compounds were present in the oil both antioxidants were oxidized within 2 hours. In fact these two antioxidants could only be detected at the beginning of the oxidation experiment. The very quick degradation might be due to the fact that no other protecting compounds are present. Analogous experiments using e.g. corn oil, the β-carotene was present until 10 h or longer (Murkovic, et al., 2013). However, during the course of the oxidation the level of the formed carbonyls was lower in the samples where the antioxidants were present initially. In addition, the pattern of the chromatogram did not change significantly which means that the same carbonyls are formed but at lower amounts. The sample containing β-carotene showed a lower carbonyl formation (Fig. 12).

V. CONCLUSIONS

The formation of carbonyls was quantified during oxidation of triolein by derivatization of the formed products with DNPH which is a selective reagent for aldehydes and ketones. Some of the oxidized fragments of triolein were identified by LC-MS/MS – using APCI in negative mode – being hexanal (*m/z* = 279), heptanal (*m/z* = 293), octanal (*m/z* = 307), nonanal (*m/z* = 321), decenal (*m/z* = 333), decanal (*m/z* = 335), and undecenal (*m/z* = 347). Using a Ranzimat for reproducible oxidation experiments with a constant air flow at defined temperatures (120 °C) the production of carbonyls showed a good repeatability. The formation of carbonyls from triolein showed a maximum after 6 h with a slight or no decrease during prolonged oxidation.

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Appendix

1. Data of carbonyls compounds measurement

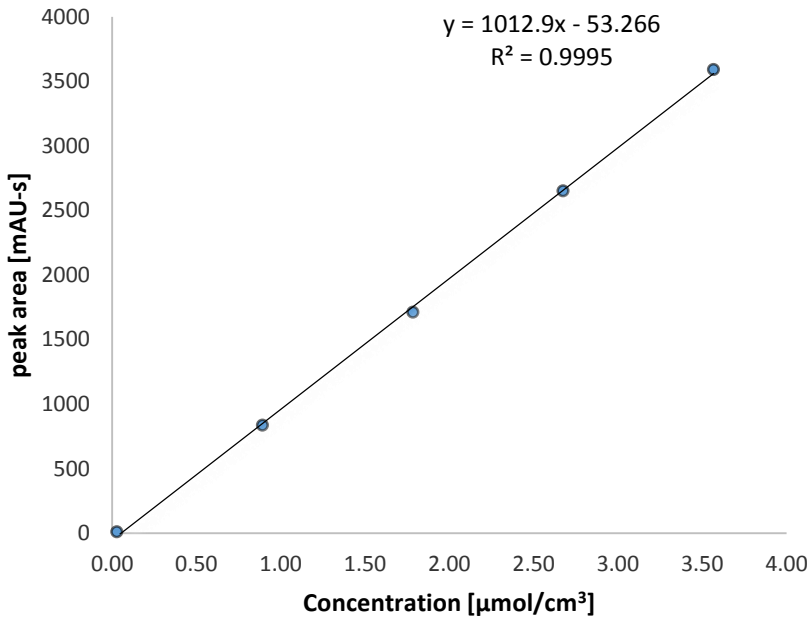

Figure 1. Standard calibration curve of hexanal. Calibration equation was used to calculate not only the total carbonyl content but also the carbonyls respectively such as hexanal, heptanal, octanal, nonanal and decanal in the oil.

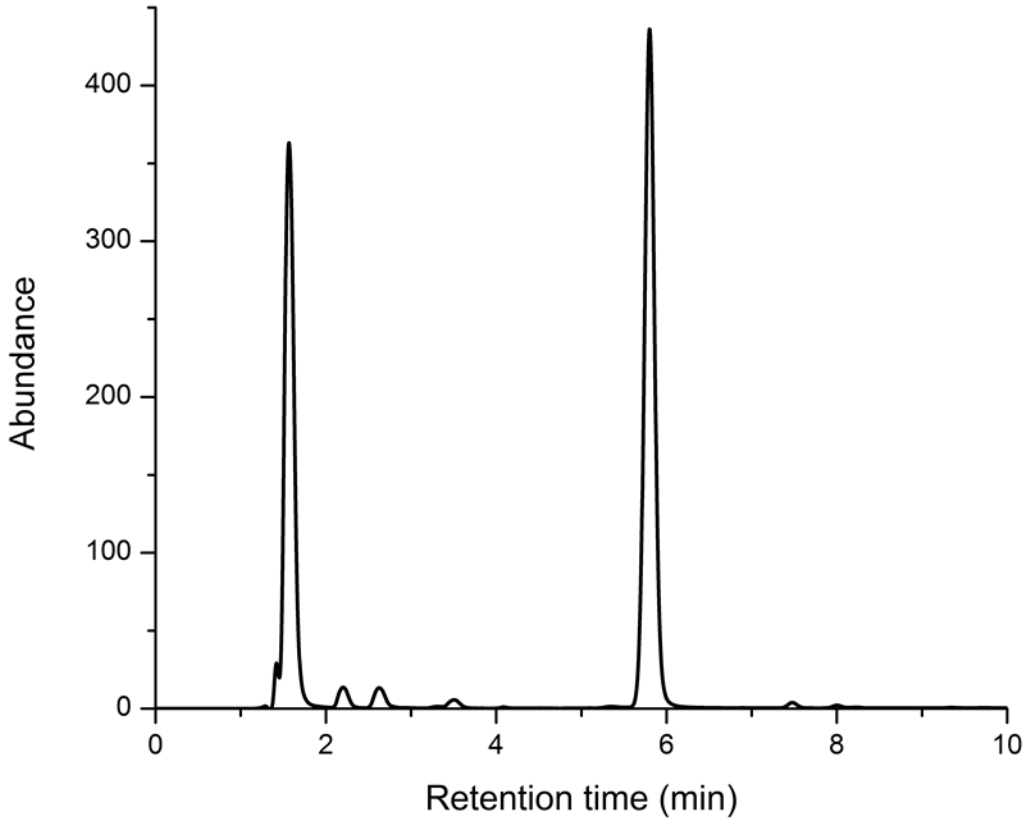

Figure 2. Standard chromatogram of hexanal

Table 1. Concentration of aldehydes in triolein (tri) and triolein + antioxidant [α-tocopherol (tritoc) and β-carotene (tribc)] during 10 h oxidation

Oxidation time (h)	The concentration of aldehydes in the oil (μmol/ml)														
	Hexanal			Heptanal			Octanal			Nonanal			Decanal		
	tri	tribc	tritoc	tri	tribc	tritoc	tri	tribc	tritoc	tri	tribc	tritoc	tri	tribc	tritoc
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0.16	0.12	0.10	0.88	0.78	0.39	0.59	0.67	0.51	0.93	0.78	0.42	1.31	1	0.47
5	0.16	0.07	0.11	0.75	0.46	0.50	0.69	0.47	0.74	1.12	0.53	0.64	1.61	0.63	0.77
6	0.24	0.07	0.12	0.63	0.41	0.52	1.19	0.60	0.92	1.86	0.73	0.83	2.74	0.89	1.05
6.5	0.19	0.13	0.14	0.49	0.54	0.57	0.96	1.11	1.11	1.39	1.33	1.12	1.95	1.68	1.50
7	0.16	0.13	0.15	0.35	0.47	0.54	0.84	1.14	1.15	1.28	1.34	1.23	1.79	1.64	1.62
7.5	0.16	0.08	0.14	0.30	0.32	0.48	0.91	0.76	1.09	1.26	1.01	1.18	1.71	1.42	1.56
8	0.16	0.11	0.14	0.24	0.32	0.42	0.67	0.76	1.01	1.11	0.96	1.01	1.56	1.39	1.49
9	0.16	0.10	0.12	0.19	0.24	0.29	0.57	0.70	0.92	0.87	0.80	0.87	1.24	1.07	1.23
10	0.18	0.09	0.12	0.20	0.21	0.19	0.63	0.60	0.94	1.04	0.53	0.78	1.48	0.74	1.10

Table 2. Concentration of antioxidants in triolein during 10h oxidation triolein (tri) + triolein with antioxidant [(α-tocopherol (tritoc) and β-carotene (tribc)] during 10 h oxidation

Oxidation time (h)	Oil + Antioxidants			
	γ-toc (mg/mg)		α-toc (mg/mg)	
	tribc	tritoc	tribc	tritoc
0	0.00074	0.0008	0.0012	0.0026
2	0.00071	0.0005	0.0011	0.0002
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
6.5	0	0	0	0
7	0	0	0	0
7.5	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0

SECTION 3

THE STABILITY OF PALM OIL DURING HEATING IN RANZIMAT

I. INTRODUCTION

The palm tree (*Elaeis guineensis*) produced palm oil are found in the tropical country. Due to its technological properties palm oil can replace animal fats which has an impact on cost and health issues (Chandra Sekharan, 1999). Malaysia and Indonesia are the leading palm oil exporting countries with around 86% of global production. Crude palm oil is produced from palm fruits. The crude palm oil is known as red palm oil (RPO) because of the very high content of β-carotene (Choo and Gapor, 1990).

On the other hand, crude unrefinded palm oil (CPO) has a typical aroma reminding on overripe mushrooms and a very pungent taste. This is the reason for its limited use in the kitchen. In addition, it is high in free fatty acids (FFA), moisture, trace metals and other impurities that limit its shelf life. The way how to increase the utility is to refine the oil by removing odours, flavours, and other impurities, as well as the red colour. After removing the adverse aroma and the colour the oil is better accepted by the consumers (Mancini et al., 2015).

Crude palm oil is extracted either by wet or dry processes. It consists of healthy beneficial compounds, such as triacylglycerols (TAGs), vitamin E, carotenoids phytosterols, as well as impurities, such as phospholipids, free fatty acids (FFAs), gums, and lipid oxidation products. These undesirable compounds can be remove by refining (Sambanthamurthi et al., 2000). Hence, the CPO is purified by centrifugation and drying; the dried oil is then cooled and stored in appropriate containers (Obibuzor, et al., 2012).

It has a high carotenoid content (500-700 mg/kg) which is responsible for the dark orange colour (Gee, 2007). The content of vitamin E (tocopherols and tocotrienols) is 600–1200 ppm, which comprises approximately 24% of tocopherols and 76% of tocotrienols. This contributes to its stability and nutritional properties (Mba et al., 2015; Sambanthamurthi et al., 2000; Edem, 2002; Sundram et al., 2003; Souganidis et al., 2013; Ong, 1993). Crude palm oil has to be refined to produce palm oil which has desirable characteristics, such as a light colour, bland taste and good oxidative stability (Gibon et al., 2007; Sampaio et al., 2011). In literature it was shown that their antioxidant properties, act mainly against reactive oxygen species (ROS), play a role in aging, in cardiovascular diseases (CVD) and in cancer prevention (Edem, 2002; Ong et al., 2002; Sen et al., 2007). Furthermore, tocotrienols have been reported to be natural inhibitors of cholesterol synthesis (Edem, 2002). γ-Tocotrienol may reduce blood pressure by reducing lipid peroxides and enhancing the total antioxidant status. Melanomas, also on the increase, can be inhibited with the delta fraction of tocotrienols. In addition, γ– and δ–tocotrienols derived from palm oil exhibit a strong activity against tumour promotion. They can also inhibit certain types of cancer, including both the estrogen-positive and estrogen-negative breast cancer cell. It is not only the cancer cells that are inhibited by the tocotrienols but also normal/healthy cells, which however needs extremely high amounts for this effect (Imoisi, 2015).

Dietary and supplemental sources of vitamin E compounds might influence critical pathways involved in cancer. Tocotrienol (T3) are mainly present in palm, rice and annatto. Pierpaoli and co-workers (2013) investigated the effect of dietary supplementation with T3 extracts from annatto seeds and the development of mammary tumours in HER-2/neu transgenic mice and observed that annatto-tocotrienol may exert important anti-tumour effects delaying the development and the metastasizing capacity of tumours in mice transgenic for the HER-2/neu oncogene. T3 have been reported to exert potent anti-proliferative effects on human breast cancer

cells (Ahn, et.al., 2007). Recently, Loganathan and co-workers (2013) compared anti-malignant effects of pure vitamin E analogues (tocotrienol analogues (α, δ and γ) and α tocopherol), a tocotrienol-rich fraction (TRF) and a tocotrienol enriched fraction (TEF) isolated from palm oil on two human breast cancer cell lines and they found a marked induction of apoptosis in both cell lines by tocotrienols compared to treatment with paclitaxel, which was used as positive control. In view of their findings, palm tocotrienols seem to induce apoptosis in human BC cells, through specific genetic pathways.

The main aim of this work was to establish an analytical method for a quantitative determination of the carbonyls in the lipid (oxidized oil) phase for determination of the alimentary exposure. The formation of carbonyls during oxidation of unrefined palm oil (UPO) and palm oil (PO) in the oil phase and not the volatile phase in which these compounds are mostly related to sensory sensation. On basis of reversed phase chromatography with UV and MS detection, the non-evaporating carbonyl compounds formed during oxidation of UPO and PO were identified and quantified.

As we know, the unrefined palm oil (UPO) and palm oil (PO) have a balance of saturated and unsaturated fatty acids. In addition, UPO has the highest β−carotene and vitamin E compared to others vegetable oils. Thus, we wanted to find out the relationship between the antioxidants and the stability of these oils. In this case, it focused on the carbonyl formation which was hexanal, heptanal, octanal, nonanal, and decanal. The carbonyls were analysed by 2,4-dinitrophenylhydrazine (DNPH) derivatisation – a commonly known derivatization reagent for carbonyls – that would give the possibility to identify and quantify single components as well as the total carbonyl content. These derivatives are stable and can be analysed by HPLC with ESI-MS in the negative mode or by its absorption at 400 nm (Schulte, 2002; Mottram et al., 1997; Ochs et al., 2015). The antioxidants (β-carotene, α-tocopherol) can be analysed by HPLC with UV and fluorescence detection with 292 nm (FLD: λ_{ex} = 292 nm, λ_{em} = 335 nm) for α-tocopherol, while β-carotene can be analysed at 450 nm.

II. LITERATURE REVIEW

2.1. Palm oil processing

Crude palm oil (CPO) is extracted from the pulp of the fruit of the oil palm tree (*Elaeis guineensis*). CPO is deep orange red in colour due to the high content of natural carotenes. Palm oil is a rich source of carotenoids and vitamin E which are related to the natural stability against oxidative deterioration. Fractionation separates the oil into liquid and solid fractions. Palm oil can be fractionated into liquid (olein) and solid (stearin) components. Palm olein is used as cooking oil, whereas the palm stearin – because of solid state – can be used in shortenings and butter substitutes (Mancini, 2015).

In order to produce red palm oil (RPO), most CPO is processed to remove odours, flavours, and impurities. The high saturated fat content makes RPO semisolid at room temperature and more resistant against lipid oxidation than oils that are composed of mainly unsaturated fatty acids (Mancini, 2015). The key for producing red palm oil is that you have to deodorize the palm oil at a low temperature to avoid thermal destruction of the carotenes.

As we know, palm oil is the most widely-used vegetable oil in the world. Palm oil is being one of vegetable oils which has limited cholesterol. It derived from the fruit of oil palm tree (*Elaeis guineensis*). Having a naturally semi-solid characteristic at room temperature with a specific original melting point between 33 ºC to 39 ºC, so it can replace hydrogenated oil for use as it does not require hydrogenation and contains lower amounts of *E*-fatty acids.

Furthermore, producing the palm oil as daily commodity some processes have to be done such as refining, bleaching and deodorizeing resulting in RBD palm oil. Refining is necessary to obtain desirable characteristics such as a light colour, bland taste and good oxidative stability (Gibon et

al., 2007; Sampaio et al., 2011). There are two kinds of the refining process, physical and chemical refining, physical refining is more common not only because of the reduction of the high free fatty acid content in CPO but also to avoid a neutral oil loss (Rossi et al., 2011). In addition, physical refining usually comprises bleaching and deodorization /deacidification (Gibon et.al, 2007).

2.2. Fatty acids content in palm oil

This tropical fruit is reddish in colour because of a high β-carotene content. The fruit is about the size of a large olive. The fruit has a single seed or kernel, which is used to produce palm kernel oil. Palm oil comes from the palm fruit. Each palm fruit contains about 30-35% oil, 50% are saturated fat, and 9% are polyunsaturated fat. Palm fruit oil and palm kernel oil differ significantly in their fatty acid composition but have the same botanical origin. We use palm fruit oil over palm kernel oil because of its monounsaturated fat content and high levels of vitamin E. Palm kernel oil is mainly used in cosmetics. Tocotrienol does not only occur in palm oil but is also common in certain cereals and vegetables such as rice bran, coconut, barley germ, wheat germ, and oat. (Qureshi, 2000; Tan, 1989).

Palm kernel oil is extracted from the palm seed. The fatty acid composition is almost identical to coconut oil which is high in medium-chain fatty acids such as caprylic acid, capric acid, and lauric acid and with a total saturated fat content of over 80%, monosaturated fat 17% and polyunsaturated fat 3% (Tab. 1).

2.3. Antioxidants in palm oil (Vitamin E and β-carotene)

2.3.1. Vitamin E

Vitamin E comprises at least eight "vitamers" named tocochromanols, also tocopherols or tocotrienols. In addition, dienols and monoenols are occurring. It is synthesized by photosynthetic eukaryotes and other photosynthetic organisms such as cyanobacteria. One way to prevent lipid

oxidation in plants is by activating tocochromanols in oily seeds and fruits or in young tissues undergoing active cell divisions (Colombo, 2010).

Table 1. Fatty acid composition of palm oil and palm kernel oil

Fatty Acid	Palm Oil	Palm Kernel Oil
Caproic acid (6:0)	-	0.2
Caprylic acid (8:0)	-	3.3
Capric acid (10:0)	-	3.5
Lauric acid (12:0)	0.2	47.8
Myristic acid (14:0)	1.1	16.3
Palmitic acid (16:0)	44.0	8.5
Stearic acid (18:0)	4.5	2.4
Oleic acid (18:1)	39.2	15.4
Linoleic acid (18:2)	10.1	2.4
Linolenic acid (18:3)	0.4	-
Arachidic acid (20:0)	0.1	0.1
Total SFAs	49.9	82.1
Total MUFAs	39.2	15.4
Total PUFAs	10.5	2.4

Source: (Marcini et al., 2015)

Vitamin E is the most potent fat-soluble antioxidant in human plasma. The four tocopherols (The dietary components with the activity of vitamin E include α and γ-tocopherols and γ- and δ-tocotrienol. These compounds containing a chromanol ring attached to a phytyl chain (tocopherols) unsaturated phytyl chain (tocotrienols) and vary as to the number and position of methyl groups on the chromanol (Traber, 2012). Vitamin E cannot be produced by humans and needs to be obtained from food.

Tocopherol is the major vitamin E components which present in most plant food, for example, vegetable oils and nuts. On the other hand, tocotrienol is present in certain plant products such as palm oil, rice bran oil, coconut oil, barley germ, wheat germ, and oat(Qureshi et al., 2000; Tan and Brzukiewcz, 1989; Bramley et al., 2000). The percentage of tocotrienol make up to almost 70% and the remaining percentage of 30% is tocopherol with a total amount of 945 mg/kg in palm oil. Palm oil is the richest source of tocols especially tocotrienols among all edible vegetable oils. Rice bran oil is the second richest source of tocotrienols with 465 mg/kg. Other sources of

tocotrienols in foods include grapefruit seed oil, oats, hazelnuts, maize, olive oils, sea buckthorn berry, rye, flax seed, poppy seed oil and sunflower oil (Kannpan, 2012)

Among the tocopherol homologs, the α-tocopherol has the highest biological potency, γ-tocopherol is regarded as the most potent free radical remover NO_x among the isomers of vitamin E. In addition, γ-tocopherol has an effect to the health as a strong anti-inflammatory activity and an inhibition of carcinogenesis (Ju et al., 2010).

The isomer of tocopherol and tocotrienol (α-, β-, γ-, δ-) are called vitamin E/tocols. Tocotrienol is thought to have more potent antioxidant properties than tocopherol (The eight forms of vitamin E consist of a chromanol ring and a hydrophobic side chain. The chromanol ring of tocopherol is methylated at varying degrees at the position 5,7, and 8. The side chain of tocopherol is phytyl with a saturated chain. However, the side chain of tocotrienol is isoprenyl which has a double bonds at the positions 3, 7, and 11. The number and position of the methyl groups are as follows: α-tocotrienol is 5,7,8-trimethyl, β-tocotrienol is 5,8-dimethyl, γ-tocotrienol is 7,8-dimethyl and δ-tocotrienol is 8-methyl (Ahsan et al., 2015).

During the oxidation, tocopherol and tocotrienol can act as chain-breaking antioxidants by scavenging free radicals and thus inhibiting the initiation. Tocopherols readily transfer an H-atom from their hydroxyl group on the chroman ring to lipid peroxyl radicals (LOO*) leading to the formation of lipid hydroperoxides (LOOH) and tocopheroxyl radicals (TO*). The tocopheroxyl radicals are resonance stabilized within the chromanol ring and therefore usually do not propagate the chain (Kamal-Eldlin and Appelquist, 1996). However, tocopheroxyl radicals as well as their oxidation products are reactive with lipids.

2.3.1.1. Identification and quantification of tocopherols and tocotrienols

Some different methods have been developed and technique for extraction, analysis, identification and quantification of vitamin E from various sources such as solvent extraction (direct solvent, pressure liquid and supercritical fluid), chemical method (saponification, esterification), enzymatic method, microwave-assisted extraction, molecular distillation, membrane technology, column chromatography, thin layer chromatography (TLC), normal and reversed high performance liquid chromatography (HPLC) (Maarasyid et al., 2014).

Gas chromatography (GC) could also be used to analyze tocols, but that would need derivatization of the analytes and might include a risk for decomposition due to high temperature. Yet GC is still sometimes being used for tocol analysis (Eitenmiller, 2004). However, HPLC is the most widely used technique to analyze tocols because of its robustness. Both normal phase (NP) and reversed-phase (RP) chromatography are applied (Abidi, 2000; Bramley, 2000; Kamal-Eldlin, 2000; Ruperez, 2001; Lampi, 2016). For detection UV, fluorescence, ELSD, electrochemical (amperometric, coulometric) detection can be used. In addition, fluorescence detection (FLD) and ultraviolet detectors can combine with HPLC to detect tocols. Fluorescence detection is more sensitive and selective than UV (Sebinova, 1991; 1994).

Although HPLC has been proven to be suitable for the separation, determination, and isolation of α-tocopherol oxidation products, only a few methods allow the simultaneous separation of polar and nonpolar oxidation products, although both the normal phase and the reversed phase are used for separation as the stationary phase. The detection of α-tocopherol oxidation products is generally achieved by diode array detection (DAD) and mass spectroscopy (MS) because of the additional spectroscopic information provided by these methods, and detection. However, no

method has yet been developed for the simultaneous determination of α-tocotrienol oxidation products with other tocochromanols isomers (Büsing & Ternes, 2011)

2.3.2. β-Carotene

The world's richest source of the natural plant carotenoids is crude palm oil (CPO). The name carotenoid is derived from the fact that they are the major pigments in the carrot root, (*Daucus carota* L). The most important carotenoids are α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, zeaxanthin, violaxanthin, and neoxanthin. β-Carotene is the major carotenoid present in vegetable oils. The carotenoids in RPO are primarily β-carotene (48.2%) and α-carotene (38.9%), with smaller amounts of 11 other carotenoids including lycopene, phytoene, and phytofluene (Cassiday, 2017).

β-Carotene is one of the most adequate antioxidants because it stabilises edible oils very efficiently by reducing excited single oxygen back down to its less reactive triplet state (Yanishlieva, 2001). β-Carotene is thermally more stable in unsaturated triglycerides mixture such as triolein than in vegetable oil (Zeb, 2009). Prooxidant chlorophylls can interfere with this antioxidant protection. Another study said that β-carotene is degraded faster in methyl linoleate at higher temperature (Anguelova, 2000)

β–Carotene degrades during the oxidation of the oil. At high temperatures the oxidation rate of the oil is the same as β-carotene degradation. There are several steps of thermal degradation of the oils which are conversion into *Z*-isomers, inter-molecular cyclization and degradation. In the first step of oxidation of β-carotene, there is overall no colour change and the reaction in isomerization is reversible. The isomerization rate of β-carotene is higher in non-polar solvent such as petroleum ether and toluene than in polar solvents. Furthermore, volatile compounds are produced by intra-molecular cyclization. In the degradation step, aldehydes and ketones with low

molecular weight are produced. Some authors, proposed that degradation is a first-order kinetic model for the degradation (Zeb, 2012; Chen, 1994; Takahashi, 2003; Mercandante, 2010).

Some carotenoids, such as α– and β-carotene, have some pivotal function in humans. It can be converted by the body into retinol (provitamin A activity). β-Carotene has about twice the vitamin A activity of α-carotene (Nagendran, 2000). Carotenoids act as biological antioxidants, protecting cells, tissues from the damaging effects of free radicals and singlet oxygen (Paiva et al., 1999; Montenegro et al., 2002), anti-oxidative potential includes the enhancement of the immune system (Bendich, 1989). Some studies indicate that carotenes can inhibit the proliferation of certain types of cancer. The health benefits of carotenes are in preventing vitamin A deficiency associated with skin and eye disease. Some carotenoids can act as antioxidants by scavenging oxygen and peroxyl radicals.

Carotenoids are insoluble in water and soluble in organic solvents such as acetone, tetrahydrofuran (THF), n-hexane, pentane, petroleum ether, methanol, and ethanol. The same solvent system with different ratio was used to extract the β-carotene e.g. THF-methanol (1:1) was used to extract different carotenoids from wheat grain and pumpkin by Hentschel et al., and Murkovic et al. Ethanol and hexane have been used for analysis of carotenoids in fruits and vegetables, with good recovery and precision. The extraction method was found suitable for wide range of sample matrices in carotenoid analysis (Taungbodhitham, 1998).

2.3.2.1. Identification and quantification of β-carotene

Extraction of carotenoids must be performed very quickly and by avoiding light, oxygen, high temperature, and prooxidant metal ions (iron, copper) in order to minimize autooxidation and isomerization. THF and methanol 1:1 (v/v) was used to extract carotenoids from wheat grain and

pumpkin by Hentschel et al. and Murkovic et al. Ethanol and hexane have been used successfully for analysis of carotenoids in fruits and vegetables with good recovery and precision.

Different extraction procedures are used to isolate carotenoids, including simple solvent extraction (soxhlet), lipid phase distribution, solid phase extraction, and supercritical-fluid extraction. Various technologies have been developed in order to recover β-carotene from being destroyed during commercial refining of palm oil, such as solvent extraction, transesterification and molecular distillation, and adsorption.

Solvent extraction is a mass transfer operation in which palm oil is extracted with non-miscible or nearly immiscible solvents that exhibit preferential selectivity towards carotenoids and tocols in palm oil. The common solvent which was used to extract carotene or vitamin E from crude palm oil is hexane (Latip, 2000; Chiu, 2009).

Another extraction which offers a highly selective process is supercritical fluid extraction (SFE). Supercritical fluid extraction uses supercritical carbon dioxide (SC-CO_2) as solvent. The SC-CO_2 is an inert, non-hazardous, non-flammable, recycle solvent which can be removed from the product easily leaving no residues in the products (Watkins, 1994; Johnson and Lusas, 1983). The disadvantages of SFE is that it needs operating pressures that are typically much higher than ambient conditions, compression of solvent that requires elaborate recycling measures to reduce energy costs and also high capital investment for equipment.

Transesterification and molecular distillation: The solubility of a compound in a solvent depends on its molecular weight, polarity and solvent strength (Puah, 2007). CPO has large molecular weight of 807-885, with a lower solubility in the solvent. The carotenoids are transported together with triglycerides. The fatty acids methyl esters (FAME) are more soluble than triglycerides. The

transesterification process will convert the large molecules of triglycerides into smaller molecule of FAME, making it much easier to be separated (Puah, 2008).

A process involving neutralization and transesterification of palm oil, followed by molecular distillation of the esters are used to recover valuable minor components from crude palm oil. Molecular distillation represents a special type of vaporization at low pressure and low temperature in order to separate and purify molecules which have high molecular weight and are thermally sensitive such as vitamins. The disadvantage of transesterification is the conversion from CPO to methyl esters which are not edible. The quality of the oil is also reduced (Puah, 2005). Recently an extraction process for carotenoids in CPO using adsorption on synthetic adsorbents was developed. This process significantly reduces the time needed for extraction resulting in a high quality CPO (Othman, et al., 2010 ; Kupan, et al., 2016)

For adsorption commonly ethanol and isopropanol (IPA) are used to build an initial layer on the surface area of the adsorbent in order to allow a contact of the dissolved compounds with the liquid phase. Another solvent is n-hexane, which is preferable for eluting the carotene adsorbed on the hydrophobic surface of the adsorbent. The IPA has good solvency for CPO. However the adsorption ability for carotene in IPA is lower than in ethanol. On the other hand, ethanol has poor solvency for CPO but more carotene tends to be adsorbed on the column.

Solvent design-chemical product design. Solvent plays a major role to recover carotenoids and vitamin E from crude palm oil. Traditionally, some researches try some tests which select possible solvents through trial and error. Nowadays, chemical product design is becoming more popular for finding a suitable solvent that meets desirable target properties. Some benefits of chemical product design is to reduce the search time, to predict the property from a reverse problem, to identify the molecule or the molecular structure, to calculate the set of target properties (Moggridge and Cussler, 2000)

2.4. Stability of palm oil

Vegetable oils consist of triglycerides between 95-99%. It also contains soluble vitamins (A, D, E, and K), phytosterols, natural pigments, and phospholipids from 1 to 5% (Evrard et al., 2007). The fatty acids (FA) of triglycerides differ from each other by the length of their carbon chain and the number of double bonds (Cuvelier, 2012).

As we already know, lipid oxidation has a negative impact on the sensory, nutritional quality of food and economic causes (Matthaus, 2010). Not only the appearance such a change in colour and texture but also the unpleasant flavour which is produced during the heating. In the end, lipid oxidation can lead the formation of potentially toxic oxidation products such as oxycholesterol, malonaldehyde, endoperoxides, acrolein, and polymeric peroxides (Kim, 2008; Farhoosh, 2009)

Garby and co-workers showed that the oxidative stability measured in the Ranzimat at 110 °C varied from 4.5 h to 15.5 h. This showed that rapeseed oil was the least heat stable oil, on the other hand, palm olein was the most stable (15.5 h) followed by sunflower oil (5.5 h). The high stability of palm olein is a result of the high content of saturated fatty acids (nearly 40%). Canola oil and soya oil which have high amounts of unsaturated fatty acids are not suitable for frying. One of the usual ways for increasing the oxidative stability of these oils is blending with other oils that have less unsaturated compounds (Chu & Kung, 1998). Palm oil is one of the suitable oils for blending with other sensitive oils. It was not encountered with the smoking problems induced by small chain fatty acids. Palm oil is suitable oil for frying. It has a low iodine value and therefore fewer amounts of polyunsaturated fatty acids (Idris, 1992)

III. MATERIALS AND METHODS

3.1. Materials and reagents

2,4-Dinitrophenylhydrazine (2,4-DNPH) was purchased from Sigma-Aldrich (St. Louis, USA), hydrochloric acid (HCl 37 %) was purchased from Merck (Darmstadt, Germany), all solvents (e.g. methanol, ACN, acetone) used were of HPLC grade and were purchased from Merck (Darmstadt, Germany), acetic acid was purchased from Roth (Karlsruhe, Germany), β-carotene and α-tocopherol were from Sigma Aldrich (St. Louis, USA), unrefined palm oil was purchased from Asia Markt in Graz, Austria, and palm oil was purchased in a local market in Medan, Indonesia.

3.2. Analysis of secondary oxidation products in oil

3.2.1. Standardized oxidation of unrefinded palm oil (UPO) and refined palm oil (PO)

The oil samples were oxidized in a Ranzimat (679, Metrohm, Herisau, Switzerland). For this, 11 g of oil were used. The temperature was set to 120 °C and the air flow to 20 l/h. UPO was treated for up to 18 h and PO for up to 11 h. The oxidized samples were cooled immediately after the oxidation and stored under nitrogen at -18 °C.

Derivatization with 2,4-dinitrophenylhrazine (DNPH)

To 1 ml of the oxidized oil samples 4 ml of acetonitrile were added and mixed with 3 ml of the reagent 2,4-DNPH (3.48 mg/ml in 2 N HCl). The reaction mixture was kept at room temperature in the dark for 1 h. After completion of the reaction 2 ml ethyl acetate were added for extraction and 1 g KCl for better phase separation. This mixture was thoroughly shaken for 30 s and centrifuged for phase separation. The organic layer on the top was analysed without further treatment by HPLC.

Liquid chromatography-mass spectrometry condition for aldehydes identification

The analyses of the DNPH derivatives of the carbonyls formed during oxidation were done by an HPLC (Agilent 1100, Waldbronn, Germany) using a reversed phase column Kinetex, EVO C18, 150 × 3 mm and 2.6 µm particle size (Phenomenex, Aschaffenburg, Switzerland). For elution a gradient was used starting with a mixture of methanol (45 %), water (30 %), and acetonitrile (25 %) changing to methanol (6 %), water (4 %), and acetonitrile (90 %) linearly within 15 min. The absorption of the eluent was measured at 400 nm for the presence of the DNPH derivatives.

For mass selective detection a QTRAP 2000 (AB Sciex, Framingham, MA, USA) was used. Ionization was done using the APCI mode with a gas drying temperature of 250 °C, capillary voltage of 4000 V, and a fragmentor potential of 150 V.

3.2.2. Analyses of vitamin E and β-carotene

For the analyses of α-tocopherol and β-carotene 25 mg of the oil (UPO and PO) were extracted with 1 ml of methanol in 2 ml reactions vials (Eppendorf, Wien, Austria). The samples were shaken for 2 min vigorously and centrifuged. Under these conditions both, α-tocopherol and β-carotene were extracted quantitatively. The methanolic extract was used directly for HPLC analysis on a reversed phase column (Kinetex, EVO C18, 150 × 3 mm, 2.6 µm) using a flow of 0.6 ml/min. α-Tocopherol was separated isocratically using 5 % water in methanol as eluent and UV detection at 292 nm. β-Carotene was chromatographed with DAD HPLC with Kinetex EVO C18 2.6 µm; 100 x 3 mm (Phenomenex, Aschaffenburg, Switzerland) column using a flow of 1 ml/min of 100 % acetonitrile with detection at 450 nm.

3.2.3. Gas chromatography condition for fatty acids identification

25 mg of palm oil were put in a Pyrex glass tube and hydrolysed with 1.5 ml M NaOH in methanol. The samples were closed tightly and vortexed for 1 – 2 min. After that, the samples were heated at 100 °C for 1 – 2 min. The free fatty acids were methylated with 2 ml of BF_3 in methanol at 100 °C for 30 min. After cooling to temperature 30 – 40 °C Isooctan was added to the solution, and then FAMEs (fatty acid methyl esters) were extracted in presence of 5 ml of saturated NaCl. The isooctane layer was analysed for the fatty acids by GC (Shimadzu Q Plus 2010, Columbia, USA) using column DB-23; 30 x 0.25 mm, with initial temperature 90 ^{o}C to 208 ^{o}C.

IV. RESULTS AND DISCUSSION

4.1. Fatty acids in palm oil and unrefined palm oil

It is known from literature that palm oil contains 50 % saturated fatty acids (mostly palmitic acid (ca. 44 %) and lower amounts of stearic acid (5 %) 40 % of the fatty acids are monounsaturated fatty acids (mostly oleic acid) and 10 % polyunsaturated fatty acids (mostly linoleic acid) (Sambanthamurthi, 2000 ; Edem, 2001 ; Gee, 007).

Gas chromatographical analysis showed that the refined palm oil which was used for the experiments described here had a fatty acid composition which was comparable to the literature values. It contained 44.5 % oleic acid, 12.2 % linoleic acid, 37.3 % palmitic acid, and 3.81 % stearic acid. A chromatogram of the fatty acid analysis is shown in Figure 1. The unrefined palm oil had a different composition with oleic acid 41.9 %, linoleic acid 9.30 %, linolenic acid 0.21 %, palmitic acid 43.1%, and stearic acid 4.18%.

The presence of vitamin E (tocopherols and tocotrienols) and the small percentage of linoleic acid (ca. 12 %) increase the stability of palm oil and palm olein.

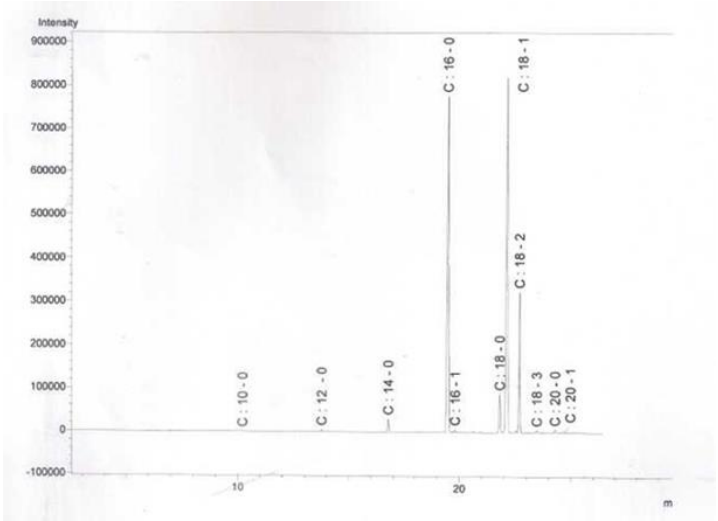

Figure 1. GC chromatogram of refined palm oil or determination of the fatty acid composition.

4.2. Secondary oxidation products in palm oil and unrefined palm oil

The oxidative and thermal stability of palm oil and unrefined palm oil was evaluated using Ranzimat 679. During oxidation formic acid is formed which is transferred with the air stream to the measurement vessel and detected by an increase of the conductivity. In previous experiments with triolein it was shown that DNPH reactive substances (carbonyls) were formed. A chromatogram of the DNPH derivatives is shown in Fig. 2. The oxidation of unrefined palm oil resulted in a very similar pattern of carbonyl in the triolein which were degradation products from oleic acid. When measuring the absorption of the eluent at 400 nm practically no other substances could be observed.

The dominant peaks that could be identified were hexanal, heptanal, octanal, nonanal, and decanal. These are degradation products of oleic acid which was show earlier. The result from previous experiment showed that the similar concentration range with maximum concentrations of 1.6 µmol/ml of hexanal, 2.3 µmol/ml of heptanal, 2.5 µmol/ml of octanal, 3.2 µmol/ml of nonanal, 4.0 µmol/ml of decanal after 6 hours. The total amount of carbonyls reached a maximum after 6 h being 27 µmol/ml for triolein without antioxidant. All these results referred to the calibration with hexanal. This is not surprising as palm oil is rich in oleic acid with ca. 45 % of the total fatty acids.

Carbonyl compounds (aldehydes and ketones) are the most abundant secondary oxidation products formed in edible oils such as, hexanal which is the main product from oleic acid, followed by octanal, nonanal, 2-nonanal and 2,4-decadienal. The heptadienal content resulting the oxidation of linolenic, heptanal is from linoleic acid and 2,4-nonadienal from arachidonic acid (Reindl, 1982).

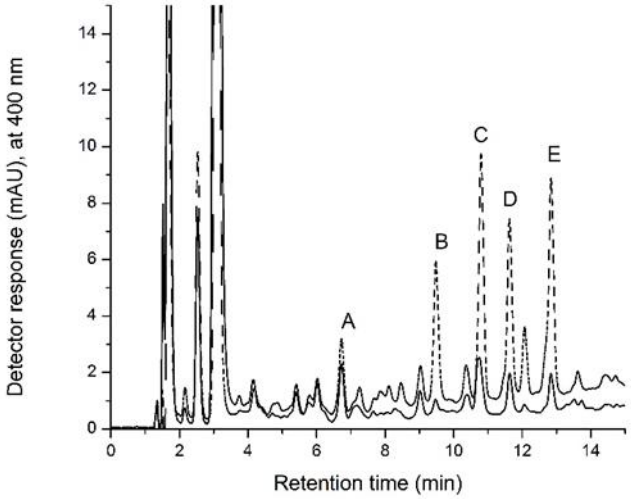

Figure 2. Carbonyls profile of triolein (------) and unrefined palm oil (——) (A: hexanal, B: heptanal, C: octanal, D: nonanal, E: decanal)

From the kinetic profile, the carbonyls compound still reach the highest amount until 18 h oxidation times with Ranzimat with 120 °C. For 18 h of oxidation, the carbonyls which were detected were hexanal 1.6 mmol/g, heptanal 0.61 mmol/g, octanal 1.36 mmol/g, nonanal 0.72 mmol/g and decanal 0.95 mmol/ g for UPO (Fig. 3). As we already know that the percentage of oleic acid in UPO was 41.9% , linoleic acid 9.30% and palmitic acid with 43.1%. This corresponds to the research undertaken by Reindl and co-workers (1982) focused on the oxidative degradation of unsaturated fatty acids which were isolated from meat. In his experiments he had shown that hexanal is the main product from oleic, linoleic and arachidonic acid.

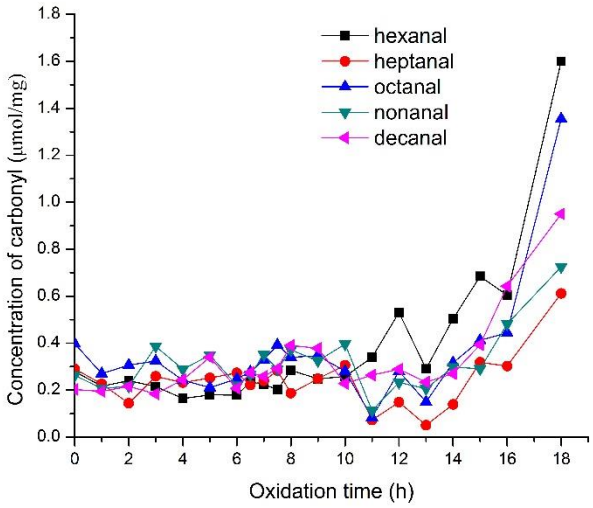

Figure 3. Formation kinetics of single compounds during oxidation of UPO in presence of lipid soluble antioxidants at 120 °C with constant air sparging for 18 h (hexanal; heptanal; octanal; nonanal; decanal).

On the other hand, oleic acid produced octanal, nonanal, 2-nonenal, and 2,4-decadienal. Thus, the main secondary oxidation product of oleic acids in the UPO was hexanal, octanal and decanal which has the higher percentage than others. The decanal is produced by homolytic cleavage B of the alkoxyl intermediate group from the 8-hydroperoxide. While octanal from the 11-hydroperoxide (Frankel, 2005). However, the percentage of linoleic acid just 9.3% so the production of heptanal was lower with 0.61 mmol/g. The other carbonyls which produced from linolenic acid, was 2,4-heptadienal from, and 2,4-nonadienal from arachidonic acid.

Autoxidation of 2,4-decadienal at ambient conditions has been shown to produce a mixture of volatiles including hexanal, butenal, heptenal, octenal, benzaldehyde, and glyoxal [21] while oxidation of 2-nonenal produced C2, C3, C7, C8 alkanals, glyoxal, and a mixture of C7, C8 and C9 α-keto aldehydes (Frankel, 1998)

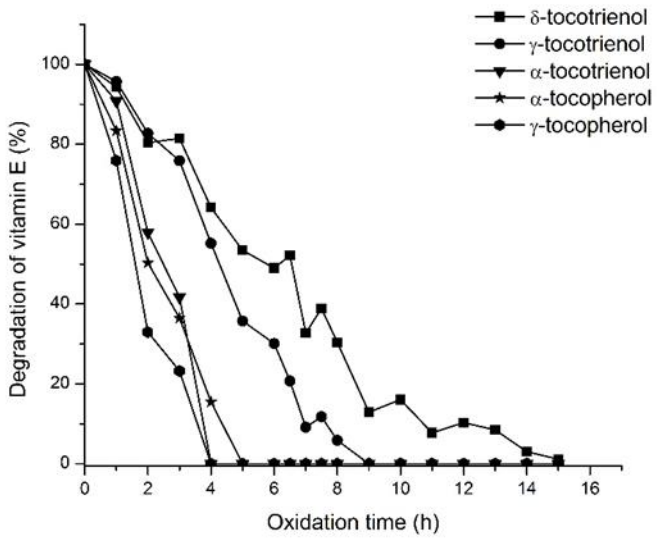

Figure 4. Degradation of vitamin E in unrefined palm oil

Crude Palm Oil (CPO) is intensively orange-red coloured and is semi-solid at room temperature, which can be separated into liquid (olein) and solid (stearin) fractions based on their melting point difference (Blekas and Boskou, 1999). CPO was separated into 82 % olein which was red liquid oil form, 39-45 % oleic acid and 10-13 % linoleic acid. However, the percentage of stearin is just 18 % w/v, which has a yellow solid fat form that contains 47-74 % of palmitic acid after centrifugation (Lin, 2011). In addition, vitamin E enriches in the palm olein with which is 30 % higher than in palm stearin. 85.7 % of total tocopherols were recovered in palm oil. (Abd and Gapor, 1990).

The results show that the tocotrienols in unrefined palm oil are more stable than the tocopherols. The δ-tocotrienol content was 3.01 mg/g in the fresh oil. It still remaining in the oxidized oil after 15 h in the Ranzimat with 0.03 mg/g δ-tocotrienol or 1.13 % compare to the fresh oil. It has been shown that half of the δ-tocotrienol was destroyed after 5 h of oxidation. The γ-tocotrienol with an original content of 11.48 mg/g was less stable. 50 % of it was degraded after 4 h of oxidation

with remaining 0.68 mg/g after 8 h (5.89 %) (Fig. 4). As we already know, tocotrienols have been found to have antioxidant and anti-cancer activities. γ and δ-tocotrienol derived from palm oil exhibit a strong activity not only against tumor promotion but also inhibit certain types of cancer, such as breast cancer cell (Imoisi, 2015).

In addition, the α-tocotrienol was completely degraded after 4 hours being 0.48 mg/g in the fresh oil. Furthermore, for α-tocopherol at 0 h with 39.96 mg/g was eliminated completely within 5 h. Both α-tocotrienol/tocopherol were degraded by 50 % after 2 h of oxidation. γ-Tocopherol just remained until 3h with 0.22mg/g compared to the fresh oil with 0.94 mg/g. At low concentration ≤ 100 µg/g for tocopherol, α–Tocopherol appeared to be the most effective antioxidant (Huang, et al., 1994; Kamal-Eldin, et al., 1996; Lampi, et al., 1999, Yanishlieva, et al., 2002; Evans, et al, 2002; Ohm, et al., 2005; Zuta, et al., 2007).

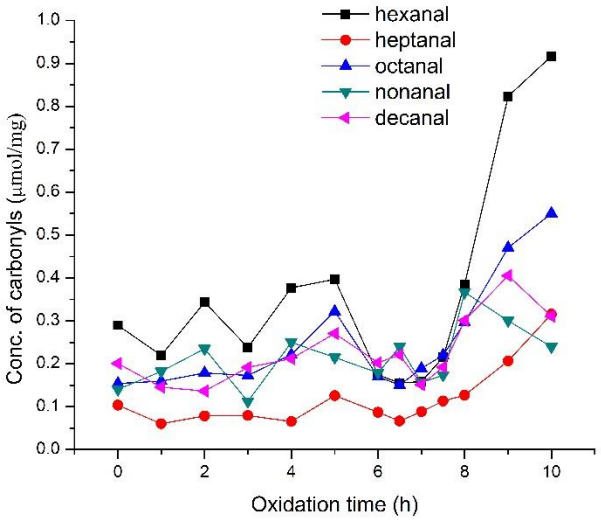

Figure 5. Formation kinetics of single compounds during oxidation of refined palm oil in presence of lipid soluble antioxidants at 120 °C with constant air sparging for 10 h (hexanal; heptanal; octanal; nonanal; decanal).

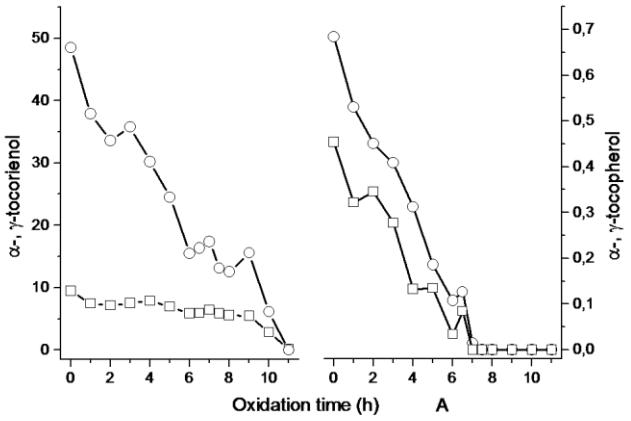

Figure 6. Degradation of vitamin E in refined palm oil

In addition, the kinetic profile of the carbonyls compound in refined palm oil until 11 h oxidation still want to reach the highest amount of secondary oxidation products with Rancimat at 120 °C (Fig. 5). For 11h oxidation, the carbonyls which detected were hexanal 0.92 mmol/g, heptanal 0.32 mmol/g, octanal 0.55 mmol/g, nonanal 0.41 mmol/g and decanal 0.31 mmol/ g for UPO (Fig.5).

This is not surprising as palm oil is rich in oleic acid with 45 % of the total fatty acids. Carbonyls can be produced by homolytic cleavage A and B on the either side of the alkoxyl intermediate group from 8-, 9- , 10-, and 11- from autoxidized oleate. The octanal is formed from the 11-hydroperoxide and the nonanal from either 9- or 10- hydroperoxide (Frankel, 2005). The other fatty acids in palm oil are linoleic acid 12.2% and palmitic acid 37.3%.

During the heating of palm oil, δ-tocotrienol and γ-tocotrienol stayed until 11 h oxidation with 0.5 mg/g and 0.2 mg/g, respectively. However, α-tocotrienol stayed until 10 h oxidation with 6.1 mg/g. α-Tocopherol and γ-tocopherol were present until 6.5 h with 0.1 mg/g.

The oxidation experiments of PO showed that δ-tocotrienol was 2.10 mg/g at 0 h to 0.49 mg/g at 11 h . On the other hand, γ-tocotrienol was present until 11 h with 0.18 mg/g from the starting point at 0 h with 9.45 mg/g while, α-tocotrienol was completely oxidized after 10 h of oxidation. The δ-tocotrienol, γ-tocotrienol and α-tocotrienol has a good stability against the oxidation of refined palm oil. The tocotrienols still remained in the oil until 10 h until 11 h oxidation in the Ranzimat. The δ-tocotrienol was stable throughout the experiment with an oxidation of 23 %. α-Tocopherol and γ-tocopherol were completely oxidized within 6.5 h (Fig. 6)

The HPLC-UV method was developed for determination of α- and γ-tocopherol in the palm oil. The validation showed that the method could be used for palm oil. The coefficient of correlation (0.999) of the linear regression indicates a good correlation between the peak area and the amount of α-tocopherol and γ-tocopherol, respectively. The limit of detection (LOD) was 0.14 μg/cm^3 and limit of quantification (LOQ) was 0.47 μg/cm^3 for γ-tocopherol. In addition for α-tocopherol, the limit of detection (LOD) was 460 μg/cm^3 and the limit of quantification (LOQ) was 1530 μg/cm^3.

The absence of carotenoids in refined palm oil is not only due to the various refining steps (bleaching). It could also be reduced because of thermal deterioration which is able to remove the β-carotene from crude palm oil (Cmolik, et al., 2000; Vogel, et al 1977; Franzke, et al., 1971; Rossi, et al., 2001; Su, et al., 2002; Moreau, et al., 2007)

4.3. β-Carotene in unrefined palm oil

The carotenoids α-carotene, β-carotene, and lycopene contribute to the color of red palm oil. Red palm oil is a form of processed palm oil in which ca. 80% of the original carotenoids are retained. This means that it is a remarkable source of vitamin A (Sutapa & Analava, 2009). The quantitative

analysis showed that β-carotene is completely oxidized in the oil decreased with a natural content of 2.34 mg/g within 8 h of the Ranzimat treatment (Fig.7).

β-carotene plays an important role during the oxidation of oils and fats. Some studies have been done to the stability of β-carotene. Henry and co-workers (1998) studied the thermal and oxidative stability of all-E-β-carotene, 9-Z-β-carotene, lycopene, and lutein in safflower seed oil at 75, 85 and 95 °C for 24, 12 and 5 h, respectively. The carotenoids were found to degrade following a first-order kinetic model. The rates of degradation were lycopene > all-E-β-carotene ≈ 9-Z-β-carotene > lutein. Other studies showed that carotenoids were degraded with a first-order kinetics model, which was dependent on concentration. The highest degradation rates were observed in extracts prepared with linoleic acid containing oils such as sunflower, soybean oil and grape-seed oil (Bezbradica, et al., 2005)

β-Carotene was found to degrade in the first few hours of thermal treatment (Zeb & Murkovic, 2011b). This statement can be strongly supported from the experiment which has been done. The β-carotene is completely oxidized after 8 h oxidation of UPO. The concentration of β-carotene at 0 h oxidation was 2.34 mg/g. However, the formation of secondary oxidation products lasts until 18 h of heating.

The previous experiments of Zeb & Murkovic, 2011, showed that β-carotene was more stable in the corn oil than in the olive and model TAGs samples. The β-carotene degraded completely before 12 h in the corn oil of the thermal treatment in the Ranzimat at 110 °C. However, β-carotene can act as prooxidant and has a lower stability compared to astaxanthin during thermal degradation in olive oil (Zeb & Murkovic, 2011b).

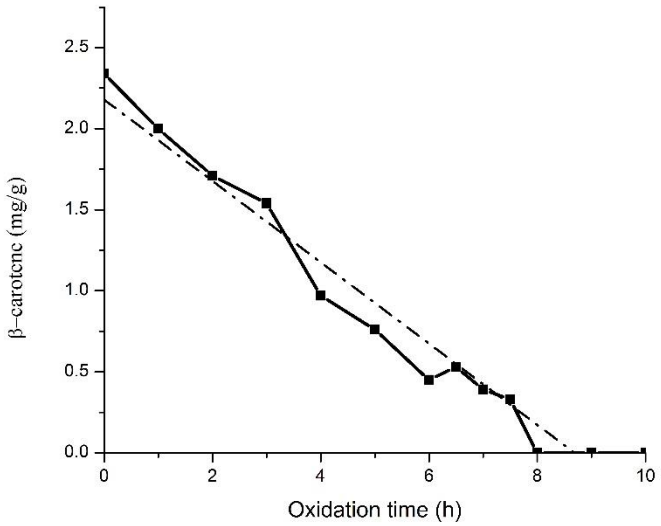

Figure 7. Degradation of β-carotene in unrefined palm oil.

The HPLC-DAD method developed for the β-carotene in the unrefined palm oil was validated. The coefficient of determination (0.999) of the linear regression indicates a good correlation between the peak area and the amount of β-carotene. The linearity of the β-carotene analysis was tested in the range 0.00078-0.05 μg/cm^3 according to Mandel. The limit of detection (LOD) was 2.65 μg/ cm^3 and the limit of quantification (LOQ) was 8.83 μg/cm^3 for β-carotene.

V. CONCLUSION

The kinetic profile of the unrefined palm oil shown that the carbonyls compound still reach the highest amount until 18 h oxidation times with Ranzimat with 120 °C (Fig. 3). For 18 h of oxidation, the carbonyls which were detected were hexanal 1.6 mmol/g, heptanal 0.61 mmol/g, octanal 1.36 mmol/g, nonanal 0.72 mmol/g and decanal 0.95 mmol/ g for UPO. As we already

know that the percentage of oleic acid in UPO was 41.9% , linoleic acid 9.30% and palmitic acid with 43.1%.

However, the kinetic profile of the carbonyls compound in palm oil until 11 h oxidation still want to reach the highest amount of secondary oxidation products. For 11h oxidation, the carbonyls which detected were hexanal 0.92 mmol/g, heptanal 0.32 mmol/g, octanal 0.55 mmol/g, nonanal 0.41 mmol/g and decanal 0.31 mmol/ g for UPO. This is not surprising as palm oil is rich in oleic acid with 45 % of the total fatty acids

The analytical method for quantifying the vitamin E and β−carotene are quick, reliable, precise, economical and suitable for the routine analysis. For both analyses a simple dilution of the oil was necessary.

Looking at the kinetic profile of the carbonyl formation in unrefined palm oil and refined palm oil, both of the oils were still stable until 10 h of oxidation with the Ranzimat at 120 °C. The quantitative analysis showed that β-carotene in the oxidized unrefined palm oil decreased from 2.34 mg/g to 0.33mg/g within 7.5 h of oxidation. However, after 7.5 h, no more β-carotene was detected. In addition, β-carotene was not detected in refined palm oil.

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Appendix

Table 1. Concentration of aldehydes in unrefined palm oil (μmol/mg)

Oxidation time (h)	hexanal	heptanal	octanal	nonanal	decanal
0	0.28	0.29	0.40	0.26	0.20
1	0.22	0.23	0.27	0.21	0.19
2	0.24	0.14	0.31	0.22	0.22
3	0.22	0.26	0.32	0.39	0.18
4	0.16	0.23	0.24	0.29	0.24
5	0.18	0.25	0.21	0.35	0.34
6	0.18	0.27	0.25	0.22	0.21
6.5	0.23	0.22	0.28	0.27	0.28
7	0.22	0.24	0.33	0.35	0.26
7.5	0.20	0.28	0.39	0.29	0.29
8	0.28	0.19	0.34	0.37	0.39
9	0.25	0.25	0.35	0.32	0.38
10	0.26	0.31	0.28	0.40	0.23
11	0.34	0.07	0.08	0.11	0.26
12	0.53	0.15	0.28	0.23	0.29
13	0.29	0.05	0.15	0.21	0.23
14	0.50	0.14	0.32	0.30	0.27
15	0.68	0.32	0.41	0.29	0.39
16	0.60	0.30	0.44	0.48	0.64
18	1.60	0.61	1.36	0.72	0.95

Table 2. Concentration of aldehydes in refined palm oil (μmol/mg)

Oxidation time (h)	hexanal	heptanal	octanal	nonanal	decanal
0	0.29	0.10	0.15	0.14	0.20
1	0.22	0.06	0.16	0.18	0.15
2	0.34	0.08	0.18	0.24	0.14
3	0.24	0.08	0.17	0.11	0.19
4	0.38	0.07	0.22	0.25	0.21
5	0.40	0.13	0.32	0.21	0.27
6	0.17	0.09	0.17	0.18	0.20
6.5	0.16	0.07	0.15	0.24	0.22
7	0.16	0.09	0.19	0.16	0.15
7.5	0.21	0.11	0.22	0.17	0.19
8	0.39	0.13	0.30	0.37	0.30
9	0.82	0.21	0.47	0.30	0.24
10	0.92	0.32	0.55	0.41	0.31

Table 3. Concentration of β-carotene in unrefined palm oil

Oxidation time (h)	β-carotene (μmol/mg)
0	0.0023
1	0.0020
2	0.0017
3	0.0015
4	0.0010
5	0.0008
6	0.0004
6.5	0.0005
7	0.0004
7.5	0.0003
8	0
9	0
10	0

Table 4. Concentration of vitamin E in unrefined palm oil

Oxidation time (h)	Vitamin E in unrefined palm oil				
	δ-tocotrienol	γ-tocotrienol	α-tocotrienol	α-tocopherol	γ-tocopherol
0	0.0030	0.0115	0.0005	0.0400	0.0009
1	0.0028	0.0110	0.0004	0.0333	0.0007
2	0.0024	0.0095	0.0003	0.0201	0.0003
3	0.0024	0.0087	0.0002	0.0146	0.0002
4	0.0019	0.0063	0	0.0062	0.0000
5	0.0016	0.0041	0	0	0
6	0.0015	0.0035	0	0	0
6.5	0.0016	0.0024	0	0	0
7	0.0010	0.0011	0	0	0
7.5	0.0012	0.0014	0	0	0
8	0.0009	0.0007	0	0	0
9	0.0004	0	0	0	0
10	0.0005	0	0	0	0
11	0.0002	0	0	0	0
12	0.0003	0	0	0	0
13	0.0003	0	0	0	0
14	0.0001	0	0	0	0
15	0	0	0	0	0
16	0	0	0	0	0
18	0	0	0	0	0

Table 5. Concentration of vitamin E in refined palm oil

Oxidation time (h)	δ-tocotrienol	γ- tocotrienol	α- tocotrienol	α-tocopherol	γ-tocopherol
0	0.002	0.008	0.040	0.0006	0.00032
1	0.001	0.006	0.027	0.0004	0.00021
2	0.001	0.006	0.023	0.0004	0.00023
3	0.002	0.007	0.033	0.0004	0.00022
4	0.002	0.007	0.025	0.0003	0.00011
5	0.002	0.007	0.020	0.0001	0.00007
6	0.002	0.006	0.011	0.0001	0.00007
6.5	0.002	0.006	0.012	0.0001	0.00006
7	0.002	0.006	0.019	0	0
7.5	0.002	0.006	0.012	0	0
8	0.002	0.005	0.012	0	0
9	0.002	0.007	0.014	0	0
10	0.002	0.003	0.006	0	0
11	0	0	0	0	0

SECTION 4

COMPARING THE SECONDARY OXIDATION PRODUCTS OF THE RAPESEED OIL WITH THE SUNFLOWER OIL

I. INTRODUCTION

Lipid oxidation in vegetables oil is related to unsaturated and saturated fatty acids in the oil. The increasing degree of unsaturated fatty acid is increases the possibility of oxidation. The composition of oleic, linoleic, and linolenic acids in oil has an effect on the oxidative stability too. (Min & Boff, 2011; Nawar, 1996).

The mechanism of the oxidation process of edible oils largely depends on the oxidative conditions. Temperature is the most important factor to be considered in evaluating the oxidative stability of fats, especially in unsaturated fatty acids. The oxidative process ran slow in the low temperature or room temperature. It could be different if the oxidation produced at higher temperature (Guillén et al., 2005; Guillén & Goicoechea, 2007). The rate of oxidation is exponentially related to temperature, the stability of a food lipid decreases logarithmically with increasing temperature. (Frankel, 1998).

Carbonyls content of rapeseed and sunflower oil was derivatised with DNPH (2,4-dinitrophenylhydrazine) which is often used to qualitatively test for carbonyl groups. Carbonyl compounds, in particular aldehydes, are reactive volatile substances. 2,4-Dinitrophenylhydrazine can be used to qualitatively detect a complex mixture of various and ketones simultaneously. In most cases, the derivatives are separated by reverse phase liquid chromatography and detected its absorption or mass spectrometry (Osório, 2013; Pötter & Karst, 1996; Grosjean et al., 1999).

The level of rancidity in the oil can determined by measuring the colour intensity of hydrazones as a product reaction. The test for positive signal of different colour can be differentiated with a red precipitate meaning aromatic and yellow colour means aliphatic carbonyls. DNPH reacts with the carbonyl group to form stable hydrazones (DNPH-carbonyls) with a lower vapour pressure in acidic media. The measurements of the λ_{max} of DNPH derivatives of saturated aldehydes and ketones is optimal between 356 and 365 nm (Brande & Jones, 1945).

The hydrazone derivatives can also be used as evidence of the original compound. The level of oxidation and the amount of those aldehydes and ketones increases with the increasing oxidation time. The controlled oxidation of rapeseed and sunflower oil by a Ranzimat 679 leads to the formation of aldehydes and ketones which can be investigated by RP- HPLC at 400 nm. As we already know that sunflower oil has approximately 70% linolenic acid (Meydani et al., 1991) and rapeseed oil contains oleic 61%. (Bocianowski et al., 2012) The opposite amount of unsaturated fatty acids (focusing on oleic acid and linoleic acid) between sunflower oil and rapeseed oil can produce a different profile of secondary oxidation products. The formation of aldehydes as secondary product oxidation reacted with 2,4-DNPH and then to separate the derivatives of the target analyte.

II. LITERATURE REVIEW

2.1. Rapeseed Oil

Rapeseed (*Brassica napus*), also known as rape, oilseed rape, oilseed rape, rapa, rappi, rapaseed (one particular cultivar). Rapeseed oil became the second most produced oilseed behind soybeans in the early 2000's. The major producers are China, India, Canada and European Union (27). World exports of rapeseeds are dominated by Canada. However, Japan is a traditional importer, while China and the EU (27) are less regular buyers (Carré & Pouzet, 2014).

Rapeseed oil is considered to be nutritionally well-balanced because it has a low content of saturated fatty acids (SFA), a high content of monounsaturated fatty acids (MUFA), and a 2 : 1 ratio of ω-6 and ω-3 PUFA. The main component of oleic acid is rapeseed oil (± 63%) and olive oil (±66.4%). Natural rapeseed oil contains erucic acid, which is mildly toxic to humans in large doses. Erucic acid which is present in rapeseed oil and mustard oil is categorized as ω–9. Besides that, the rapeseed oil has a high content of α-linolenic acid (8-12%) compared to other vegetables oils such as soybean (8%), sunflower (0.2%), olive (0.8%) and corn (0.7%). From information stated above, the high polyunsaturated fats are easily oxidised on heating (Farhoosh & Samaneh, 2009)

For studying the oxidative stability of rapeseed oil it is needed to measure the content of oxidation products. This reaction leads to the formation of intermediate compounds i.e. hydroperoxides as primary oxidation products. Because of unstable, these products will be change fastly to the secondary product oxidation including carbonyl compound (Farhoosh & Samaneh, 2009). According to Woyewoda et al. (1986) carbonyl compounds are more stable than peroxides which are normally used for evaluating the quality of oil.

The content of primary (PO) and secondary (SO) oxidation products of rapeseed oil was measured. The peroxide values showed the highest contribution (53.69%) to the PO, whereas the SO was much less significant (0.36%). However, the acid value contribution to the production of hydroperoxides and carbonyls was 12.8% and 29.8%, respectively. Polyene index showed a relatively low contribution to the PO (6.71%) but contributed highly to the SO (21.8%). Carbonyl compounds are more stable than hydroperoxides and their measurement is a good index for oxidative changes of lipids (Farhoos & Samaneh, 2009).

2.2. Sunflower oil

The top sunflower producers in the world are Ukraine and Russia. They produce almost half of the world's sunflower seeds. Ukraine produces 14 million tons of sunflower seeds accounting for 26% of the world's production while Russia produced 11 million ton in 2016/2017 accounting for 24% of the world's production.

Sunflower oil is polyunsaturated oil with low saturated fat levels. It is predominantly (65%) polyunsaturated and low saturated fatty acid. Thus the oil is clean, light taste and high in vitamin E. The type of polyunsaturated fat it contains is linoleic acid (omega-6 acid) and α-linolenic acid (ALA), an omega-3-fatty acid, are considered essential fatty acids (EFA) because they cannot be synthesized by humans. Our bodies need this essential nutrient but cannot make it. It must be supplied by food sources (Gharby et al., 2014)

Because of the high levels of polyunsaturated fats in linoleic sunflower oil, the oil is susceptible to oxidation during commercial usage, especially frying. Like other highly polyunsaturated oils, such as soybean and canola, hydrogenation is needed to stabilise them.

Some of the health benefits of sunflower oil include its ability to improve heart health, boost energy, strengthen the immune system, improve your skin health, prevent cancer, lower cholesterol, protect against asthma, and reduce inflammation (Aftab oil, 2016)

Aldehydes and ketones which was produced from frying fats are reacted with 2,4-dinitrophenylhydrazine in acidic solution for 1 h at room temperature. Using a reversed phase HPLC column that is eluted with a very steep gradient between methanol and tert-butylmethyl ether (TBME) with wavelength set to 370 nm. This method resulted as a milliequivalents of carbonyls per kg of fat which was produced from oxidative cleavage of double bonds in unsaturated fatty acids (Schule, 2002). A wide variety of chemical reactions result in the formation of compounds with high molecular weight in the formation of compounds with high molecular weight and polarity.

2.3. Fatty acid content in rapeseed and sunflower oil

Edible oils and fats are composed primarily of triglycerides, which are the ester of one molecule of glycerol and three molecules of fatty acids. They commonly contain 18 carbons such as stearic acid, oleic acid, linoleic acid, and linolenic acid (Table 1). Unsaturated fatty acids such as oleic acid, (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) are the main target to be oxidized (Franket al., 1982; Lomanno and Nawar, 1982). The linoleic acid has been found to be 10-100 times easier to be oxidise than monoene or saturated fatty acids (Selke et al., 1980).

Fatty acids contain a carboxyl group and a hydrocarbon chain. The hydrocarbon chain can be differentiated by varying the length from 4 to 24 carbon atoms and it can be saturated, monosaturated and polyunsaturated. Fatty acids oxidize at a slightly greater rate when free compared to the glycerol ester (Fennema, 1996). Their formation is responsible for undesirable

flavours and aromas, especially in oils which have large quantities of low molecular weight fatty acids.

Rapeseed oil analyses show that the triglyceride content is between 91.8% and 99% of the total lipid (Mag, 1990). Triglycerides in rapeseed oil contain mainly oleic acid (60%), linoleic acid (20%), and linolenic acid (8%) acids with lower concentrations of some other fatty acids, such as palmitic acid (4%), gadoleic acid (2-3%) and stearic acid (1-2%) acids (Holčapek, 1999).

Jáky and Kurnik (1981) investigated the concentration of linoleic acid in the 1,3- and 2-position. They found that in high erucic acid rapeseed oil (HEAR) at least 95% of linoleic acid was concentrated in the sn-2 position whereas in canola oil the linolenic acid was placed by the plant's enzymatic system into sn-1,3 position to replace eruic acid. Ohlson et al., (1975) indicated that linoleic acid replaced erucic acid in the sn-1 position, and while only present in canola oil at low levels, gadoleic and erucic acids were preferentially esterified in the sn-3 position. The investigators also found that linolenic acid was similarly distributed as linoleic acid.

The oxidative rates of oleic acid, linoleic acid, and linolenic acid are 1:12:25 (Min & Boff, 2001). Thus, the rapeseed oil which has high oleic acid content should have better oxidative stability than the sunflower oil. However, the minor substances in the oil can support the oxidative stability such as natural antioxidants, phospholipids, free fatty acids, mono and di-glycerides, polymers and the number of double bonds in the oils (Smith, et al., 2007)

Table 1. Fatty acid composition of vegetable oils

FAs [%]	SAF	GRP	SIL	HMP	SFL	WHG	PMS	SES	RB	ALM	RPS	PNT	OL	COC
C6:0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.52
C8:0	nd	0.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	7.6
C10:0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.01	nd	nd	5.5
C12:0	nd	0.01	0.01	nd	0.02	0.07	nd	nd	nd	0.09	nd	nd	nd	47.7
C14:0	0.10	0.05	0.09	0.07	0.09	nd	0.17	nd	0.39	0.07	nd	0.04	nd	19.9
C15:0	nd	0.01	0.02	nd	nd	0.04	nd	nd	nd	nd	0.02	nd	nd	nd
C16:0	6.7	6.6	7.9	6.4	6.2	17.4	13.1	9.7	20.0	6.8	4.6	7.5	16.5	nd
C17:0	0.04	0.06	0.06	0.05	0.02	0.03	0.13	nd	nd	0.05	0.04	0.07	nd	nd
C18:0	2.4	3.5	4.5	2.6	2.8	0.7	5.7	6.5	2.1	2.3	1.7	2.1	2.3	2.7
C20:0	nd	0.16	2.6	nd	0.21	nd	0.47	0.63	nd	0.09	nd	1.01	0.43	nd
C22:0	nd	nd	nd	nd	nd	nd	nd	0.14	nd	nd	nd	nd	0.15	nd
C16:1 (n-7)	0.08	0.08	0.05	0.11	0.12	0.21	0.12	0.11	0.19	0.53	0.21	0.07	1.8	nd
C17:1 (n-7)	nd	nd	0.03	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
C18:1cis (n-9)	11.5	14.3	20.4	11.5	28.0	12.7	24.9	41.5	42.7	67.2	63.3	71.1	66.4	6.2
C18:1trans (n-9)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.14	nd	nd	nd
C20:1(n-9)	nd	0.40	0.15	16.5	0.18	7.91	1.08	0.32	1.11	0.16	9.1	nd	0.30	nd
C18:2cis (n-6)	79.0	74.7	63.3	59.4	62.2	59.7	54.2	40.9	33.1	22.8	19.6	18.2	16.4	1.6
C18:3 (n-3)	0.15	0.15	0.88	0.36	0.16	1.2	0.12	0.21	0.45	nd	1.2	nd	1.6	nd
C18:3 (n-6)	nd	nd	nd	3.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
SFAs	9.3	10.4	15.1	9.2	9.4	18.2	19.6	16.9	22.5	9.3	6.3	10.7	19.4	92.1
MUFAs	11.6	14.8	20.7	28.1	28.3	20.9	26.1	42.0	44.0	67.9	72.8	71.1	68.2	6.2
PUFAs	79.1	74.9	64.2	62.8	62.4	61.0	54.3	41.2	33.6	22.8	20.9	18.2	18.0	1.6
n-3 PUFAs	0.2	0.2	0.9	0.4	0.2	1.2	0.1	0.2	0.5	0.0	1.2	0.0	1.6	0.0
n-6 PUFAs	79.0	74.7	63.3	62.4	62.2	59.7	54.2	40.9	33.1	22.8	19.6	18.2	16.4	1.6

Data are expressed as percentages of total fatty acid methyl esters (FAMEs); nd means that the fatty acids were not determined. Abbreviations of the samples mean: SAF-safflower, GRP-grape, SIL-Silybum marianum, HMP-hemp, SFL-sunflower, WHG-wheat germ, PMS-pumpkin seed, RB-rice bran, ALM-almond, RPS-rapeseed, PNT-peanut, OL-olive, and COC-coconut oils (Orsavova, et al., 2015)

2.4. Vitamin E contain in rapeseed and sunflower oil

The tocopherols are one of the main groups regarding the portion of the minor compounds in vegetable oils. Oils originated from the same species can have variable content and composition

of these minor compounds, due to climatic and agronomic conditions, fruit quality or seed origin, oil extraction system and refining procedure (Cert et al., 2000).

The richest in α-tocopherol are the sunflower and corn oils. This information regarding the sunflower oil is in accordance with the statement that this compound is found in higher concentrations in wheat germ oil, olive oil, and sunflower oil (Traber, 2006; Gliszczyńska-Świgło et al., 2007; Smolarek & Suh, 2011; Velasco et al., 2010).

2.5. Edible oil oxidation

Lipid oxidation is a series of chemical reaction which can reduce the quality of oil because of undesirable off flavours. By mechanism, lipid oxidation can be divided into auto-oxidation, photo-oxidation, and enzymatic oxidation. The external factors that influence lipids oxidation such as light, heat, pigments, enzymes, metals ions, metallproteins, microorganism, etc (Choe & Min, 2006; Frankel, 2005)

There are three steps of lipid oxidation related to radical reactions which are initiation, propagation, and termination (Judde, 2004). In the initiation step, the unsaturated fatty acid reacts with an initiator to produce a radical. During the second step, the propagation, the radical reacts with other fatty acids to propagate as a chain reaction. Followed by the last step, termination, the radicals react to non-radical compounds (Schneider et al., 2008).

The primary oxidation products, lipid hydroperoxides, are relatively stable at room temperature in the absence of metals. The primary oxidation products formed in the first stage (form smaller volatile compounds such as aldehydes, ketones, alcohols etc.) or polymerise to dimers or oligomers). However, in the presence of metals or at high temperature they can be further decompose to alkoxy radicals and then form aldehydes, ketones, acids, esters, alcohols, and short chain hydrocarbons. The most likely pathway of hydroperoxide decomposition is a homolytic

cleavage between oxygen and the oxygen bond, in which alkoxy and hydroxy radicals are produced (Choe & Min, 2006; Frankel, 2005).

The time for secondary product formation from the primary oxidation products, hydroperoxides, varies with different oils. Secondary oxidation products are formed immediately after hydroperoxide formation in olive and rapeseed oils. However, in sunflower and safflower oils, secondary oxidation products are formed when the concentration of hydroperoxides is appreciable (Guillen and Cabo, 2002).

The decomposition of lipid hydroperoxides is always accompanied by the formation of a great variety of aldehydes. Aldehydes can react with 2,4-dinitrophenylhydrazine to produce hydrazones. The hydrazine derivatives are stable and not volatile. Moreover, the DNPHs have an intense yellow colour (molar absorption 25000-28000, λ_{max} = 360-380 nm) which facilitates their detection on TLC plates by HPLC (Esterbauer, 1989). DNPH is poorly soluble in water and most organic solvents (Schulte, 2002).

2.6. Secondary oxidation products and health effects

Lipid oxidation has a negative effect to the sensory and nutritional quality and leads to economic losses. If it still ran during process and storage, for the final production as a major products of the degradation and due to their capacity to induce toxicological effects it can produce some toxic oxidation products such as oxycholesterol ester, malonaldehyde, endoperoxides, acrolein, and polymeric peroxides. Aldehydes like acrolein are considered to have high relevance (Matthäus, et al., 2010; Kim, 2008; Farhoosh, 2009; Umano & Shibamoto, 1987; Yen & Wu, 2003; Zu et al., 2001).

Table 2. Secondary oxidation products of fatty acid methyl ester by autoxidation

Class	Oleic acid	Linoleic acid	Linolenic acid
Aldehydes	Octanal Nonanal 2-Decenal Decanal	Pentanal Hexanal 2-Octenal 2-Nonenal 2,4-Decadienal	Propanal Butanal 2-Butenal 2-Pentenal 2-Hexenal 3,6-Nonadienal Decatrienal
Carboxylic acid	Methyl heptanoate Methyl octanoate Methyl 8-oxooctanoate Methyl 9-oxononanoate Methyl 10-oxodecanoate Methyl 10-oxo-8-decenoate Methyl 11-oxo-9-undecenoate	Methyl heptanoate Methyl octanoate Methyl 8-oxooctanoate Methyl 9-oxononanoate Methyl 10-oxodecanoate	Methyl heptanoate Methyl octanoate Methyl nonanoate Methyl 9-oxononanoate Methyl 10-oxodecanoate
Alcohol	1-Heptanol	1-Pentanol 1-Octene-3ol	
Hydrocarbons	Heptane Octane	Pentane	Ethane Pentane

Source : Frankel, 1985

Unsaturated aldehydes, such as alkenal and alkadienals, are more toxic than alkanals (Meacher & Menzel, 1999; Tovar & Kaneda, 1977). In addition, aldehydes with high molecular weight such as chloroacetaldehyde, valeraldehyde, furfural, butyraldehyde, glyoxal, malonaldehyde, benzaldehyde, and synapaldehyde are less toxic than acrolein and acetaldehyde which have low molecular weight (Silva, 2008).

The most abundant secondary oxidation products which is produced in edible oil are carbonyls compounds (aldehydes and ketones) (Evrard et al., 2007). Aldehydes can be grouped as follows:

1. Short chain (unhindered aldehydes): formaldehyde, acetaldehyde

2. Long chain aldehydes: octanal, nonanal, decanal

3. Aromatic aldehydes: benzaldehyde, vanillin

4. α,β,– unsaturated aldehydes: aromatic alkenals, short and long chain alkenals, and hydroxyl or oxoalkenals

5. α-oxoaldehydes, glyoxal, and glycolaldehyde (O'brien, 2005; Feron, 1991).

Oxidized oils are absorbed in the intestine, transported in chylomicrones to the liver, and may affect unaltered hepatic cells as well as the process of hepatocarcinogenesis. Lipid hydroperoxides of dietary origin may be an important driving force for carcinogenesis in the liver (Rohr-Udilova et al., 2008). Consumption of lipid peroxides from unhealthy food may favour the progression from fatty liver to NASH (non-alcoholic fatty liver disease). Ingestion of peroxidized fatty acids carries a considerable pro-imflammatory stimulus into the body which reaches the liver and may trigger the development of hepatic inflammation (Böhmet al., 2013).

Some aldehydes are produced during heating in the frying temperature at 190 °C in the headspace of virgin olive, sunflower and virgin linseed oils like 4-oxo-(*E*)-2-decenal, 4-oxo-(E)-2-undecenal, not only in frying oil but also in foods. Other aldehydes are also produced such as 4-hydroxy-(*E*)-2-nonenal, (E)-4,5-epoxy-(*E*)-2decenal, 4-hydroxy-(*E*)-2-hexenal, and 4-oxo-(*E*)-2-nonenal (Guillen, 2012).

Researchers from the University of the Basque Country (UPV/EHU, Spain) have found some toxic aldehydes in heated at a frying temperature such as 4-hydroxy-(*E*)-2-nonenal, 4-oxo-(*E*)-2-decenal and 4-oxo-(*E*)-2-undecenal which can lead to some neurodegenerative diseases and some types of cancer. They stated that in sunflower oil the most toxic aldehydes are produced due to the high content of polyunsaturated fatty acids (linoleic acid and linolenic acid) (Science Daily, 2012).

Others studies stated that unsaturated carbonyl compounds are known to be formed by lipid peroxidation in many foods (e.g., milk, butter, and vegetable oils). These compounds are directly

associated with various diseases, including cancer, mutagenesis, Alzheimer's, aging, arthritis, inflammation, diabetes, atherosclerosis and AIDS (Shibamoto, 2006)

With increasing degree of unsaturation the number of oxidation products increases. For example, in rapeseed oil, the secondary oxidation products which have lower molecular weight oxygenated constituents including carbonyls (e.g. hexanal, *Z*-2-octenal, *E*-2-nonenal, 2,4-nonadienal, 2,4-decadienal, *Z*-2-octenal, 3-octene-2-one, *E*-2-octenal) are mainly responsible for the rancid aroma (Farhoosh, 2009).

The most reactive and toxic end products formed in situ during lipid peroxidation are 4-hydroxyalkenals. These can inhibit DNA, RNA and protein synthesis, block proliferation of various cell lines. These are acutely cytotoxic towards most cells and show various genotoxic effects at concentrations above 100 μM.

2.7. Major fragments (m/z) of MS^2 spectrum formed by fragmentation of the precursor $[M-H]^-$ ions of hydrazones.

Brombacher and co-workers published their work on carbonyl-hydrazone fragmentation. The main product ions formed are *m/z* 120, 122, 152, and 179 in the DNPH-part when using negative ionization (Brombacher et al., 2001). Aldehyde and ketone hydrazones can be differentiated by their different fragmentation pathways. Other DNPH-product ions are *m/z* 163, 167, and 182 were studied by Kölliker in 2001. Ketones showed low or no formation of the fragment of *m/z* 163 and 152, as was shown in the MS/MS spectra of acetone and cyclohexanone (Fig. 1; Fig. 2). Ketones also showed a highly abundant fragment of $[M-H-30]^-$, as shown in the spectra of acetone (*m/z* 207) and cyclohexanone (*m/z* 247). This ion was also found in the mass spectra of aldehydes, but with a relative low abundance. Typical fragments of α,β−unsaturated aldehydes corresponded to ions of $[M-H-31]^-$ and $[M-H-17]^-$, this is the result of a neutral loss of NH_3 (Kolliner et al., 1998).

The relative intensity of *m/z* 163 is more than 50% for aromatic aldehydes. The aldehyde DNPH adducts also show an intense *m/z* 179 ion. Further, straight chain aldehydes and ketones are characterized by the presence of a fragment ion of 191, which contributes to nearly 10% of the base peak (Kolliker & Oehme, 1998).

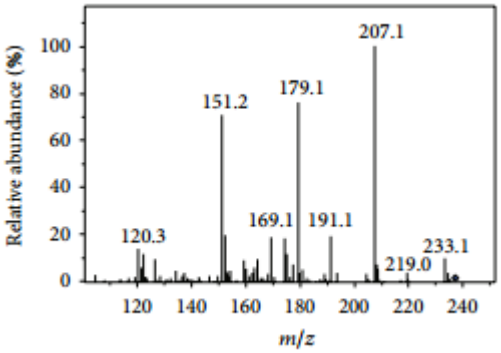


Figure 1. MS/MS spectrum of acetone

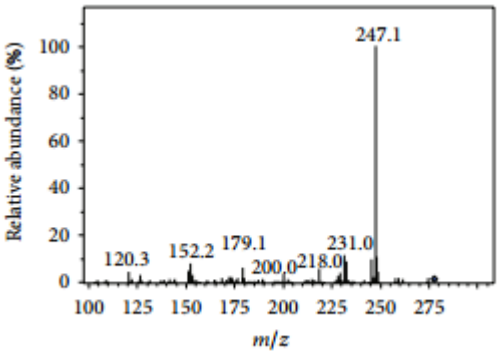


Figure 2. MS/MS spectrum of cyclohexane

Source : Ochs,2015

From the literature data, the ion at *m/z* 163 is observed as the most characteristic fragment for mono-DNPH derivatives of the aldehydes formaldehyde, acetaldehyde, myrtanal, campholene aldehyde, perillaldehyde, pinonaldehyde, endolim, and caronaldehyde (Kölliker, 1998).

2.8. Using gradient steps for high performance liquid chromatography (HPLC) with C18 reversed-phase columns

Some studies have been done related to separate the C_1 – C_{10} hydrazone by HPLC with C18 reversed-phase columns. Selim (1977) used acetonitrile-water mixtures using isocratic elution. Vigh and co-workers (1980) successfully separated saturated (C_1-C_{12}) as well as of mono-unsaturated (C_3-C_8) aldehyde hydrazones using methanol-water mixtures with isocratic elution. On the other hand, Demko and Nakamura et al (1979), used gradient elution to separate straight-chain saturated C_1-C_{10} aldehydes hydrazone with acetonitrile-water.

There is a benefit to use step gradient with a rapid change of the solvent composition since the higher carbonyl derivatives are eluting nearly at the same time. The way that we have to compare the substance peaks to internal the internal standard with integration has to be evaluated. It is also possible to use shorter columns to reduce analysis time and solvent consumption used without losing the quality of separation.

III. MATERIALS AND METHODS

3.1. Reagents and solvents

2,4-Dinitrophenylhydrazine (DNPH) was purchased from Sigma-Aldrich (St. Louis, USA), hydrochloric acid (HCl 37%) was purchased from Merck (Darmstadt, Germany), all solvents (e.g. methanol. ACN, acetone) used were of HPLC grade and were purchased from Merck (Darmstadt, Germany), acetic acid was purchased from Roth (Karlsruhe, Germany), rapeseed oil was purchased from Spar-Austria, β-carotene and α-tocopherol were from Sigma Aldrich (St. Louis, USA).

3.2. Analysis of secondary oxidation products in oil

3.2.1. Standardized oxidation of rapeseed

The rapeseed oil samples were subjected to oxidation in a Ranzimat (679, Metrohm, Herisau, Switzerland). 11 g of sample were used for the oxidation experiments. The temperature was set to 120 °C and the air flow to 20 l/h. Rapeseed oil was treated for up to 10 h. The oxidized samples were cooled immediately after the oxidation and stored under nitrogen below -18 °C.

3.2.2. Derivatization with 2,4-dinitrophenylhrazine (DNPH)

To 1 ml of the oxidized oil samples 4 ml of acetonitrile were added and mixed with 3 ml of the reagent 2,4-DNPH (3.48 mg/ml). The reaction mixture was kept in the dark for 1 h. After completion of the reaction 2 ml ethyl acetate were added for extraction and 1 g KCl for better phase separation. This mixture was thoroughly shaken for 30 s and centrifuged for phase separation. The organic layer was analysed without further treatment by HPLC.

3.2.3. Liquid chromatography-mass spectrometry condition for aldehydes identification.

The analyses of the DNPH derivatives of the carbonyls formed during oxidation were done by HPLC (Agilent 1100, Waldbronn, Germany) using a reversed phase column (Kinetex, EVO C18, 150 × 3 mm, 5 µm, Phenomenex, Aschaffenburg, Switzerland). For elution a gradient was used starting with methanol (45 %), water (30 %), and acetonitrile (25 %) changing to methanol (6 %), water (4 %), and acetonitrile (90 %) linearly within 15 min. The absorption of the eluent was measured at 400 nm for the presence of the DNPH derivatives.

For mass selective detection a QTRAP 2000 (AB Sciex, Framingham, MA, USA) was used. Ionization was done using the APCI mode with a gas drying temperature of 250 °C, capillary voltage of 4000 V, and a fragmentor potential of 150 V.

3.3. Analyses of α-tocopherol and β-carotene

For the analyses of α-tocopherol and β-carotene 25 mg of the oil were extracted with 1 ml of methanol in 2 ml reactions vials (Eppendorf, Wien, Austria). The samples were shaken for 2 min vigorously and centrifuged. Under these conditions both, α-tocopherol and β-carotene were extracted quantitatively. The methanolic extract was used directly for HPLC analysis on a reversed phase column (Kinetex, EVO C18, 150 × 3 mm, 5 µm, Phenomenex, Aschaffenburg, Switzerland) using a flow of 0.6 ml/min. α-Tocopherol was separated isocratically using 5 % water in methanol detecting it at 292 nm. β-Carotene was chromatographed with 100 % acetonitrile with detection at 450 nm.

3.4 Statistical methods

All experiments were carried out in triplicate and are calculated as means and standard deviation with the software package Origin 8 for windows (Origin lab Corporation). In addition the one-way analysis of variance (ANOVA) followed by Bonferroni test was done. The differences were considered to be significant at a probability level of $P < 0.05$.

IV. RESULTS AND DISCUSSION

4.1 Fatty acid content of rapeseed oil and sunflower oil

Gas chromatography analysis showed that rapeseed oil contains oleic acid 66.22%, linoleic acid 18.54%, linolenic acid 8.19%, palmitic acid 4.07%, and stearic acids 1.31%. Warner and Mounts (1993) found that some amount of linolenic acid is required for good flavour formation in fried foods. On the other hand, sunflower oil consists of linoleic acid 60.66%, oleic acid 29.28%, linolenic acid 0.09%, palmitic acid 6.03%, and stearic acid 2.86%. This is favourable for the formation of oxidation products which are important flavour compounds. Thus elimination of linolenic acid from oil can cause negative changes in the flavour of fried products.

4.2. The total amount carbonyls in rapeseed oil and sunflower oil

The oil was heated at 120 °C for 10 h in the formation of primary and secondary oxidation. At the starting time the oil is yellow, then finally becoming pale yellow to white in a liquid form after 10 h for oxidation (Fig. 3)

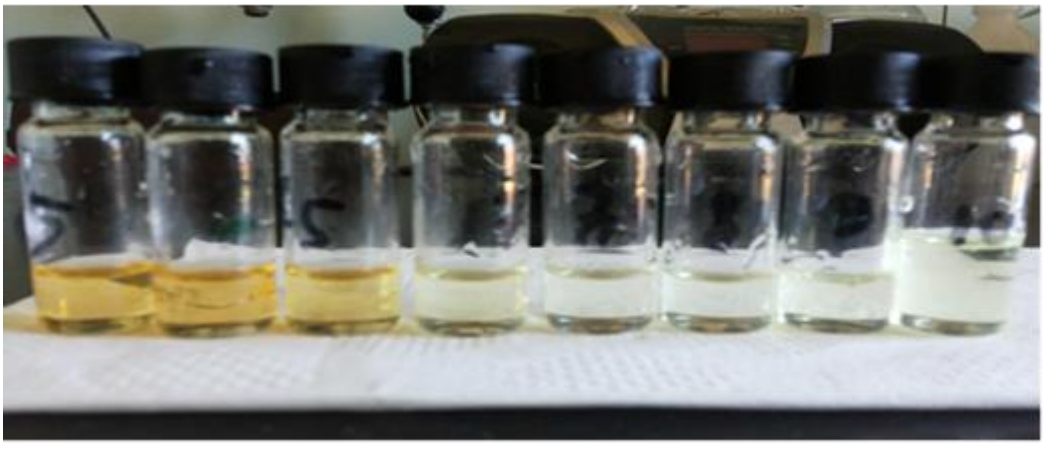


Figure 3. The colour results of oxidation process from 5 h to 10 h by Ranzimat 679 in rapeseed oil

The composition of sunflower oil with 70% of linoleic made highly susceptible to lipid oxidation (Meydana et.al., 1991). The composition of unsaturated fatty acid such as oleic acid, linoleic acid and linolenic acid in oil has an effect on the oxidative stability (Min & Boff, 2001; Nawar, 1996). In our research, the sunflower oil which was used contains 60.66% of linoleic acid compared to rapeseed oil which just has 18.54%. It means that sunflower oil is highly susceptible to lipid oxidation (Jeleń et al., 2000).

The total amount of carbonyls formed in rapeseed oil and sunflower oil is shown in Fig. 4. Similar to the single aldehydes, a maximum concentration of aldehydes in rapeseed is reached at 7.5 hours with maximum concentration is 60.5 mM and a decrease afterwards. On the other hand, the sunflower oil reached the maximum carbonyl content at 6.5 h with 56.3 mM based on a calibration with hexanal.

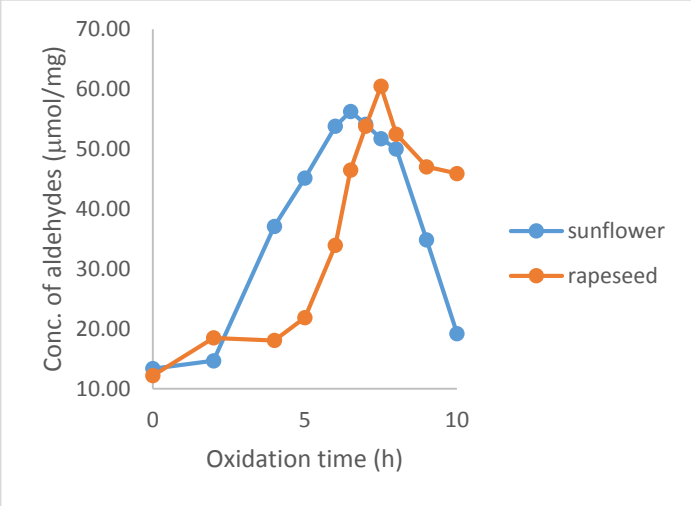


Figure 4. Formation of the total amount of aldehydes during oxidation of sunflower and rapeseed at 120 °C with constant air sparging

Oxidation of edible oils occurs when MUFA and PUFA, which are mainly glycerol bound, react with atmospheric O_2. Linoleate was 40 times more reactive than oleate, linolenate was 2.4 times more reactive than linoleate, and arachidonate was 2 times more reactive than linolenate. Primary oxidation products, hydroperoxides, are formed through different chemical mechanisms. Even though fresh vegetable oils were not oxidized extensively, heating altered the content of lipid oxidation products considerably (Frankel, 2005).

4.3. The carbonyls detection in rapeseed and sunflower oil

The formation of secondary lipid oxidation products, alkenals, increased in all vegetable oils that were heated. The increase in alkenal concentration of heated oils was in the range 2.9-11.2 times compared to the corresponding fresh oil (Halvorsen & Blomhoff, 2011)

For some of the peaks we assumed that the concentration of the carbonyls will be reduced during the oxidation time. The fifth points of selected peaks which are hexanal (A), octanal (B), nonanal (C), decenal (D), and 2-undecanal (E) showed that after 7.5 h and 6.5 h respectively for rapeseed and sunflower oils produced the highest peak area with a decrease after 10 h.

In previous work (Damanik & Murkovic, 2017) identified some carbonyls in the triolein which were hexanal, heptanal, octanal, nonanal, and decanal. As we already know that triolein just contains oleic acid. It means that secondary oxidation product are produced from oleic acid itself. There are some similar profile of secondary oxidation products of rapeseed oil and sunflower oil (Fig. 5)

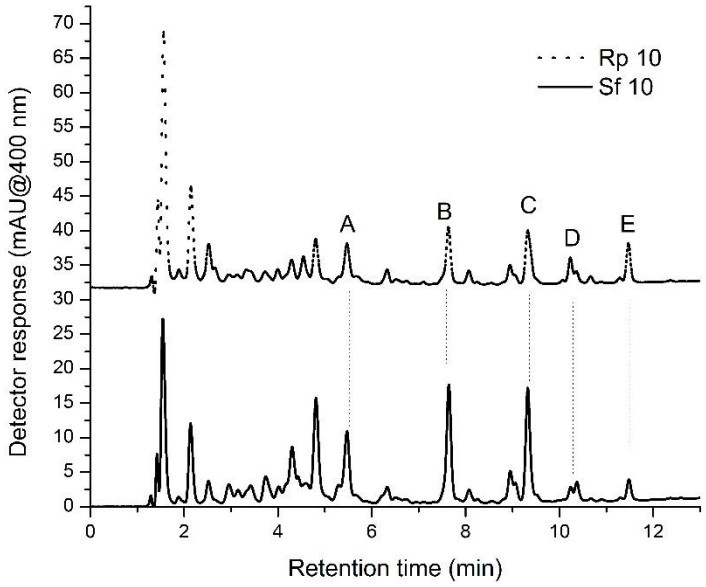


Figure 5. The profile of oxidation value because of the increasing of temperature

With the LC-MS analysis, we detected some aldehydes with molecular mass *m/z* 279 (hexanal), *m/z* 305 (octanal), *m/z* 321 (nonanal), *m/z* 333 (decenal) and *m/z* 347 (2-undecanal) in rapeseed oil. (Fig. 6).

The main product from oleic, linoleic, linoleic and arachidonic acid is hexanal. Besides that, octanal and nonanal are derived from oleic acid. They have a fruity and floral odour. In addition, hexanal and 2-hexenal derive from linoleic acid and linolenic acid, which has an unpleasant grassy odour (Shiraki, 1965)

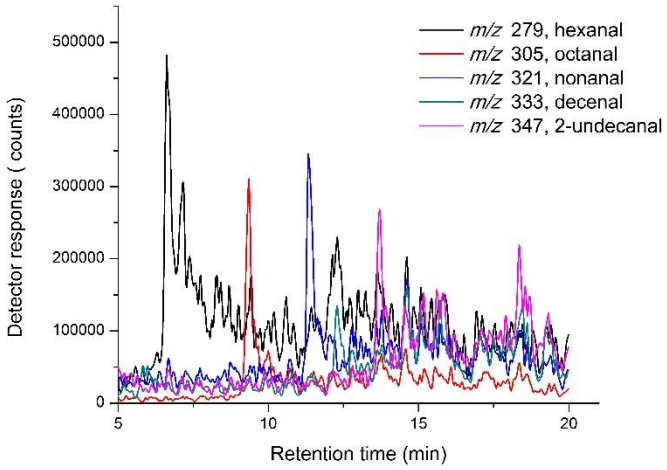
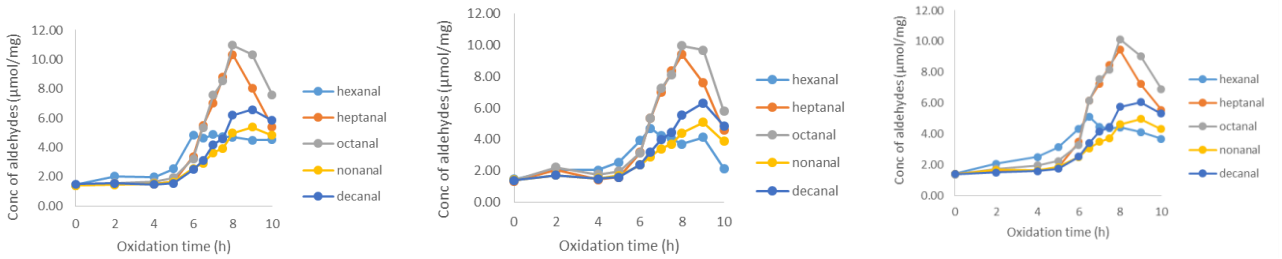


Figure 6. Triple quadrupole LC-MS chromatogram of rapeseed oil oxidized for 10 h

4.4. Influence of lipid soluble antioxidants on carbonyl formation

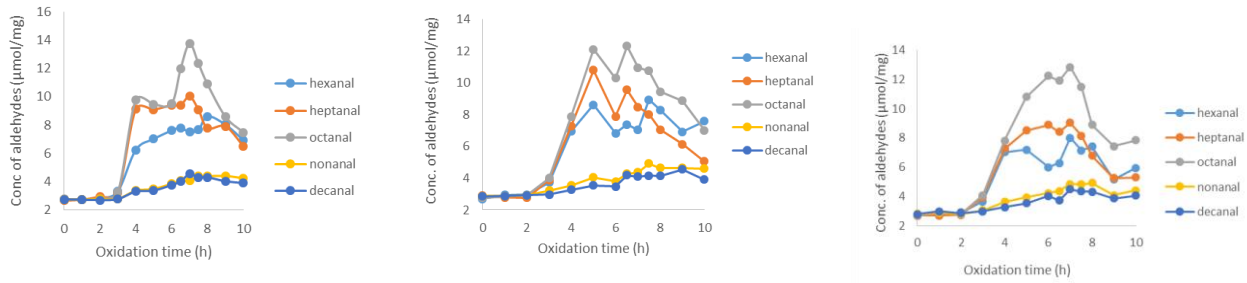
In general, β-carotene was an effective antioxidant to reduce the amount of carbonyls during the heating process Figure 7a for rapeseed oil. Both antioxidants worked well in the long term heating process. With β-carotene addition, some kinds of aldehydes such as hexanal, octanal, nonanal, decenal and 2-undecanal can be reduced by 53%; 15%; 24%; 19%; 17%, respectively at 10 h in rapeseed oil. However, the tocopherol did not give a higher percentage of reducing aldehydes, such as hexanal with 19% and decenal with 10% at 10 h, nonanal with 13% and octanal with 9.8% at 9 h compared to the rapeseed oil without antioxidants (Figure 7b; Figure 7c). There are no significant differences between rapeseed oil, rapeseed oil with β-carotene and rapeseed oil with α-tocopherol from the statistical analysis with ANOVA at the level 0.05. However, one point shown at 2 h, there is significant difference between rapeseed with β-carotene and rapeseed w/o antioxidant resulting in reduced levels of carbonyls.



(a) rapeseed (b) rapeseed + β-carotene (c) rapeseed + α-tocopherol

Figure 7. The profiles of carbonyls from rapeseed with and without antioxidants

On the other hand, sunflower with β–carotene can reduce heptanal with 22.47% compared to sunflower without antioxidants at 10h oxidation (Fig. 8b). β-carotene provides stability to vegetable oils because it's less reactive triplet state. Another study shown that β-carotene is degraded faster in methyl linoleate at higher temperature (Zeb & Murkovic, 2009 ; Anguelova & Warthesen, 2001)



(a) sunflower (b) sunflower + β-carotene (c) sunflower + α–tocopherol

Figure 8. The profiles of carbonyls from sunflower with and without antioxidants

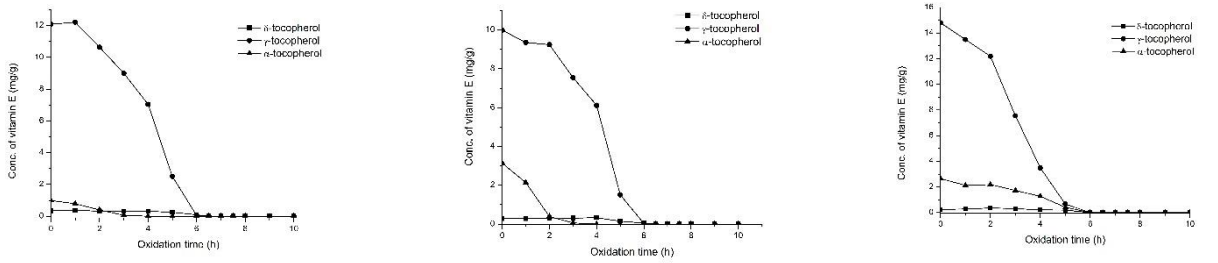
In addition, sunflower with α-tocopherol reduced the hexanal and the heptanal with 35.74% and 33.44 % respectively at 9h oxidation (Figure 8c). The off-flavour such as hexanal, with γ and δ-

tocopherol can exhibit the highest efficiency to reduce the oxidative breakdown of vegetable oils in frying system (Wagner, et.al., 2001 ; Wagner & Elmadfa, 2000 ; Warner et.al., 2003).These results are consistent with the assumption that MUFA are more resistant to oxidation and heat than PUFA (Fig. 8c)

4.5.Antioxidants contain in rapeseed and sunflower oil

4.5.1. Vitamin E in rapeseed and sunflower oil

The natural vitamin E present in the rapeseed oil and sunflower oil comprises δ-tocopherol, α-tocopherol and γ-tocopherol. The total tocopherol content in fresh rapeseed oil and fresh sunflower oil is 0.13 mg/mg and 0.0046 mg/mg respectively with γ-tocopherol> α-tocopherol> δ-tocopherol in both oils, however, no tocotrienol were detected.



(a) Rapeseed (b) Rapeseed with α-tocopherol (c)Rapeseed with β-carotene

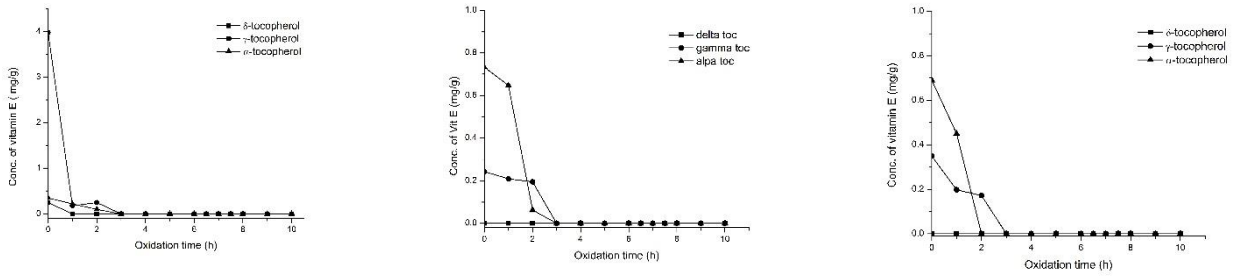
Figure 9. The vitamin E contain in rapeseed oil

The levels of the formed carbonyls were lower in the samples with added antioxidants. γ–tocopherol was more stabil and effective antioxidant than α-tocopherol in rapeseed oil (Figure 9a-c). However, the stability of rapeseed oil was still mantained even no antioxidant added. This can be proven by the data that the oil without antioxidant and with γ-tocopherol added were still remained until 6h with 0.04 mg/g and 0.03 mg respectively. γ-tocopherol was a more effective

antioxidant than α-tocopherol at levels above 100 μg/g. As long as there were tocopherols present, the hydroperoxide were quite stable and non volatile aldehydes were formed (Lampi, 1999)

In addition, the α-tocopherol added to the rapeseed oil made the increasing amount of α-tocopherol. However, the data shown that the stability of rapeseed oil without antioxidant and with α-tocopherol addition remained in the oil until 4h oxidation with 0.06 mg/g respectively.

On the other hand, rapeseed oil with β-carotene increased the stability of α-tocopherol in the oil. It involved the synergism among antioxidants. The synergism occurs when one free radical scavenger has a higher reduction potential than the other. For example : tocopherol (E^o = 550 mV) are primary antioxidants and ascorbic acid (E^o= 330 mV) is a synergist (Lieber, 1993). Tocopherol (TH) act as antioxidant by donating hydrogen to alkyl (R **.**) or alkyl peroxy (ROO .) radicals in foods and become tocopherol radical (T.) which does not have antioxidant activity. While, ascorbic acid (AsH) gives hydrogen to tocopherol radical to regenerate tocopherols and it becomes semihydroascorbyl radical (As.), and then dehydroascorbic acid (DHAs) (Buettner 1993).



(a) Sunflower (b) Sunflower with α-tocopherol (c)Sunflower with β-carotene

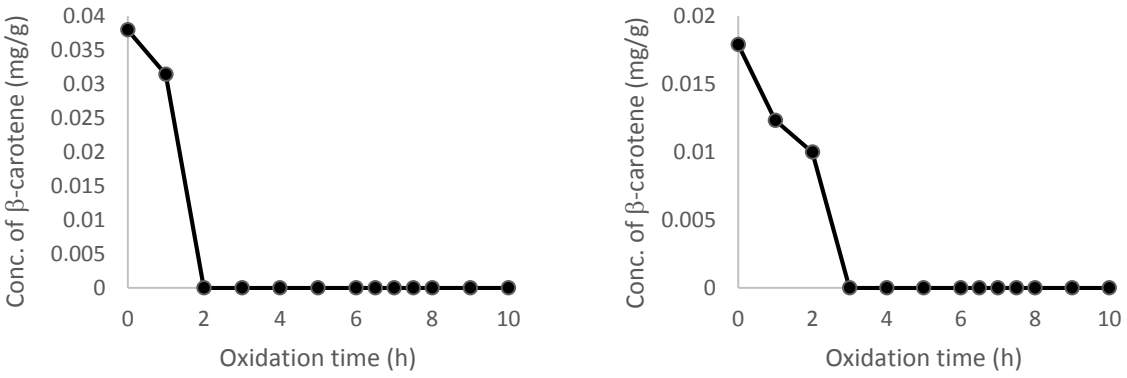
Figure 10. The vitamin E contain in sunflower oil

γ-Tocopherol was a more effective antioxidant than α-tocopherol at levels above 100 μg/g. As long as there were tocopherols present, the hydroperoxides were quite stable and non-volatile aldehydes were formed (Lampi, 1999)

The addition of antioxidants in the sunflower oil gave no effect not only to the stability of oil but also to the quantity of the vitamin E itself. The raw sunflower oil has 3.98 mg/g γ-tocopherol in the oil. Addittional antioxidants such as α-tocopherol and β-carotene reduced the γ-tocopherol in the oil too.

4.5.2. β-Carotene in rapeseed and sunflower oil

β-Carotene is degraded by thermal treatment in a different temperatures. The oxidation of β-carotene can be separated with some steps such as isomerization into *Z*-isomers, intra-molecular cyclization to form volatile compounds and degradation to form aldehydes. Isomerisation is a reversible reaction and no colour change during this process. However, degradation is a non-reversible reactions (Chen, et al., 1994; Takahashi et al., 2003).



(a) rapeseed (b) sunflower

Figure 11. The β-carotene contain in rapeseed and sunflower oil

Analysis with HPLC was successful when the β-carotene added to the fresh oil. The amount of β-carotene is added 300 mg/g to each type of oils. The 0h oxidation of rapeseed oil was 0.04 mg/g and just stayed until 1h oxidation. On the other hand, the sunflower oil was 0.02 mg/g on the 0h oxidation and still remained in the oxidized oil until 2h oxidation with 0.01 mg/g. Most of β-carotene was found to degrade faster than TAGs (Zeb and Murkovic, 2011b).

V. CONCLUSION

RP HPLC with gradient coupled to orbitrap LC-MS was used for the analysis the aldehydes as a secondary oxidation products. The fifth points of selected peaks which are hexanal (A), octanal (B), nonanal (C), decenal (D) and 2-undecanal (E) showed that in the 7.5 h produced the highest value of detector area and it would be decreased significantly at the 10 h.

The β-carotene addition can reduced some kinds of aldehydes such as hexanal, octanal, nonanal, decenal and 2-undecanal with 52.73%; 15.43%; 23.91%; 19.38%; 16.48% respectively at 10 h for rapeseed oil. However the tocopherol didn't give a higher percentage of reducing aldehydes, such as hexanal with 18.57% and decenal with 10.31% at 10h, nonanal with 12.50% and octanal with 9.83% at 9h compared to the rapeseed without antioxidants.

The sunflower oil with β–carotene can reduce octanal with 22.47% compared to sunflower without antioxidants at 10h oxidation. In addition, sunflower with α-tocopherol reduced the hexanal and the octanal with 35.74% and 33.44 % respectively at 9h oxidation.

The stability of rapeseed oil and sunflower oil was still mantained even no antioxidant added.

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Apendix

Table 1. Concentration of aldehydes in rapeseed (rps) and rapeseed + antioxidant [(α-tocopherol (rpstoc) and β-carotene(rpsbc)] during 10h oxidation

Oxidation time (h)	The concentration of aldehydes in the oil (μmol/ml)														
	Hexanal			Heptanal			Octanal			Nonanal			Decanal		
	rps	rpsbc	rpstoc	rps	rpsbc	rpstoc	rps	rpsbc	rpstoc	rps	rpsbc	rpstoc	rps	rpsbc	rpstoc
0	1.78	1.80	1.75	1.73	1.65	1.74	1.70	1.73	1.66	1.68	1.71	1.67	1.79	1.69	1.68
2	2.49	2.54	2.55	1.82	2.51	1.93	1.93	2.72	2.11	1.74	2.09	2.02	1.87	2.08	1.81
4	2.44	2.51	3.09	1.82	1.78	1.96	2.00	2.15	2.38	1.82	1.85	1.97	1.77	1.80	1.92
5	3.11	3.13	3.86	2.03	1.95	2.21	2.34	2.40	2.73	1.99	2.05	2.23	1.85	1.92	2.11
6	5.95	4.84	5.32	4.11	3.86	4.26	3.93	3.83	4.00	3.07	2.87	3.01	2.99	2.89	3.05
6.5	5.68	5.76	6.28	6.68	6.51	7.51	6.51	6.51	7.47	3.50	3.49	3.70	3.72	3.85	4.09
7	5.98	5.20	5.47	8.55	8.54	8.84	9.22	8.81	9.19	4.36	4.10	4.19	5.04	4.78	5.01
7.5	5.78	4.95	5.34	10.72	10.17	10.32	10.40	9.88	9.97	4.74	4.48	4.48	5.55	5.36	5.36
8	5.75	4.56	5.43	12.60	11.46	11.53	13.37	12.11	12.34	5.98	5.32	5.56	7.45	6.67	6.93
9	5.51	5.08	5.04	9.79	9.25	8.83	12.58	11.77	11.00	6.47	6.12	6.00	7.94	7.61	7.29
10	5.55	2.62	4.52	6.60	5.58	6.76	9.26	7.04	8.40	5.80	4.68	5.21	7.02	5.86	6.42

Table 2. Concentration of aldehydes in sunflower (sfo) and sunflower + antioxidant [(α-tocopherol (sfotoc) and β-carotene(sfobc)] during 10h oxidation

Oxidation time (h)	The concentration of aldehydes in the oil (μmol/ml)														
	Hexanal			Heptanal			Octanal			Nonanal			Decanal		
	sfo	sfobc	sfotoc	sfo	sfobc	sfotoc	sfo	sfobc	sfotoc	sfo	sfobc	sfotoc	sfo	sfobc	sfotoc
0	2.72	2.69	2.70	2.66	2.92	2.69	2.76	2.76	2.72	2.75	2.88	2.81	2.76	2.86	2.79
2	2.75	2.95	2.72	2.72	2.80	2.71	2.74	2.85	2.89	2.75	2.88	2.90	2.73	2.89	2.98
4	2.84	2.97	2.91	2.95	2.77	2.75	2.80	2.92	2.80	2.77	2.93	2.82	2.70	2.94	2.87
5	3.32	3.72	3.65	3.20	3.89	3.94	3.26	4.00	4.09	2.78	3.24	3.05	2.77	2.98	2.99
6	6.25	6.95	7.04	9.12	7.27	7.28	9.78	7.87	7.81	3.36	3.55	3.64	3.30	3.28	3.27
6.5	7.02	8.63	7.20	9.09	10.80	8.51	9.47	12.11	10.81	3.49	4.05	3.95	3.38	3.55	3.54
7	7.61	6.83	6.00	9.41	7.88	8.89	9.50	10.30	12.24	3.86	3.81	4.23	3.77	3.48	4.05
7.5	7.80	7.37	6.30	9.40	9.57	8.42	11.97	12.32	11.90	4.07	4.28	4.36	4.02	4.19	3.73
8	7.51	7.05	8.01	10.02	8.49	9.04	13.75	10.97	12.83	4.06	4.37	4.86	4.54	4.10	4.50
9	7.66	8.95	7.15	9.07	8.04	8.15	12.33	10.76	11.51	4.38	4.92	4.82	4.30	4.17	4.34
10	8.62	8.29	7.40	7.78	7.05	6.78	10.89	9.44	8.90	4.39	4.66	4.93	4.31	4.13	4.34

Table 3. Concentration of vitamin E in sunflower oil during 10h oxidation

Vit. E (mg/g) in sfo oil	Oxidation time (h)												
	0	1	2	3	4	5	6	6.5	7	7.5	8	9	10
δ-toc	0.25	0	0	0	0	0	0	0	0	0	0	0	0
γ-toc	3.98	0.18	0.25	0	0	0	0	0	0	0	0	0	0
α-toc	0.35	0.22	0.1	0	0	0	0	0	0	0	0	0	0

Table 4. Concentration of vitamin E in rapeseed oil during 10h oxidation

Vit. E (mg/g) in rps oil	Oxidation time (h)												
	0	1	2	3	4	5	6	6.5	7	7.5	8	9	10
δ-toc	0.34	0.38	0.32	0.31	0.32	0.25	0.09	0	0	0	0	0	0
γ-toc	12.08	12.21	10.64	9	7.04	2.51	0.04	0	0	0	0	0	0
α-toc	0.99	0.79	0.4	0.06	0	0	0	0	0	0	0	0	0

Table 5. Concentration of vitamin E in sunflower with β-carotene (sfobc) oil during 10h oxidation

Vit. E (mg/g) in sfobc oil	Oxidation time (h)												
	0	1	2	3	4	5	6	6.5	7	7.5	8	9	10
δ-toc	0	0	0	0	0	0	0	0	0	0	0	0	0
γ-toc	0.35	0.2	0.17	0	0	0	0	0	0	0	0	0	0
α-toc	0.69	0.45	0	0	0	0	0	0	0	0	0	0	0

Table 6. Concentration of vitamin E in rapeseed with β-carotene (rpsbc) oil during 10h oxidation

Vit. E (mg/g) in rpsbc oil	Oxidation time (h)												
	0	1	2	3	4	5	6	6.5	7	7.5	8	9	10
δ-toc	0.24	0.28	0.36	0.31	0.21	0.2	0	0	0	0	0	0	0
γ-toc	14.82	13.49	12.19	7.55	3.49	0.67	0	0	0	0	0	0	0
α-toc	2.65	2.14	2.19	1.73	1.27	0.42	0	0	0	0	0	0	0

Table 7. Concentration of vitamin E in sunflower with α-toc (sfotoc) during 10h oxidation

Vit. E (mg/mg) in sfotoc oil	Oxidation time (h)												
	0	1	2	3	4	5	6	6.5	7	7.5	8	9	10
δ-toc	0	0	0	0	0	0	0	0	0	0	0	0	0
γ-toc	0.24	0.21	0.19	0	0	0	0	0	0	0	0	0	0
α-toc	0.73	0.65	0.06	0	0	0	0	0	0	0	0	0	0

Table 8. Concentration of vitamin E in rapeseed with α-toc (rpstoc) during 10h oxidation

Vit. E (mg/mg) in rpstoc oil	Oxidation time (h)												
	0	1	2	3	4	5	6	6.5	7	7.5	8	9	10
δ-toc	0.28	0.28	0.32	0.31	0.33	0.15	0.05	0	0	0	0	0	0
γ-toc	9.99	9.36	9.24	7.55	6.12	1.51	0.03	0	0	0	0	0	0
α-toc	3.14	2.14	0.39	0.06	0	0	0	0	0	0	0	0	0

Table 9. Concentration of b-carotene in sunflower with β-carotene (sfobc) and rapeseed with β-carotene (rpsbc) oil during 10h oxidation

Oxidation time (h)	Conc. of β-carotene (mg/g)	
	sfobc	rpsbc
0	0.018	0.038
1	0.012	0.031
2	0.010	0.000
3	0	0
4	0	0
5	0	0
6	0	0
6.5	0	0
7	0	0
7.5	0	0
8	0	0
9	0	0
10	0	0

SECTION 4

PUBLICATIONS

Formation of potentially toxic carbonyls during oxidation of triolein in presence of alimentary antioxidants

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Abstract

A relation between the oil uptake and cancer as well as induction of hepatic inflammation was shown earlier. It is discussed that the main oil oxidation products – hydroperoxides and carbonyls – might be the reason for the mentioned diseases. In this manuscript the quantitative determination of aldehydes which are formed during oxidation of triolein – as a model substance – using the Rancimat 679 is described. The oxidation of 11 g of triolein is carried out at 120 °C sparging air with a flow of 20 dm^3/h for 10 h. A series of aliphatic aldehydes starting from hexanal to decanal as well as decenal was identified by LC-MS/MS and quantified as DNPH derivatives. In addition the total amount of carbonyls was determined. Based on the calibration with hexanal, all other dominant substances were in the similar concentration range with maximum concentrations of 1.6 µmol/cm^3 of hexanal, 2.3 µmol/cm^3 of heptanal, 2.5 µmol/cm^3 of octanal, 3.2 µmol/cm^3 of nonanal, 4.0 µmol/cm^3 of decanal after 6 hours. The total amount of carbonyls reached a maximum after 6 h being 27 µmol/cm^3 for triolein without antioxidant. The results of this investigation will be a basis for further toxicological studies on oxidized oils.

Keywords Triolein ● Lipid oxidation ● Aldehydes ● DNPH ● Antioxidants ● β-Carotene ● α- Tocopherol

Introduction

Prolonged exposure to oxidized lipids can be detrimental to human health. It has been reported that lipid hydroperoxides from processed dietary oils can enhance the growth of hepatocarcinoma cells [1]. Another issue was published by Böhm and co-workers [2] who found that oxidized fat might result in liver inflammation which was shown in experimental rats. In the latter publication no relation to a specific substance or group of substances was established. Therefore, a quantitative characterisation of oxidised oils is a prerequisite to start further mechanistic investigations on the toxic principles of oxidised oils.

In earlier manuscripts the formation of different peroxides (hydroperoxides, epidioxides) and epoxides was described in detail [3, 4]. A second group of reactive products from oil oxidation are the carbonyls which are formed during further reactions of the primary peroxides.

The formation of carbonyls during oil oxidation was investigated earlier in rapeseed oil [5] when it was shown that as a result of the degradation of the intermediate hydroperoxides a great variety of aldehydes and ketones are formed. It is well known that medium and short chain aldehydes which are formed from higher unsaturated fatty acids are the key aroma active components which are responsible for a rancid aroma. These are C_7-C_{11} monounsaturated aldehydes, or C_6-C_9 dienals, or C_5 branched aldehydes or some C_8 ketones which are important contributors to the oil aroma having negative attributes (rancid, winey-vinegary, fusty, muddy sediment, musty) [6].

These carbonyls and other compounds such as aldehydes, terminal alkenes, carboxylic acids and aliphatics are formed by a β-cleavage of lipid alkoxyl radicals [7]. The fragmentation reactions of allylic hydroperoxides by the Hock rearrangement create two different carbonyls from the same lipid peroxide, i.e. aldehydes or ketones [8]. Mechanistically, this is achieved by the insertion of one oxygen atom into the lipid carbon backbone and delocalisation of a positive charge on a carbon or oxygen atom.

The main aim of this work was to establish an analytical method for a quantitative determination of the carbonyls in the lipid (oxidised oil) phase for determination of the alimentary exposure. For the easier identification and assignment of the reaction products a defined matrix was used (triolein) which did not contain any interfering compounds. It was decided to use the derivatisation with 2,4-

dinitrophenylhydrazine (DNPH) – a commonly known derivatisation reagent for carbonyls – that would give the possibility to identify and quantify single components as well as the total carbonyl content. These derivatives are stable and can be analysed by HPLC with ESI-MS in the negative mode or by its absorption at 400 nm [9-11].

With this background we have started to characterize the formation of carbonyls during oxidation of triolein in the oil phase and not the volatile phase in which these compounds are mostly related to sensory sensation. On basis of reversed phase chromatography with UV and MS detection the non-evaporating carbonyl compounds formed during oxidation of triolein were identified and quantified. In this case triolein was used as model oil. The results of these experiments will be a basis for future work on food oil oxidation. In addition, the interaction of antioxidants (β-carotene, α-tocopherol) with the carbonyl formation was analysed.

Results and Discussion

Secondary oxidation products analysis

The derivatization of carbonyls is commonly used in chromatographic analysis. The hydrazones formed are stable enough for MS experiments. In some cases the carbonyls from fatty acid oxidation are too volatile to be analysed by LC/MS. Therefore the DNPH adds molecular weight to increase sensitivity and selectivity [12]. Negative ionization using APCI was the most sensitive MS method which could be used. The hydrazones formed were analysed using visible light absorption (400 nm) or by MS. The advantage of detection at 400 nm is that practically all peaks that appeared in the chromatogram were DNPH derivatives. In the control chromatograms only the baseline could be observed at 400 nm (Fig. 1). The semiquantitative evaluation of the carbonyls was based on a calibration using hexanal with the peak area relating to the molar concentration of hexanal. The linear calibration of hexanal was done in the range of 8 – 1000 µg/cm^3 (0.08 – 10 mM). The formation of carbonyls could be explained by a Hock cleavage which was reviewed by [7]. This might explain the formation of e.g. decanal and 2-undeceneal which are produced by homolytic cleavages on either sides of the 8-hydroperoxide and nonanal from either the 9- or

10-hydroperoxide. The linearity of the hexanal analysis was tested in the range of 5 – 1300 µg/cm^3 according to Mandel [13] with a limit of detection of 4.1 µg/cm^3 and a limit of quantification of 13 µg/cm^3.

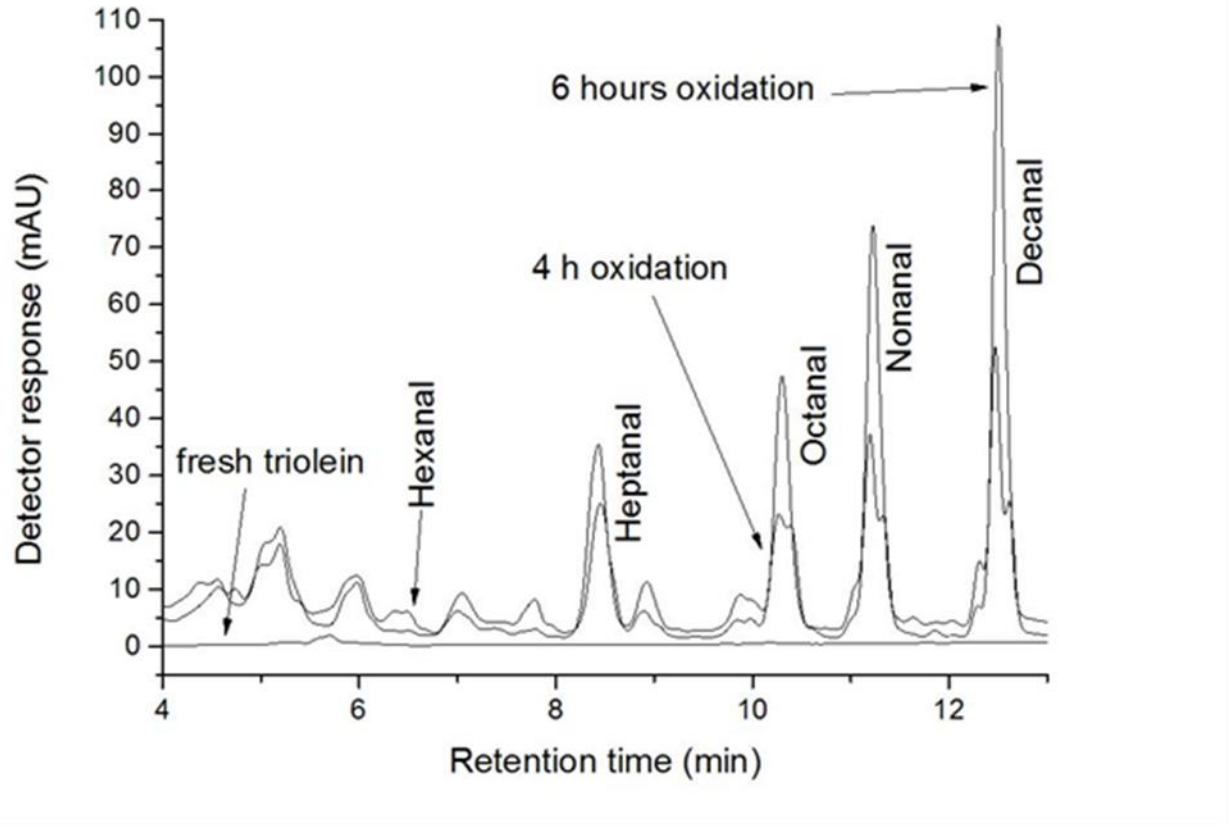

Fig. 1 Chromatogram of DNPH derivatives of fresh triolein and after 4 as well as 6 hours of oxidation in the Rancimat.

The formation of carbonyls began after 2 hours of oxidation at 120 °C while bubbling air through the oil. A strong increase of carbonyl formation was observed between 2 and 4 hours with a maximum after 6 hours and a slight decrease or constant concentrations afterwards. A series of linear aldehydes (from 6 carbons to 10 carbons) could be identified positively by LC-MS. Based on the calibration with hexanal all other dominant substances were in the similar concentration range with maximum concentrations of 1.6 µmol/cm^3 of hexanal, 2.3 µmol/cm^3 of heptanal, 2.5 µmol/cm^3 of octanal, 3.2 µmol/cm^3 of nonanal, 4.0 µmol/cm^3 of decanal after 6 hours (Fig. 2). These identified aldehydes were volatile and undergo further degradation reactions which may explain the reduced concentration.

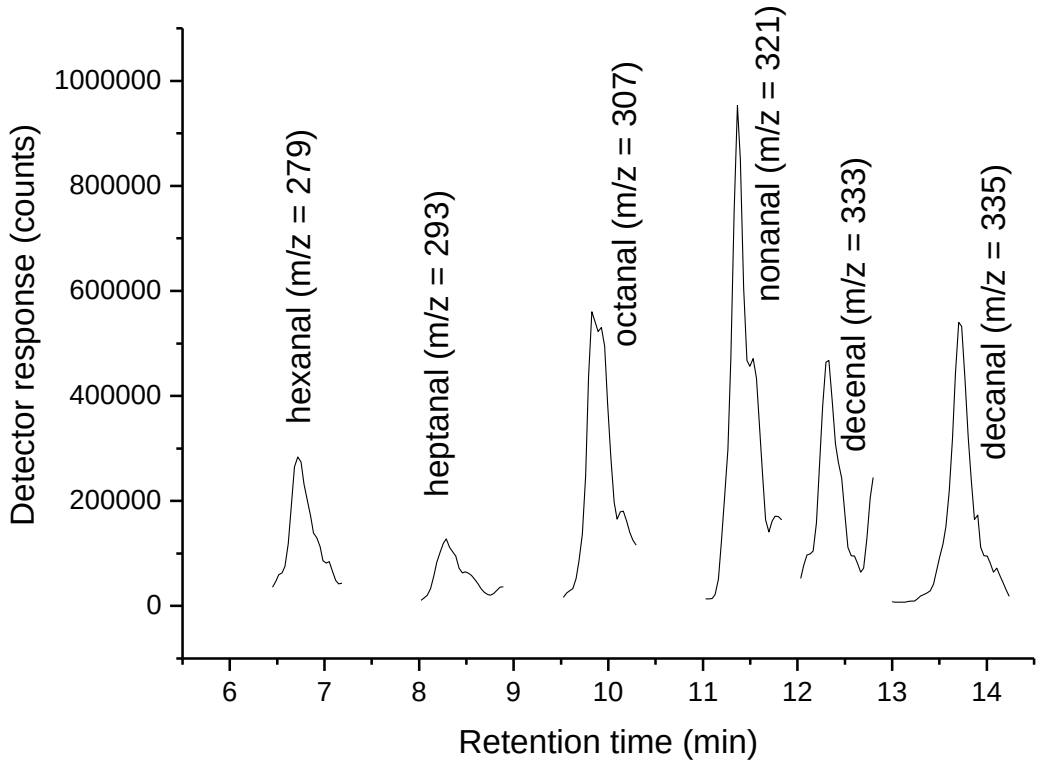

Fig. 2 Selected ion traces of the DNPH conjugates of the saturated and unsaturated aldehydes found in oxidised triolein.

The double peaks which could be seen after 4 hours of oxidation were attributed to Z/E isomer formation since no difference in the mass spectrum and UV absorption could be observed. After 6 h of oxidation the double peaks disappear resulting in a shift in the relative concentrations (Fig 1).

The identification of the peaks was done by LC-MS/MS as is shown in Fig. 2. The earlier mentioned saturated aldehydes (hexanal – decanal) including decenal could be identified. The fragmentation of all of these compounds was similar with a specific fragment of *m/z* = 182 originating from the DNPH moiety.

In this experiment, nonanal and octanal were the main products. 2-Nonenal, and 2,4-decadienal which are known degradation products of linoleic acid were not detected. Additional compounds (heptane, heptanol, octane, 1-undecene, and 2-undecenal) that were described earlier are produced by a homolytic fission of

the intermediary formed R-O-bond [14-19]. However, non-carbonyls could not be detected with the method described here.

The total amount of carbonyls formed is shown in Fig. 3. Similar to the single aldehydes, a maximum concentration is reached after 6 hours with a decrease afterwards. The maximum concentration is 27 mM which is based on a calibration with hexanal.

Influence of lipid soluble antioxidants on carbonyl formation

In a second series of experiments, two lipid soluble antioxidants were added, namely α-tocopherol and β-carotene. The earlier experiments showed that the best stabilization was obtained by addition of 300 ppm of ascorbyl palmitate to soybean oil (SO). In addition, a combination of ascorbyl palmitate (300 ppm) and α-tocopherol (1000 ppm) was able to limit hydroperoxide and hexanal formation in SO at 35 °C for 12 weeks [20].

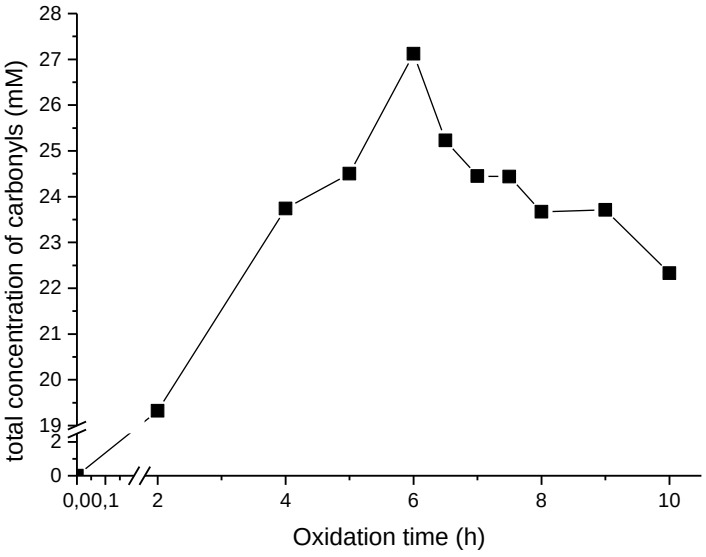

Fig. 3 Formation of the total amount of carbonyls during oxidation of triolein at 120 °C with constant air sparging.

Antioxidants inhibit lipid oxidation by quenching free radicals and physically stabilizing the micelles at reaction sites [21]. As described in [22] the oxidation starts after 5 h in both, the refined and fortified oils (containing β-carotene), with the formation of hydroperoxides. In this case, the addition of β-carotene resulted in a pro-oxidant effect. β-Carotene was still measurable after 10 hours [22]. During the course of the oxidation in the experiments described here, the levels of the formed carbonyls were lower in the samples with added antioxidants (Fig. 4). In addition, the pattern of the chromatograms did not change significantly indicating the same carbonyls are formed but at lower amounts. The sample containing α-tocopherol showed a higher formation of octanal after 10 h (Fig.5).

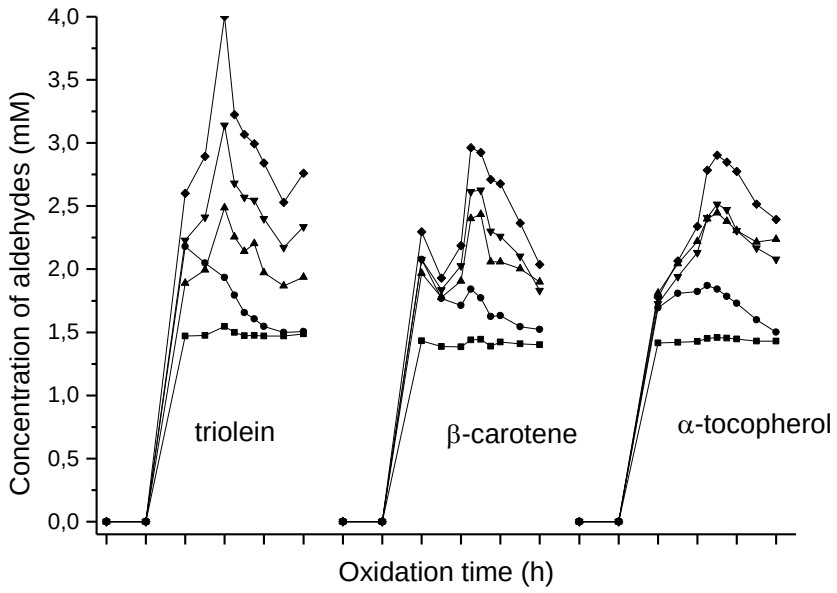

Fig. 4 Formation kinetics of single compounds during oxidation of triolein in presence of lipid soluble antioxidants at 120 °C with constant air sparging for 10 h (-●-hexanal; -■-heptanal; -▲-octanal; -▼-nonanal; -♦-decanal).

For this, β-carotene and α-tocopherol were dissolved in acetone and added to the triolein. The concentrations of the antioxidants were 300 µg/g ± 0.5 µg. The fortified triolein was stirred for 15 min and then flushed with nitrogen for 30 min to remove the acetone before sealing airtight in glass bottles. For the

oxidation in the Rancimat 20 g of triolein were used. The concentrations which were applied were in the same range as it can be expected in natural oils. The α-tocopherol concentration was 300 µg/g and the level of β-carotene was 500 µg/g.

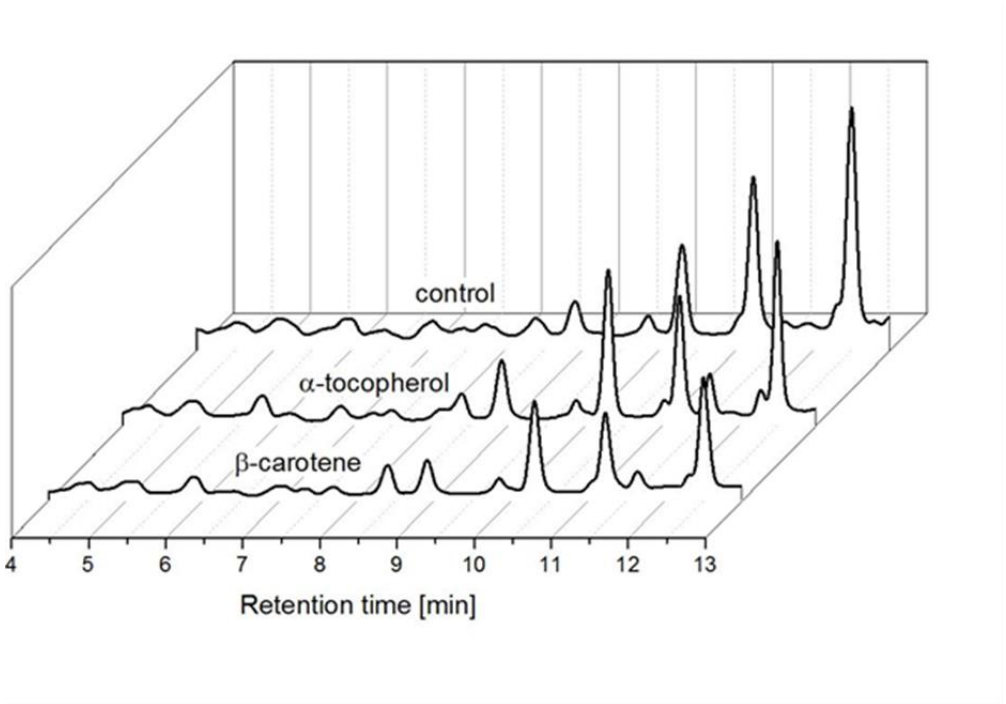

Fig. 5 Comparison of the carbonyl formation in presence of lipid soluble antioxidants (α-tocopherol, β-carotene) after 10 h of oxidation at 120 °C. The carbonyls are measured as DNPH derivatives detected at 400 nm.

The concentrations were selected according to the concentrations normally occurring in red palm oil which are in the range of 600 – 1000 µg/g for vitamin E and 400 – 3500 µg/g for α- and β-carotene [23].

Conclusion

The formation of carbonyls was quantified during oxidation of triolein by derivatization of the formed products with DNPH which is a selective reagent for aldehydes and ketones. Some of the oxidized fragments of triolein were identified by LC-MS/MS – using APCI in negative mode – being hexanal (*m/z* = 279), heptanal *(m/z* = 293), octanal (*m/z* = 307), nonanal (*m/z* = 321), decenal (*m/z* = 333), decanal (*m/z* = 335), and undecenal (*m/z* = 347). Using a Rancimat for reproducible oxidation experiments with a

constant air flow at defined temperatures (120 °C) the production of carbonyls showed a good repeatability. The formation of carbonyls from triolein showed a maximum after 6 h with a slight or no decrease during prolonged oxidation.

Experimental

2,4-Dinitrophenylhydrazine (2,4-DNPH) was purchased from Sigma-Aldrich (St. Louis, USA), hydrochloric acid (HCl 37%) was purchased from Merck (Darmstadt, Germany), all solvents (e.g. methanol, ACN, acetone) used were of HPLC grade and were purchased from Merck (Darmstadt, Germany), acetic acid was purchased from Roth (Karlsruhe, Germany), triolein was purchased from FLUKA (Buchs, Switzerland), β-carotene and α-tocopherol were from Sigma Aldrich (St. Louis, USA).

Oxidation of triolein with Rancimat

Standardized oxidation of triolein

The triolein oil samples were subjected to oxidation in a Rancimat (679, Metrohm, Herisau, Switzerland). Eleven grams of sample were used for the oxidation experiments. The temperature was set to 120 °C and the air flow to 20 l/h. Triolein was treated for up to 10 h. The oxidized samples were cooled immediately after the oxidation and stored under nitrogen below -18 °C.

Derivatization with 2,4-dinitrophenylhrazine (DNPH) (reference)

To 1 ml of the oxidized oil samples 4 ml of acetonitrile were added and mixed with 3 ml of the reagent 2,4-DNPH (3.48 mg/ml). The reaction mixture was kept in the dark for 1 h. After completion of the reaction 2 ml ethyl acetate were added for extraction and 1 g KCl for better phase separation. This mixture was thoroughly shaken for 30 s and centrifuged for phase separation. The organic layer was analysed without further treatment by HPLC.

Liquid chromatography-mass spectrometry condition for aldehydes identification

The analyses of the DNPH derivatives of the carbonyls formed during oxidation were done by HPLC (Agilent 1100, Waldbronn, Germany) using a reversed phase column (Kinetex, EVO C18, 150 × 3 mm, 5

µm, Phenomenex, Aschaffenburg, Switzerland). For elution a gradient was used starting with methanol (45%), water (30%), and acetonitrile (25%) changing to methanol (6%), water (4%), and acetonitrile (90%) linearly within 15 min. The absorption of the eluent was measured at 400 nm for the presence of the DNPH derivatives.

For mass selective detection a QTRAP 2000 (AB Sciex, Framingham, MA, USA) was used. Ionization was done using the APCI mode with a gas drying temperature of 250 °C, capillary voltage of 4000 V, and a fragmentor potential of 150 V.

Analyses of α-tocopherol and β-carotene

For the analyses of α-tocopherol and β-carotene 25 mg of the oil (triolein, triolein with β-carotene and triolein with α-tocopherol) were extracted with 1 ml of methanol in 2 ml reactions vials (Eppendorf, Wien, Austria). The samples were shaken for 2 min vigorously and centrifuged. Under these conditions both, α-tocopherol and β-carotene were extracted quantitatively. The methanolic extract was used directly for HPLC analysis on a reversed phase column (Kinetex, EVO C18, 150 × 3 mm, 5 µm, Phenomenex, Aschaffenburg, Switzerland) using a flow of 0.6 ml/min. α-Tocopherol was separated isocratically using 5% water in methanol detecting it at 292 nm. β-Carotene was chromatographed with 100% acetonitrile with detection at 450 nm.

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The authors have declared no conflict of interest

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The stability of palm oils during heating in Rancimat

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Abstract

The unrefined palm oil (UPO) and refined palm oil (PO) not only have a balance of saturated and unsaturated fatty acids but also have the highest β–carotene and vitamin E compared to others vegetable oils. A series of aliphatic aldehydes starting from hexanal to decanal was identified by LC-MS/MS and quantified as DNPH derivatives. In addition the total amount of carbonyls was determined. Based on the calibration with hexanal, The kinetic profile of the unrefined palm oil shown that the carbonyls compound still reach the highest amount until 18 h oxidation times with Ranzimat with 120 °C . While, palm oil until 11 h oxidation still want to reach the highest amount of secondary oxidation products.

The analytical method for quantifying the vitamin E and β–carotene are quick, reliable, precise, economical and suitable for the routine analysis. For both analyses a simple dilution of the oil was necessary. Looking at the kinetic profile of the carbonyl formation in unrefined palm oil and refined palm oil, both of the oils were still stable until 10 h of oxidation with the Ranzimat at 120 °C. The quantitative analysis showed that β-carotene in the oxidized unrefined palm oil decreased from 2.34 mg/g to 0.33mg/g within 7.5 h of oxidation. However, after 7.5 h, no more β–carotene was detected. In addition, β-carotene was not detected in refined palm oil.

Keywords: Unrefined palm oil, refined palm oil, lipid oxidation, aldehydes, DNPH, antioxidants, β-carotene, α-tocopherol

Introduction.

The palm tree (*Elaeis guineensis*) produced palm oil are found in the tropical country. Palm oil consumed in the world so it can replaced animal fats related to the cost and health concerns [1]. The leading country which exported of palm oil are Malaysia and Indonesia around 86% of global production. Crude palm oil is produced from palm fruits. The crude palm oil is known as red palm oil, RPO because it represents the richest natural of β-carotene. It contributes to the stability and nutritional value of the oil [2].

On the other hand, crude palm oil has a typically smell like an overripe mushroom and taste very pungent without any processing so, it has not many using in the kitchen. In addition, it has free fatty acids (FFA), moisture, trace metals and other impurities that limit its shelf life. The way how to increase the utility with refined which can remove odors, flavors, and impurities, as well as that some consumers didn't like the red color because it unappetizing [3]. Palmitic acid is the main saturated fatty acid naturally occurring in animal fats and vegetable oil, as well as the main component of human breast milk fat [4].

Crude palm oil (CPO) is extracted either by wet or dry processes. It contains 1% of minor components which can be healthy or somehow beneficial compounds, such as, vitamin E, carotenoids, phytosterols, as well as impurities, such as phospholipids, free fatty acids (FFAs), gums, and lipid oxidation products. During the refining unwanted components can be removed [5]. Hence, the CPO is purified by centrifugation and drying; the dried oil is then cooled and stored in appropriate containers [6].

It has a high carotenoid content (500-700 mg/kg) which is responsible for the dark orange color [7] The content of tocopherols and tocotrienols is 600-1200 ppm, which is comprises approximately 24% of tocopherols and 76% of tocotrienols. The vitamin E and the carotenoids contribute to its stability and nutritional properties [5, 8-12). Crude palm oil has to be refined to

produce palm oil which has desirable and technological appropriate characteristics, such as a light color, bland taste and good oxidative stability [13, 14]

The main aim of this work was to establish an analytical method for a quantitative determination of the carbonyls in the lipid (oxidized oil) phase for determination of the alimentary exposure. As we know, the unrefined palm oil (UPO) and refined palm oil (PO) not only have a balance of saturated and unsaturated fatty acids but also have the highest β−carotene and vitamin E compared to others vegetable oils. The formation of carbonyls during oxidation of unrefined palm oil (UPO) and refined palm oil (PO) in the oil phase and not the volatile phase in which these compounds are mostly related to sensory sensation. On basis of reversed phase chromatography with UV and MS detection, the non-evaporating carbonyl compounds formed during oxidation of UPO and PO were identified and quantified. A comparison of the two oils is difficult since it originally from two different sources.

In addition, we wanted to find out the relationship between a reduction of the antioxidants and the stability of the oils. In this case, it focused on the carbonyls formed which were hexanal, heptanal, octanal, nonanal, and decanal. The carbonyls were analyzed by 2,4-dinitrophenylhydrazine (DNPH) derivatisation with –a commonly known derivatization reagent for carbonyls – that would give the possibility to identify and quantify single components as well as the total carbonyl content. These derivatives are stable and can be analyzed by HPLC with ESI-MS in the negative mode or by its absorption at 400 nm [15-17]. The antioxidants (β-carotene, α-tocopherol) were be analyzed by HPLC with UV detection (292 nm for α-tocopherol and for β-carotene 450 nm) and additionally fluorescence detector (λ_{em}: 292 nm, λ_{ex}: 335 nm).

2 Materials and Methods

2.1 Materials and reagents

2,4-Dinitrophenylhydrazine (2,4-DNPH) was purchased from Sigma-Aldrich (St. Louis, USA), hydrochloric acid (HCl 37 %) was purchased from Merck (Darmstadt, Germany), all solvents (e.g. methanol, ACN, acetone) used were of HPLC grade and were purchased from Merck (Darmstadt, Germany), acetic acid was purchased from Roth (Karlsruhe, Germany), β-carotene and α-tocopherol were from Sigma Aldrich (St. Louis, USA), unrefined palm oil was purchased from Asia Markt in Graz, Austria, and refined palm oil was purchased in a local market in Medan-North Sumatera, Indonesia.

2.2 Analysis of secondary oxidation products in oil

2.2.1 Standardized oxidation of unrefined palm oil (UPO) and refined palm oil (PO)

The oil samples were oxidized in a Ranzimat (679, Metrohm, Herisau, Switzerland). For this, 11 g of oil were used. The temperature was set to 120 °C and the air flow to 20 l/h. UPO was treated for up to 18 h and PO for up to 11 h. The oxidized samples were cooled immediately after the oxidation and stored under nitrogen at -18 ° C.

Derivatization with 2,4-dinitrophenylhrazine (DNPH)

To 1 ml of the oxidized oil samples 4 ml of acetonitrile were added and mixed with 3 ml of the reagent 2,4-DNPH (3.48 mg/ml in 2 N HCl). The reaction mixture was kept at room temperature in the dark for 1 h. After completion of the reaction 2 ml ethyl acetate were added for extraction and 1 g KCl for better phase separation. This mixture was thoroughly shaken for 30 s and centrifuged for phase separation. The organic layer on the top was analyzed without further treatment by HPLC.

Liquid chromatography-mass spectrometry condition for aldehydes identification

The analyses of the DNPH derivatives of the carbonyls formed during oxidation were done by an HPLC (Agilent 1100, Waldbronn, Germany) using a reversed phase column (Kinetex, EVO C18, 150 × 3 mm (Phenomenex, Aschaffenburg, Switzerland). For elution a gradient was used starting with a mixture of methanol (45 %), water (30 %), and acetonitrile (25 %) changing to methanol (6 %), water (4 %), and acetonitrile (90 %) linearly within 15 min. The absorption of the eluent was measured at 400 nm for the presence of the DNPH derivatives.

For mass selective detection a QTRAP 2000 (AB Sciex, Framingham, MA, USA) was used. Ionization was done using the APCI mode with a gas drying temperature of 250 °C, capillary voltage of 4000 V, and a fragmentor potential of 150 V.

2.2.2 Analyses of vitamin E and β-carotene

For the analyses of α-tocopherol and β-carotene 25 mg of the oil (UPO and PO) were extracted with 1 ml of methanol in 2 ml reactions vials (Eppendorf, Wien, Austria). The samples were shaken for 2 min vigorously and centrifuged. Under these conditions both, α-tocopherol and β-carotene were extracted quantitatively. The methanolic extract was used directly for HPLC analysis on a reversed phase column (Kinetex, EVO C18, 150 × 3 mm,) using a flow of 0.6 ml/min. α-Tocopherol was separated isocratically using 5 % water in methanol as eluent and UV detection at 292 nm. β-Carotene was chromatographed with DAD HPLC with Kinetex EVO C18 2.6 μm; 100 x 3 mm (Phenomenex, Aschaffenburg, Switzerland) column using a flow of 1 ml/min of 100 % acetonitrile with detection at 450 nm.

2.2.3 Gas chromatography condition for fatty acids identification

25 mg of palm oil were put in a Pyrex glass tube and hydrolyzed with 1.5 ml M NaOH in methanol. The samples were closed tightly and vortexed for 1 – 2 min. After that, the samples were heated at 100 °C for 1 – 2 min. The free fatty acids were methylated with 2 ml of BF_3 in methanol at 100 °C for 30 min. After cooling to temperature 30 – 40 °C iso-octane was added to the solution, and then FAMEs (fatty acid methyl esters) were extracted in presence of 5 ml of saturated NaCl. The isooctane layer was analyzed for the fatty acids by GC (Shimadzu Q Plus 2010, Columbia, USA) using column DB-23; 30 m × 0.25 mm.

3 Results and Discussion

It is known from literature that palm oil contains 50 % saturated fatty acids (mostly palmitic acid (ca. 44 %) and lower amounts of stearic acid (5 %); 40 % of the fatty acids are monounsaturated fatty acids (mostly oleic acid) and 10 % polyunsaturated fatty acids (mostly linoleic acid) [5,7,9].

The gas chromatographical analysis showed that the refined palm oil which was used for the experiments described here had a fatty acid composition which was comparable to the literature values. It contained 44.5 % oleic acid, 12.2 % linoleic acid, 37.3 % palmitic acid, and 3.81 % stearic acid. The unrefined palm oil had a different composition with 41.9 % oleic acid, 9.30 % linoleic acid, 0.21 % linolenic acid, 43.1 % palmitic acid, and 4.18 % stearic acid. The presence of vitamin E (tocopherols and tocotrienols) and the comparably low content of linoleic acid increase the stability of palm oil and palm olein

The oxidative and thermal stability of palm oil and unrefined palm oil was evaluated using Ranzimat 679. During oxidation formic acid is formed which is transferred with the air stream to the measurement vessel filled with water and detected by an increase of the conductivity. In previous experiments [18] using triolein it was shown that DNPH reactive substances (carbonyls)

were formed. A chromatogram of the DNPH derivatives is shown in Fig. 2. The oxidation of unrefined palm oil is similar to triolein. The dominant peaks of the derivatized carbonyls eluted at the same time resulting in a similar pattern. When measuring the absorption of the eluent at 400 nm practically no other substances could be observed.

The dominant peaks that could be identified were hexanal, heptanal, octanal, nonanal, and decanal. These are degradation products of oleic acid which were described earlier in the triolein. The results from previous experiment show that the similar concentration range with maximum concentrations of 1.6 µmol/ml of hexanal, 2.3 µmol/ml of heptanal, 2.5 µmol/ml of octanal, 3.2 µmol/ml of nonanal, 4.0 µmol/ml of decanal after 6 hours. The total amount of carbonyls reached a maximum after 6 h being 27 µmol/ml for triolein without antioxidant. All these results referred to the calibration with hexanal to find the concentration of all others dominant carbonyls which presented in the oxidize oil.

Carbonyl compounds are the most abundant secondary oxidation products formed in edible oils at higher temperatures. The position of double bond in unsaturated fatty acid was correctly localized based on the fact that double bonds are more prone to cleavage than as single bond [19]. The ease of hydroperoxidation depends on the number of double bonds present [20].

Some of aldehydes and carboxy aldehydes that could possibly result from fatty acid oxidation have been identified in sea buckthorn berry (Hippophae rhamnoides) samples. Mathew and co-workers [21] concluded that the lower carbonyls such as formaldehyde, acetaldehyde and acetone were found to predominate in the berries, whereas longer aldehydes and carboxy aldehydes were dominant in oxidized oil. Our results shown that the longer aldehydes are formed to a greater extent. Both of the oils, UPO and PO shown that octanal produced after hexanal.

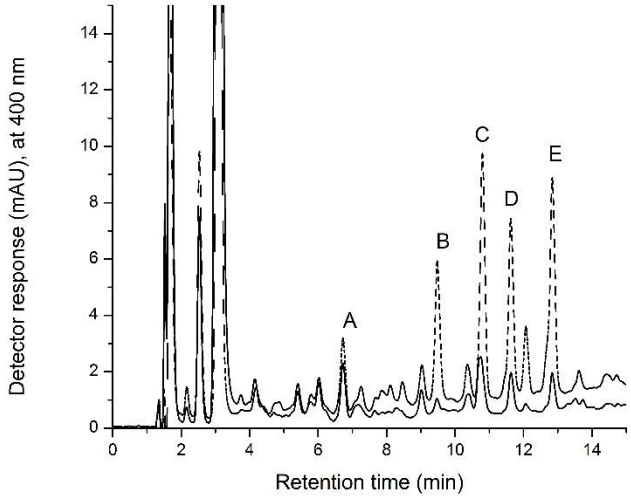

Figure 2. Carbonyls profile of triolein (------) and unrefined palm oil (——) (A: hexanal, B: heptanal, C: octanal, D: nonanal, E: decanal) after oxidation at 120 °C for 18 hours.

The reason might be that the longer aldehydes are less volatile and remain in the oil phase during air sparging. These aldehydes were produced by thermal decomposition via homolytic cleavage meaning a further oxidation of functional groups such as hydroperoxy, keto, epoxides, and dimer oxygen linkages [22-26]. The oxidized oleic volatile produced octanal and nonanal. The aldehydes such as pentanal and hexanal are usually formed from linoleic acid hydroperoxide. Another compound which is associated with oxidized linoleic acid such as 2-pentyl furan and 4-octen-3one were produced in heated triolein [24]. Those secondary products decompose the same way as monohydroperoxides to produce similar volatile compounds.

From the kinetic profile, the carbonyls compound still reach the highest amount until 18 h oxidation times with Ranzimat with 120 °C (Fig. 3). For 18 h of oxidation, the carbonyls which were detected were hexanal 1.6 mmol/g, heptanal 0.61 mmol/g, octanal 1.36 mmol/g, nonanal 0.72 mmol/g and decanal 0.95 mmol/ g for UPO (figure 3) As we already know that the percentage

of oleic acid in UPO was 41.9% , linoleic acid 9.30% and palmitic acid with 43.1%. This corresponds to the research undertaken by Reindl [27] focused on the oxidative degradation of unsaturated fatty acids which were isolated from meat. In his experiments he had shown that hexanal is the main product from oleic, linoleic and arachidonic acid.

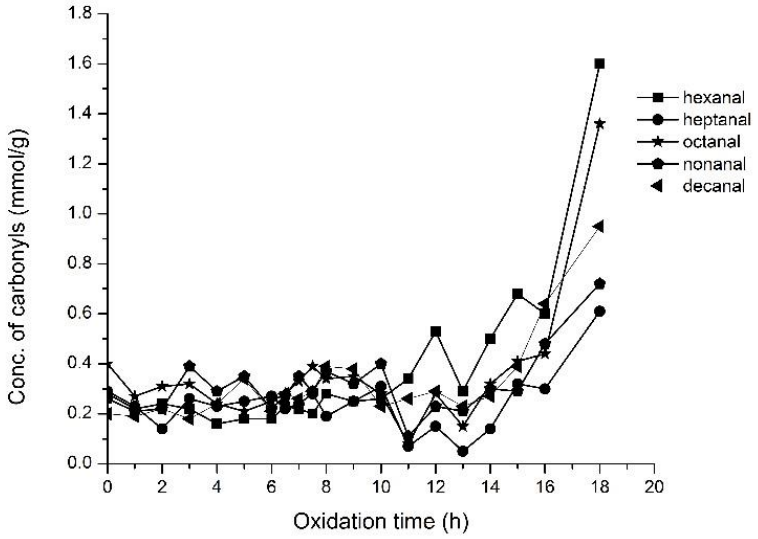

Figure 3. Formation kinetics of single compounds during oxidation of UPO (containing the natural antioxidants vitamin E and β-carotene) at 120 °C with constant air sparging for 18 h.

On the other hand, oleic acid produced octanal, nonanal, 2-nonenal, and 2,4-decadienal. Thus, the main secondary oxidation product of oleic acids in the UPO was hexanal, octanal and decanal which has the higher percentage than others. The decanal is produced by homolytic cleavage B of the alkoxyl intermediate group from the 8-hydroperoxide. While octanal from the 11-hydroperoxide [28]. However, the percentage of linoleic acid just 9.3% so the production of heptanal was lower with 0.61 mmol/g. The other carbonyls which produced from linolenic acid, was 2,4-heptadienal from, and 2,4-nonadienal from arachidonic acid.

Autoxidation of 2,4-decadienal at ambient conditions has been shown to produce a mixture of volatiles including hexanal, butenal, heptenal, octenal, benzaldehyde, and glyoxal [21] while oxidation of 2-nonenal produced C_2, C_3, C_7, C_8 alkanals, glyoxal, and a mixture of C_7, C_8 and C9 α-keto aldehydes [29].

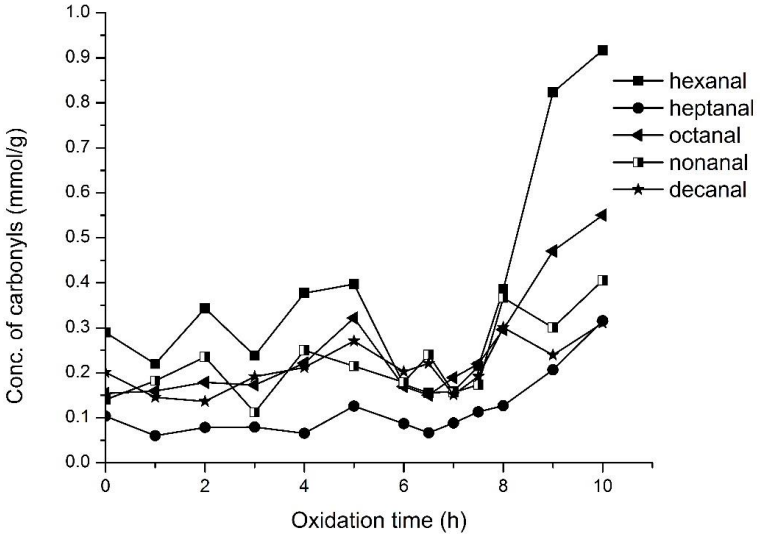

Figure 4. Formation kinetics of single compounds during oxidation of refined palm oil in presence of lipid soluble antioxidants at 120 °C with constant air sparging for 10 h (hexanal; heptanal; octanal; nonanal; decanal).

In addition, the kinetic profile of the carbonyls compound in palm oil until 11 h oxidation still want to reach the highest amount of secondary oxidation products with Rancimat at 120 °C (Fig. 4). For 11h oxidation, the carbonyls which detected were hexanal 0.92 mmol/g, heptanal 0.32 mmol/g, octanal 0.55 mmol/g, nonanal 0.41 mmol/g and decanal 0.31 mmol/ g for UPO (figure 3).This is not surprising as palm oil is rich in oleic acid with 45 % of the total fatty acids. Carbonyls can be produced by homolytic cleavage A and B on the either side of the alkoxyl intermediate group from 8-, 9- , 10-, and 11- from autoxidized oleate. The octanal is formed from the 11-

hydroperoxide and the nonanal from either 9- or 10- hydroperoxide (Frankel, 2005). The other fatty acids in palm oil are linoleic acid 12.2% and palmitic acid 37.3%.

The carotenoids α-carotene, β-carotene, and lycopene contribute to the color of red palm oil. Red palm oil is a form of processed palm oil in which ca. 80% of the original carotenoids are retained. This means that it is a remarkable source of vitamin A [30]. The quantitative analysis showed that β-carotene is completely oxidized in the oil decreased with a natural content of 2.34 mg/g within 8 h of the Ranzimat treatment (Fig.5).

β-carotene plays an important role during the oxidation of oils and fats. Some studies have been done to the stability of β-carotene. Henry et al [31] studied the thermal and oxidative stability of all-*E*-β-carotene, 9-*Z*-β-carotene, lycopene, and lutein in safflower seed oil at 75, 85 and 95 °C for 24, 12 and 5 h, respectively. The carotenoids were found to degrade following a first-order kinetic model. The rates of degradation were lycopene > all-*E*-β-carotene ≈ 9-*Z*-β-carotene > lutein. Other studies showed that carotenoids were degraded with a first-order kinetics model, which was dependent on concentration. The highest degradation rates were observed in extracts prepared with linoleic acid containing oils such as sunflower, soybean oil and grape-seed oil [32]

β-Carotene was found to degrade in the first few hours of thermal treatment [33]. This statement can be strongly supported from the experiment which has been done. The β-carotene is completely oxidized after 8 h oxidation of UPO. The concentration of β-carotene at 0 h oxidation was 2.34 mg/g. However, the formation of secondary oxidation products lasts until 18 h of heating.

The previous experiments of [34], showed that β-carotene was more stable in the corn oil than in the olive and model TAGs samples. The β-carotene degraded completely before 12 h in the corn oil of the thermal treatment in the Rancimat at 110 °C. However, β-carotene can act as prooxidant and has a lower stability compared to astaxanthin during thermal degradation in olive oil [33].

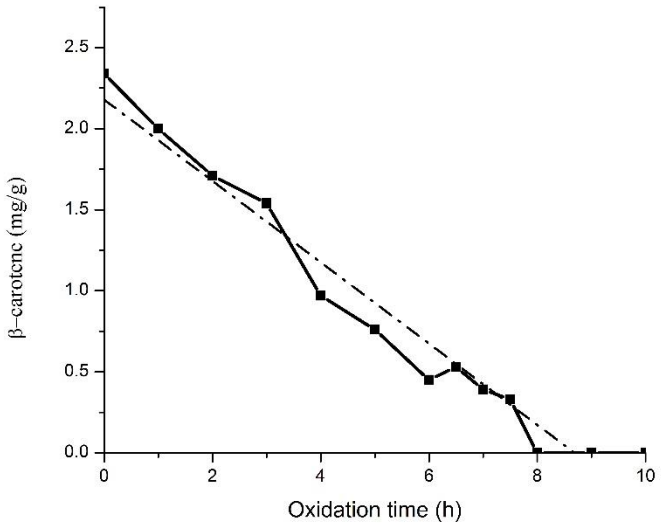

Figure 5. Degradation of β-carotene in unrefined palm oil

The HPLC-DAD method developed for the β-carotene in the unrefined palm oil was validated. The coefficient of determination (0.999) of the linear regression indicates a good correlation between the peak area and the amount of β-carotene. The linearity of the β-carotene analysis was tested in the range 0.00078-0.05 μg/cm^3 according to Mandel [35]. The limit of detection (LOD) was 2.65 μg/ cm^3 and the limit of quantification (LOQ) was 8.83 μg/cm^3 for β-carotene.

Crude Palm Oil (CPO) is intensively orange-red colored and is semi-solid at room temperature. This can be separated into liquid (olein rich) and solid (stearin rich) fractions based on their melting point difference [36]. CPO is separated into palm olein (82 %) which is a red liquid oil containing 39 – 45 % oleic acid and 10 – 13 % linoleic acid. The second fraction palm stearin is just 18 % w/v which is a yellow solid fat that contains 47 – 74 % palmitic acid [37]. In addition, in palm olein vitamin E enriches which is 30 % higher than in palm stearin. 85.7 % of total tocopherols are recovered during the production of palm oil [38].

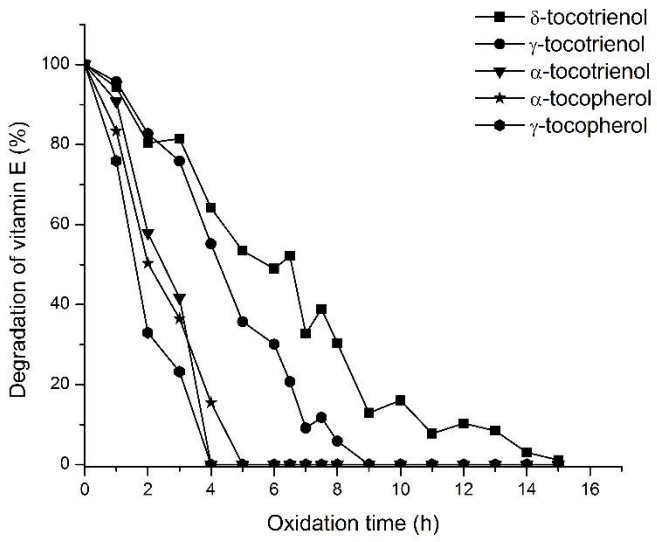

Figure 6. Degradation of vitamin E in unrefined palm oil

The results show that the tocotrienols are more stable than the tocopherols. The δ-tocotrienol content was 3.01 mg/g in the fresh oil. It still remaining in the oxidized oil after 15 h in the Ranzimat with 0.03 mg/g δ-tocotrienol or 1.13 % compare to the fresh oil. It has been shown that half of the δ-tocotrienol was destroyed after 5 h of oxidation. The γ-tocotrienol with an original content of 11.48 mg/g was less stable. 50 % of it was degraded after 4 h of oxidation with remaining 0.68 mg/g after 8 h (5.89 %) (Fig. 6). As we already know, tocotrienols have been found to have antioxidant and anti-cancer activities. γ and δ-tocotrienol derived from palm oil exhibit a strong activity not only against tumor promotion but also inhibit certain types of cancer, such as breast cancer cell [39].

In addition, the α-tocotrienol was completely degraded after 4 hours being 0.48 mg/g in the fresh oil. Furthermore, for α-tocopherol at 0 h with 39.96 mg/g was eliminated completely within 5 h. Both α-tocotrienol/tocopherol were degraded by 50 % after 2 h of oxidation. γ-Tocopherol just

remained until 3h with 0.22mg/g compared to the fresh oil with 0.94 mg/g. At low concentration ≤ 100 μg/g for tocopherol, α–Tocopherol appeared to be the most effective antioxidant [40-46].

The oxidation experiments of PO showed that δ-tocotrienol was 2.10 mg/g at 0 h to 0.49 mg/g at 11 h . On the other hand, γ-tocotrienol was present until 11 h with 0.18 mg/g from the starting point at 0 h with 9.45 mg/g while, α-tocotrienol was completely oxidized after 10 h of oxidation. The δ-tocotrienol, γ–tocotrienol and α-tocotrienol has a good stability against the oxidation of refined palm oil. The tocotrienols still remained in the oil until 10 h until 11 h oxidation in the Ranzimat. The δ-tocotrienol was stable throughout the experiment with an oxidation of 23.35 %. α-Tocopherol and γ-tocopherol were completely oxidized within 6.5 h (Fig. 7).

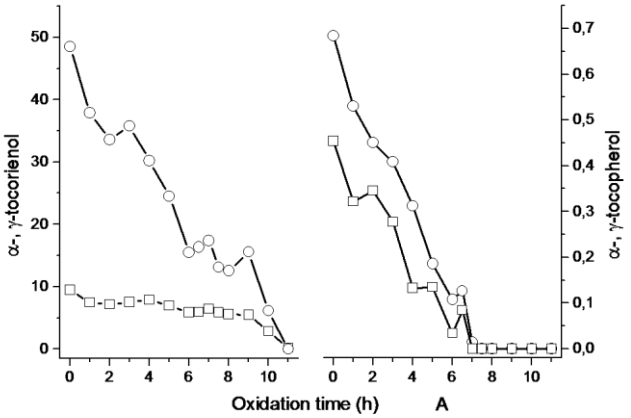

Figure 7. Degradation of tocotrienols (A) and tocopherols (B) in refined palm oil.

The HPLC-UV method was developed for determination of α- and γ-tocopherol in the palm oil. The validation showed that the method could be used for palm oil. The coefficient of correlation (0.999) of the linear regression indicates a good correlation between the peak area and the amount of α-tocopherol and γ-tocopherol, respectively. The limit of detection (LOD) was 0.14 μg/cm^3 and

limit of quantification (LOQ) was 0.47 μg/cm^3 for γ-tocopherol. In addition for α-tocopherol, the limit of detection (LOD) was 460 μg/cm^3 and the limit of quantification (LOQ) was 1530 μg/cm^3.

The absence of carotenoids in refined palm oil is not only due to the various refining steps (bleaching). It could also be reduced because of thermal deterioration which is able to remove the β-carotene from crude palm oil [47-52].

Conclusion

The kinetic profile of the unrefined palm oil shown that the carbonyls compound still reach the highest amount until 18 h oxidation times with Ranzimat with 120 °C. For 18 h of oxidation, the carbonyls which were detected were hexanal 1.6 mmol/g, heptanal 0.61 mmol/g, octanal 1.36 mmol/g, nonanal 0.72 mmol/g and decanal 0.95 mmol/ g for UPO. As we already know that the percentage of oleic acid in UPO was 41.9% , linoleic acid 9.30% and palmitic acid with 43.1%.

However, the kinetic profile of the carbonyls compound in refined palm oil until 11 h oxidation still want to reach the highest amount of secondary oxidation products. For 11h oxidation, the carbonyls which detected were hexanal 0.92 mmol/g, heptanal 0.32 mmol/g, octanal 0.55 mmol/g, nonanal 0.41 mmol/g and decanal 0.31 mmol/ g for UPO. This is not surprising as palm oil is rich in oleic acid with 45 % of the total fatty acids

The analytical method for quantifying the vitamin E and β−carotene are quick, reliable, precise, economical and suitable for the routine analysis. For both analyses a simple dilution of the oil was necessary.

Looking at the kinetic profile of the carbonyl formation in unrefined palm oil and refined palm oil, both of the oils were still stable until 10 h of oxidation with the Ranzimat at 120 °C. The quantitative analysis showed that β-carotene in the oxidized unrefined palm oil decreased from

2.34 mg/g to 0.33mg/g within 7.5 h of oxidation. However, after 7.5 h, no more β-carotene was detected. In addition, β-carotene was not detected in refined palm oil.

The two tocotrienols (δ and γ) are more stable than the α- and γ-tocopherol. The δ–tocotrienol is degraded slowly and can be found even after 15 h of oxidation with 0.035 mg/g remaining in the oxidized unrefined palm oil. However, in refined palm oil, the δ-tocotrienol could be determined until 11 h of oxidation with 0.49 mg/g before disappearing completely.

In unrefined palm oil γ-tocotrienol was less stable than δ-tocotrienol with 0.68 mg/g remaining in unrefined palm oil after 8 h. On the other hand, in palm oil the γ-tocotrienol still remained with 0.2 mg/g after 11 h of oxidation. Furthermore, α-tocopherol was measureable for 4 h and γ-tocopherol for 3 h in unrefined palm oil. The tocopherols (α and γ) disappeared within 7 h.

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The authors have declared no conflict of interest

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SECTION 5

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