Carotenoids and Triacylglycerols Interactions during Oxidation

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I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and I have explicitly marked all materials which have been quoted either literally or by content from the used sources.

Furthermore, I declare that this work has so far neither been submitted to the Faculty of Technical Chemistry at the Technical University of Graz, nor to any other scientific institution for the purpose of Doctorate or any other degree program.

Graz, 08.11.2010 **Alam Zeb**

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Alam Zeb

To my

Mother

Who's Love and Prayers were the

Sources of all my

Strengths and Happiness

ABBREVIATIONS AND SYMBOLS

HPTLC	*High performance thin layer chromatography*
HPLC	*High performance liquid chromatography*
ELSD	*Evaporative light scattering detector*
LC-MS	*Liquid chromatography with mass spectrometry*
APCI-MS	*Atmospheric pressure chemical ionization mass spectrometry*
ESI-MS	*Electro-spray ionization mass spectrometry*
PDA	*Photo diode array*
BHT	*Butylated hydroxy toluene*
MTBE	*Methyl tert-butyl ether*
AMVN	*2,2'-Azobis, 2,4-dimethylvaleronitrile*
TAGs	*Triacylglycerols*
DAGs	*Diacylglycerols*
MAGs	*Mono-acylglycerols*
PUFA	*Poly unsaturated fatty acid*
LOOH	*Lipid hydroperoxide*
M	*Molecular ion*
β-	*Beta-*

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Abstract

Carotenoids (β-carotene and astaxanthin) were oxidized in high oleic model triacylglycerols and edible oils such as corn and olive oils. The main techniques used in this dissertation were HPTLC and HPLC coupled to DAD and mass spectrometry. The previous literature on the uses of TLC suggests that HPTLC have the potential to be the first choice in the analysis of carotenoids in foods. We also found that HPTLC is a useful tool in the study of degradation of β-carotene in model triacylglycerols and edible oils.

The isocratic HPLC-ESI-MS method was very useful for the fast screening and identification of triacylglycerols in edible oils. We correctly identify and separated thirteen; fourteen, fifteen and sixteen TAGs in refined olive oil, rapeseed oil, corn oil and sunflower oil, respectively. The oxidation products of TAGs were also studied using this method. Epoxy epidioxides, hydroxy bis-hydroperoxides and epidioxy bis-hydroperoxides were identified as major oxidized compounds that have been identified for the first time in model triacylglycerols and edible oils under similar conditions. Other triacylglycerols oxidized species were hydroxy hydroperoxides, mono-hydroperoxides, bis-hydroperoxides, epoxy-epidioxides, and epoxides.

Significant degradation of β-carotene was observed in sunflower oil. In high oleic model TAGs, β-carotene degraded significantly in the first three hours, however, in olive oil of relatively similar triacylglycerols composition, β-carotene degraded slowly. Astaxanthin degradation was much slower than β-carotene in olive oil. The HPLC method for the degradation and oxidation of carotenoids reveal a total of eight oxidized compounds of β-carotene in corn oil. The degradation of all-*E*-β-carotene in corn oil was relatively similar to model TAGs and olive oil.

The interactions of carotenoids and triacylglycerols reveal the pro-oxidant action of both β-carotene and astaxanthin. The pro-oxidant action of β-carotene was much stronger than astaxanthin. These findings help us to understand the structural characterization of triacylglycerols using mass spectrometry and the possible role and interactions of carotenoids or its oxidation products with the normal and oxidized triacylglycerols during thermal oxidation.

Kurzfassung

Die beiden Carotinoide (β-Carotin und Astaxanthin) wurden in einer definierten Mischung von Triglyceriden mit einem besonders hohen Anteil an Ölsäure bzw. in Maiskeimöl und Ölivenöl oxidiert.

Für die Analysen wurden hauptsächlich HPTLC sowie HPLC-DAD und HPLC-MS verwendet. Aus früheren Publikationen geht hervor, dass insbesondere die HPTLC gut geeignet ist, die Carotinoide in Lebensmitteln zu analysieren. In der hier vorgelegten Arbeit konnte ebenfalls gezeigt werden, dass die HPTLC für diesen Zweck verwendet werden kann. Damit können die Carotinoide in den Modell-Triacylglyceriden und den Speiseölen analysiert werden. Eine isokratische Trennung auf der HPLC mit einer massenselektiven Detektion kann als schnelle Screening-Methode verwendet warden, welche zusätzlich eine Identifikation der Triacylglyceride in den Speiseölen ermöglicht. Es wurden bis zu 16 TAGs in raffinierten Ölen (Olivenöl, Rapsöl, Maiskeimöl, Sonnenblumenöl) mittels der HPLC getrennt und mit Hilfe der ESI-MS eindeutig identifiziert. Die Oxidationsprodukte der TAGs wurden ebenfalls mit dieser Methode untersucht. Dabei konnten Epoxy-epidioxide, Hydroxy-bis-hydroperoxide und Epidioxy-bis-hydroperoxide als die Hauptoxidationsprodukte zum ersten Mal im Modellsystem und in den Speiseölen identifiziert werden. Weitere Oxidationsprodukte umfassten Hydroxy-hydroperoxide, mono-Hydroperoxide, bis-Hydroperoxide, Epoxy-epidioxide sowie Epoxide.

Ein ausgeprägter Abbau von β-Carotin wurde in Sonnenblumenöl beobachtet. Im Modellsystem wird das β-Carotin innerhalb der ersten drei Stunden abgebaut, währenddessen im Olivenöl der Abbau von β-Carotin wesentlich langsamer vonstatten ging. Der Abbau von Astaxanthin in Olivenöl war signifikant langsamer im Vergleich zum Olivenöl. Mittels der HPLC-MS konnten insgesamt 8 oxidierte Verbindungen von β-Carotin identifiziert werden. Der Abbau von all-E-β-Carotin war im Maiskeimöl und im Modellsystem vergleichbar.

Die Wechselwirkungen der Carotinoide mit den Triacylglyceriden weisen auf eine prooxidative Wirkung von beiden Carotinoiden hin. Die prooxidative Wirkung von β-Carotin war wesentlich ausgeprägter als die von Astaxanthin.

Diese Ergebnisse erleichtern die strukturelle Identifizierung von Triacylglyceriden mittel Massenspektrometrie und zeigen auch eine mögliche Interaktion von Carotinoiden oder ihren Oxidationsprodukte mit den Triacylaglyceriden bzw. deren Oxidationsprodukten bei der thermischen Oxidation hin.

1. INTRODUCTION

1.1 Carotenoids

Carotenoids are among the most widespread and important pigments in living organisms. They are fat soluble and present as food components. Carotenoids are derived from a 40-carbon polyene chain, which is considered to be the backbone of the molecule. They are a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) consisting of eight isoprenoid units, which are joined in such a manner that the arrangement of isoprenoid units is reversed at the centre of the molecule. Thus two central methyl groups are in 1,6-position and the remaining non-terminal methyl groups are in a 1,5-positional relationship (Britton et al., 1995). Carotenoids are formally derived from the acyclic $C_{40}H_{56}$ structure with a long central chain of conjugated double bonds, by hydrogenation, dehydrogenation, cyclization, oxidation or any combination of these processes. With a few exceptions (phytofluene, ζ-carotene and lycopene) the hydrocarbon chain is terminated by cyclic end-groups, which may also be complemented with oxygen-containing functional groups (Britton, 1995).

Carotenoids are found in common foods of plant and animal origin (Rock, 1997). Most xanthophylls are found in green leafy vegetables and nearly all carotenes are found in yellow vegetables (Rodriguez-Amaya, 2001). Carotenoids are present in some non-photosynthetic bacteria, yeasts, and molds, where they may act as protective function against damage by light and oxygen. However, in contrast to this, animals are incapable of synthesizing carotenoids. They incorporate carotenoids from their diet, which are accumulated in certain tissues. In animals, carotenoids give bright coloration, serve as antioxidants, and are a source of vitamin A (Ong & Tee, 1992; Britton, 1995). Carotenoids are responsible for many colors like red, orange, and yellow of plant leaves, fruits, and most of the flowers, as well as the colors of some birds, insects, fish, and crustaceans (Gordon & Bauernfeind, 1981). Some familiar examples of carotenoids coloration are the oranges of carrots and citrus fruits, the reds of peppers and tomatoes, and the pinks of flamingoes and salmon (Pfander, 1992). This thesis deals only with the β-carotene and astaxanthin oxidation as examples of carotene and xanthophyll.

1.1.1 β-Carotene

β-Carotene (β, β-carotene) is the most important and widely studied carotenoid. It is a strongly red-orange colored compound. It is a good source of vitamin A (Goodman, 1984). It serves as a biological antioxidant and is helpful in maintaining human health (Paiva & Russel, 1999). In food industries it is used as colorant and also as a source of pro-vitamin A. β-Carotene is commercially produced in large scale by chemical synthesis and also from different plant sources. Red palm oil is now one of the main sources of the plant oil containing large amount of β-carotene. β-Carotene is also produced by fermentation and from microalgae (Dufosse, 2009). In fruits and vegetables β-carotene may occur as a mixture of its geometrical isomers (Figure 1.1). During processing *E*/*Z* isomerization is more frequent. Thus some other antioxidants like BHT or ascorbic acid are added to prevent the isomerization and oxidation of carotenoids.

Naturally the *Z*-β-carotene isomers are present in mango fruits (Godoy and Rodriguez-Amaya, 1994, Pott et al., 2003 and Vásquez-Caicedo et al., 2005), which suggests that their exposure to heat and light may result in to *E*-*Z*-isomerization. It has been reported that 13-*Z*- and 9-*Z*–β-carotene are formed during vegetable preparations due to heat and illumination, respectively (Chen et al., 1994, 1996 and Marx et al., 2003).

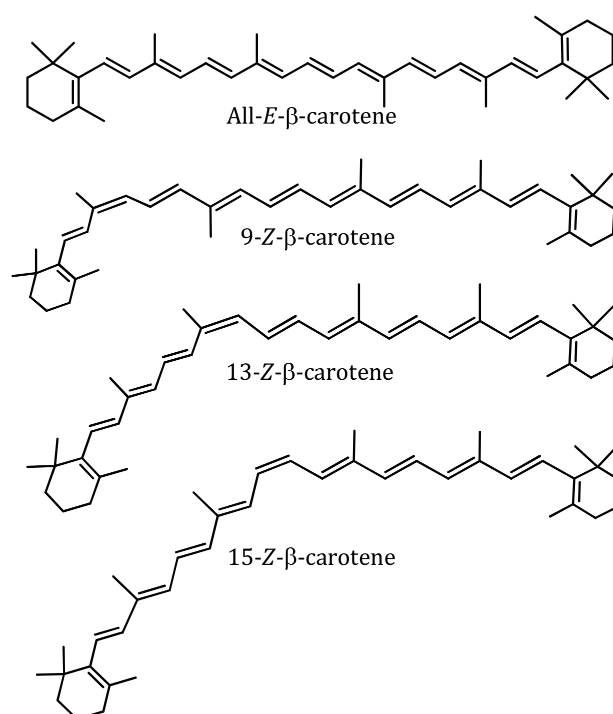


Figure 1.1: All-E-β-Carotene and its Z-isomers.

It was reported that 13-*Z*–β-carotene formation was enhanced in carrot juice in the presence of lipids at temperatures of 80–100 °C for 30–60 min, and also using the addition of grape seed oil prior to thermal preservation (Marx et al., 2003). The thermal oxidative degradation of β-carotene has been found to decrease the nutritional value of vitamin A and the activity as antioxidant. The thermal oxidation of β-carotene also causes the loss of natural flavor and chromophores of foods. The food products are thus less acceptable or unacceptable to consumers (Ager & Schroeder, 1993). β-Carotene is added to some commercial fruit and vegetable beverages (Rodríguez-Comesana et al., 2002). It is thus extremely important to protect β-carotene, in fruits, vegetable juices and beverages during processing and storage in order to maintain the high quality of nutrition and flavor of products. Thus the study of biosynthesis, reactions, antioxidant functions and degradation or oxidations of β-carotene are important tasks for the present day food chemist.

1.1.2 Astaxanthin

Astaxanthin is a keto-carotenoid (3,3’-dihydroxy-β-β’-carotene-4,4’-dione). It belongs to the family of the xanthophylls, biosynthesized in plants from lycopene. It is important and economically valuable carotenoids due to its biological function as pro-vitamin A (Gobantes et al., 1998). Astaxanthin is one of the main pigments in crustacean, salmons and sea foods. It provides the desirable reddish-orange color in these organisms and serves as natural antioxidants (Higuera-Ciapara et al., 2006). It is also an essential nutritional component for adequate growth and reproduction. Astaxanthin has a strong antioxidant property which has been reported to surpass those of β-carotene or even α-tocopherol (Miki, 1991). It helps to protect the organism against cardiovascular problems, different types of cancer and immunological diseases.

Synthetic astaxanthin is an identical molecule to that produced in living organisms and it consists of a mixture 1:2:1 of isomers (3S, 3S’), (3R, 3S’), and (3R, 3R’), respectively (Figure 1.2). It is the main carotenoid used worldwide in the aquaculture industry. About 90% of the astaxanthin is produced by chemical synthesis, while the rest is produced from algal products.

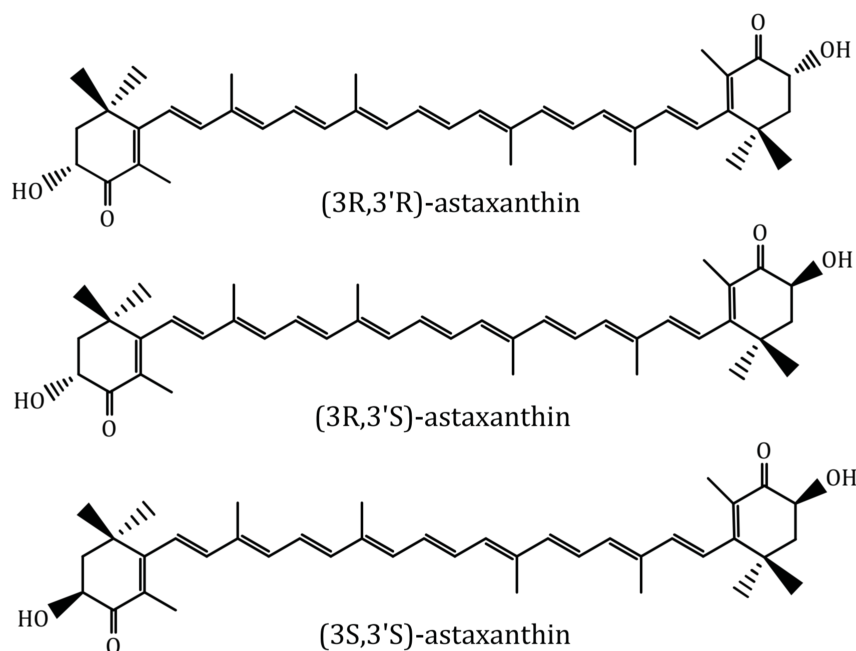


Figure 1.2: The three possible R & S isomers of all-E-astaxanthin present in nature.

A large scale production of synthetic astaxanthin was started by Roche in 1990, which practically fulfilled the world market requirement, estimated at 150–200 million dollars. However, the high cost of synthetic pigments and the growing demand for natural foods has tremendously stimulated the search for natural sources of astaxanthin with potential for industrialization. So far only a few sources of microbial origin were found to compete economically with synthetic astaxanthin. They are the green microalgae *Haematococcus pluvialis* and the red yeast *Phaffia rhodozyma*. Their production method has been presented by Parajo et al. (1998). However, because of the limited production, these microbial methods take up a very small fraction of the market production (Higuera-Ciapara et al., 2006; Bjerkeng, 2008).

Astaxanthin was found to possess an unusual antioxidant activity, health benefits such as cardiovascular disease prevention, immune system boosting, bioactivity against *Helicobacter pylori*, and cataract prevention. Thus research on the health benefits of astaxanthin is very recent and has mostly been performed in vitro or at the pre-clinical level with humans. However, less attention has been given to the fate of astaxanthin during daily cooking and its interactions with other food ingredients such as triacylglycerols. A part of this thesis thus deals with the thermal interactions of astaxanthin in triacylglycerols, which would give us information about what we eat during astaxanthin containing cooked foods.

1.1.3 Chromatographic Methods for Carotenoids Analysis

Different chromatographic methods are used for the separation and identification of carotenoids. A number of recent review articles (Oliver & Palou, 2000; Moret & Conte, 2000; Cserhati & Forgacs, 2001; Schoefs, 2002; Su et al., 2002; Sun et al., 2005; Quiros & Costa, 2006; Marston, 2007) describe the uses of different chromatographic techniques for the analyses of carotenoids or related compounds. However, the work reported in this thesis is based on high performance thin layer chromatography (HPTLC) and largely on high performance liquid chromatography (HPLC) for the qualitative and quantitative analysis of carotenoids.

1.1.3.1 Thin Layer Chromatographic Analysis

Thin-layer chromatography (TLC) is widely used for food and pharmaceutical analyses. The applications of TLC in food analyses comprises studies of composition, adulteration, contaminants, degraded and decomposed products of carbohydrates, proteins, lipids and vitamins (Sherma, 2000). The technique is often used for the separation and isolation of individual classes of molecules as TLC is fast, effective and relatively cheap. Earlier TLC methods used silica stationary phase and a non-polar mobile phase for the separation of carotenoids (Buckle & Rahman, 1979; Cserhati et al., 1993; Cserhati & Forgacs, 2001). Non-oxygenated carotenoids like β-carotene have higher R_F-values than oxygenated carotenoids, due to the absence of polar interactions as in the case of xanthophyll (Figure 1.3). Davis (1976) described some important and practical aspects like choice of adsorbents, preparation and running of chromatograms, detection and elution of carotenoids by old TLC techniques.

The progress and commercialization of new stationary TLC phases (octyl-, octadecyl-, cyano-, diol-, and aminopropyl silica) highly increased the scope of applications of TLC. The separation of color pigments of paprika has been performed on a considerable number of stationary phases and the results have been compared (Cserhati et al., 1993). A recent review by Poole (2003) showed some of characteristics of different thin layers and its eluents for various TLC systems. Thus TLC is classified into normal and reversed phase on the basis of types of thin layers.

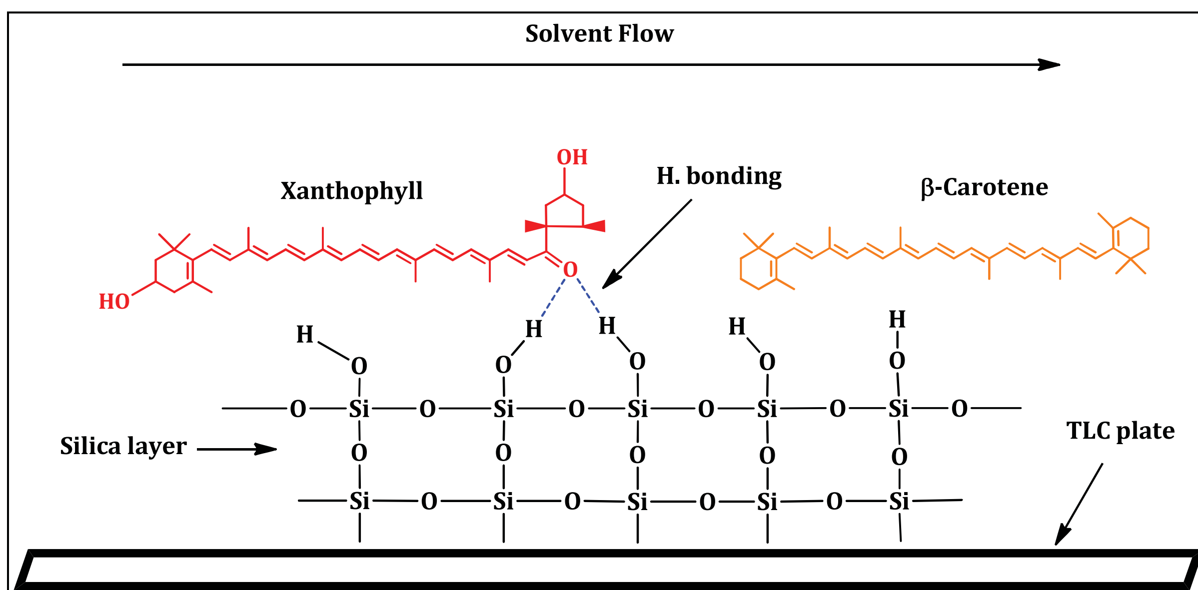


Figure 1.3: Schematic representation of the separation of oxygenated and non-oxygenated carotenoids on the surface of a TLC plate.

1.1.3.2 High Performance Liquid Chromatographic Analysis

High performance liquid chromatography (HPLC) is the most important and widely used separation technique used in carotenoids analysis. HPLC has significant advantages over other techniques, in terms of robustness, speed, sensitivity, specificity, precision, and sample preservation (Su et al., 2002). For carotenoids analysis both isocratic and gradient mobile phase systems are used. The separation can be carried out using normal phase as well as reversed phase stationary phases. In order to avoid the degradation or oxidation of carotenoids, the separation is achieved at a controlled temperature and the uses of antioxidants like butylated hydroxytoluene (BHT) in the mobile phase (Rodriguez-Amaya, 2001; Murkovic et al., 2006).

HPLC with reversed-phase C_8 and C_{18} bonded phase columns are commonly used for the separation of carotenoids. Recently the polymeric C_{30} stationary phase has been developed, which greatly improved the separations of carotenoids. The properties of the C_{30} column were modified specifically for the analysis of carotenoids. The adequate retention time and excellent selectivity of both polar and non-polar carotenoids, including structural and geometrical isomers has been achieved (Craft, 1992; Sander et al., 1994; Emenhiser et al., 1995, 1996; Clinton et al., 1996). Excellent separations of all-*E* carotenoids from mixtures of standards as well as several extracts have been achieved by Sander et al. (1994). They showed that the C_{30} stationary phase possesses a high degree

of selectivity toward geometrical isomers of β-carotene. Later on Emenhiser et al. (1995) explored more extensively the shape selectivity of the C_{30} column and separated geometrical isomers of six common carotenoids on the C_{30} column. Selectivity of the C_{30} phase toward these isomers was especially good and superior to that of the other stationary phases tested.

Among reversed phase columns, the C_{30} stationary phase has a unique property of resolving geometrical isomers of asymmetrical carotenoids in which *Z* bonds are present at the same carbon number but at opposite ends of the molecule. For example, a good separation of 9 & 13-*Z* and all-*E* isomers of β-carotene was achieved with YMC-C_{30} column in cassava (Rodriguez-Amaya & Kimura, 2004) as shown in Figure 1.4. This separation was obtained using isocratic mobile phase consisting of methanol-*tert*-butyl ether (MTBE) (80:20) at the flow rate of 0.8mL/min. The previous C_{30} carotenoid separations were obtained using either gradient programmed elution (Craft, 1992; Sander et al., 1994) or geometrical isomers of individual carotenoids under isocratic conditions of chromatography (Clinton et al., 1996; Emenhiser et al., 1996).

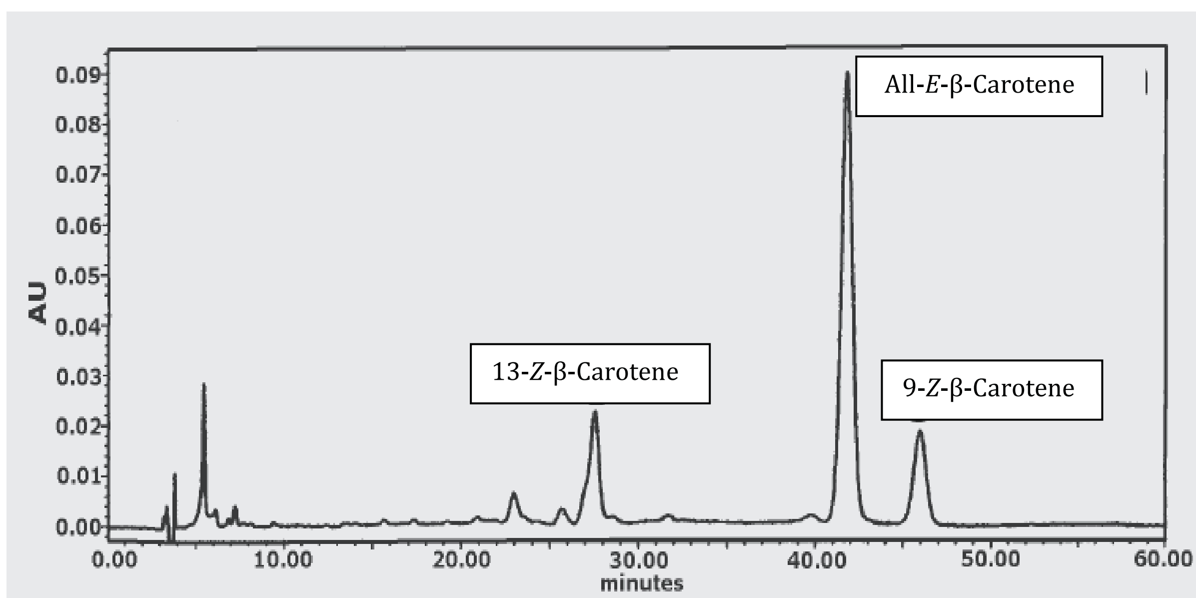


Figure 1.4: Separation of geometrical isomers of β-carotene using YMC C_{30} column with isocratic methanol-MTBE (80:20) in cassava (Rodriguez-Amaya & Kimura, 2004).

Several different types of detectors have been used for the detection of the separated carotenoids in HPLC. Electrochemical array detector (Ferruzzi et al., 2001) has been used for separation and identification of 13 lycopene isomers. Standard ultraviolet-visible (UV-Vis) detector of single wavelength has been used for many years for the

quantification of carotenoids in foods. However, the coupling of photodiode array detector (PDA) to the HPLC allows us to collect a large set of spectrophotometric data of carotenoids during the analysis (Su et al., 2002).

Mass spectrometric detector (MSD) coupled to liquid chromatography is the most acceptable choice for characterization and structure elucidation of carotenoids. Atmospheric chemical ionization mass spectrometric (APCI-MS) techniques have been widely used for characterization of carotenoids and its oxidation products. Van Breeman et al. (1996) published the first report on the LC-APCI-MS analysis of carotenoids. Carotenoids were separated using C_{30} column and a gradient elution of methanol and MTBE. Ammonium acetate was added to the mobile phase. It has been found that the solvent composition greatly influences the relative abundances of the molecular ions and its protonation (Van Breeman, 1997). Polar solvents especially alcohols enhance the formation of protonated carotenoids. MTBE was found to increase the abundance of molecular ions. The fragmentation of carotenoids molecules may also be helpful in explaining the exact structure. For example α-carotene forms $[M-56]^+$ of m/z 480, while β-carotene eliminates toluene from the molecular ion $[M-92]^+$ (Pajkovic & van Breeman, 2005). Astaxanthin monoesters had characteristic fragment ions at m/z 597 $[M+H\text{-fatty acid}]^+$ and m/z 579 and 561 that resulted from a continuous loss of water (Miao et al., 2006). LC coupled to APCI-MS and PDA was used for the analysis of carotenoids in vegetable juice using C_{30} reversed phase column. A gradient elution of methanol and MTBE was used for separation. APCI-MS revealed protonated carotenoids however, *Z*-carotenoids have been identified using the PDA detector.

1.1.4 Thermal Degradation of Carotenoids

β-Carotene is oxidized by thermal treatment at different temperatures. Increasing temperature will increase the oxidation rate. The first step in the oxidation of β-carotene is the conversion to its *Z*-isomers. However, there is no change in overall color during isomerization. Other reactions include the intra-molecular cyclization to form volatile compounds; degradation to form aldehydes and ketones with low molecular weight. Isomerization is a reversible reaction, while the degradation or oxidation is non-reversible reactions. Carotenoids degradation in food or similar model systems is a highly complex phenomenon. However, some authors proposed a first order kinetic model for

the degradation (Capellos & Bielski, 1972; Rios et al., 2005). Borsarelli and Mercadante (2010) showed a simplified mechanism of overall changes occurring in carotenoids during heating (Figure 1.5).

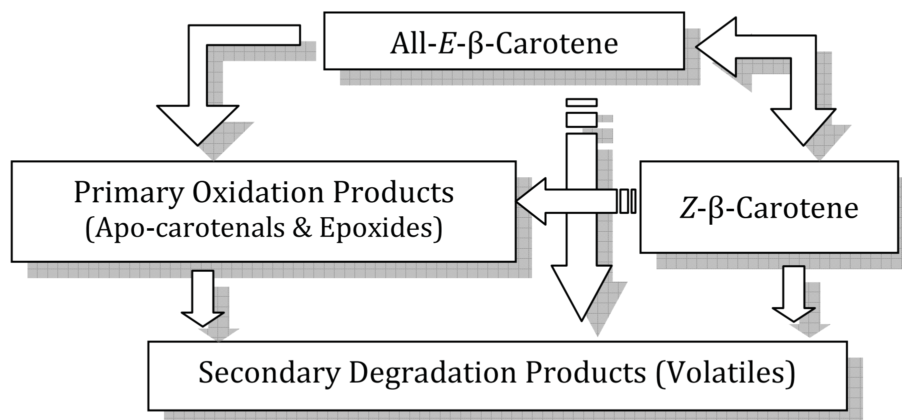


Figure 1.5: Schematic representation of overall structural changes occurring in carotenoids during heating (modified from Borsarelli & Mercadante, 2010).

The mechanism however, does not say anything about the susceptibility of *Z*-isomers towards oxidation or degradation. *Z*-isomers are commonly found in various foods systems. This mechanism fits to the carotenoids in solution. In solutions *E* & *Z* isomers are in equilibrium. However, the solvent also affects the isomerization induced by heating. For example, the isomerization rate of β-carotene is higher in non-polar solvents (petroleum ether and toluene) than in polar solvents (Zechmeister, 1944).

1.1.4.1 Carotenoids Oxidation in Solutions

Several studies have been carried out on the carotenoids oxidation in experimental models by using only organic solvents and a flow of oxygen. However, only the oxidation of β-carotene and astaxanthin as model carotenoids will be presented here. El-Tinay and Chichester (1970) studied the reaction between β-carotene and oxygen in toluene at 60°C in the dark. The products of the reaction were identified as 5,6- and 5,8-epoxides, 5,6; 5´,6´- and 5,8; 5´,8´-diepoxides of β-carotene. They proposed that the site of "initial attack" of oxygen was on the terminal carbon–carbon double bond with the highest electron density in the polyene chain. They also found overall zero-order reaction kinetics. Similarly Handelman et al. (1991) oxidized β-carotene in toluene at 60°C, in the presence of oxygen for 120 min. The 5,6-epoxide of β-carotene and apo-carotenals were identified using HPLC and mass analysis. Using similar experimental conditions but lower

temperatures and longer times (β-carotene, benzene or tetra chloromethane, 30°C, oxygen, dark, 48 and 77 h), Mordi et al. (1993) identified mono- and diepoxides of β-carotene, Z-isomers, apo-carotenals, volatile short compounds, and minor or oligomeric compounds not previously identified. They proposed a free radical-mediated reaction in which the initiation process involves the formation of a diradical of β-carotene. In all these cases very similar types of products were found, which are mono- and diepoxides and apo-carotenals.

Numerous studies have been reported in order to quantify carotenoids remaining during thermal degradation, and also the oxygenated cleavage products formed. Bonnie & Choo (1999) presented an excellent review on the thermal degradation of carotenoids. They showed that thermal treatments generate not only oxygenated cleavage compounds but also oxidized compounds that do not necessarily undergo a cleavage reaction of the hydrocarbon chain, such as epoxides or furanoxides of the parent carotenoids. Thermal treatments can also produce non-oxygenated compounds formed by cleavage of the polyenic chain followed by a rearrangement, by means of a radical mechanism.

Caris-Veyrat et al. (2001) studied the oxidative cleavage of β-carotene by dioxygen induced by a ruthenium porphyrin catalyst. The complete range of β-apo-carotenals and β-apo-carotenones formed were identified using HPLC-DAD and HPLC-MS. They found *Z*-isomers as first products formed after 1 h of reaction. *Z*-isomers, epoxides and apo-carotenals were identified after 6h of reaction. They found that after 24h there was no more β-carotene present in the reaction mixture. They also proposed a reaction mechanism as shown in Figure 1.6. They proposed that β-carotene may be first isomerized and then oxidized and cleaved to form apo-carotenals. Apo-carotenals may either undergo a second cleavage to produce diapo-carotendials or which may be oxidized into 5,6-epoxide. These epoxides may either isomerize to give an epoxy-apo-carotenals, or may cleave into diapo-carotendials, or it may be directly cleaved to produce a diapo-carotendial. Apo-carotenals bearing epoxide or furanoxide functions may also be formed by the cleavage of the corresponding epoxide/furanoxide β-carotene.

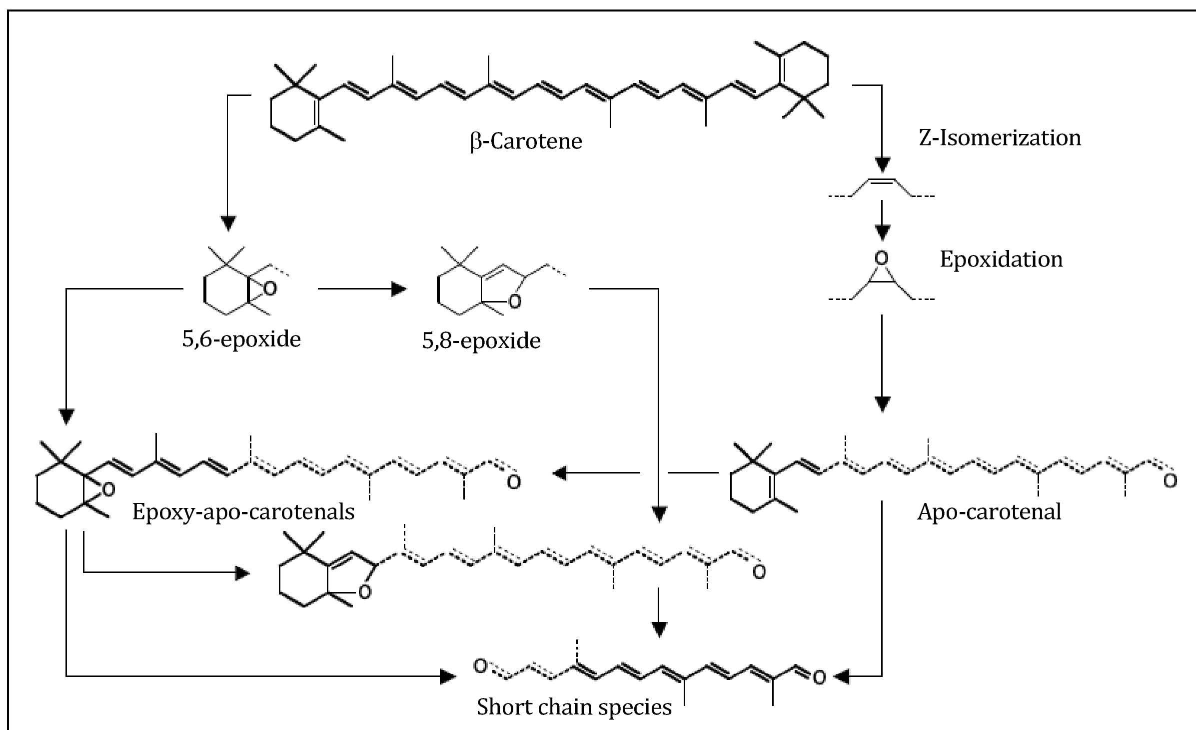


Figure 1.6: Caris-Veyrat et al. (2001) proposed oxidative degradation of β-carotene by molecular oxygen in the presence of ruthenium tetramesitylporphyrin. Dotted line represents varied chain length.

Different oxidizing reagents have been used in order to obtain oxygenated derivatives such as epoxides (Rodriguez & Rodriguez-Amaya, 2007), dihydrooxepin (Zurcher et al., 1997), ozonides (Zurcher & Pfander, 1999), or oxo-carotenoids (Molnar et al., 2006). These reactions may also produce carotenoid oxygenated cleavage compounds discussed above as by-products (Molnar et al., 2006). The presence of carotenoids epoxides can be confirmed by exposing a silica thin-layer (developed with 5% methanol in toluene) chromatograms to HCl fumes, which gives blue-green and blue spots of mono-epoxides and diepoxides, respectively (Rodriguez & Rodriquez-Amaya, 2007).

1.1.4.2 Carotenoids Oxidation in Lipids

Carotenoids play an important role during the auto-oxidation of oil and fats. The results obtained during the early investigations show that the behavior of β-carotene in the oxidation process depends strongly on its concentration, on the lipid medium, as well as on whether the process proceeds in the dark or in the light and in the presence and absence of other antioxidants and proxidants. Hunter & Krackenberger (1947) oxidized β-carotene at 50 °C in benzene and in peanut oil. They found that the rate of the oxidation

process was much higher in benzene than in the oil. They attributed this fact to the presence of a natural antioxidant in the peanut oil. Earlier studies of carotenoid auto-oxidation described the influence of lipids or other antioxidants on the auto-oxidation of carotenoids (Lisle, 1951; Budowski & Bondi 1960). Budowski & Bondi (1960) showed the influence of fat was found to be a "pro-oxidant" towards carotenoids. The carotenoids were believed to be oxidized by molecular oxygen and also by lipid oxidation products. Warner and Frankel (1987) showed that β-carotene had a significant effect in protecting soybean oil against light induced oxidation. Similarly, the effects of 0, 5, 10, and 20 ppm of β-carotene on the oxidation of soybean oil/methylene chloride model system containing 4 ppm chlorophyll in light (4000 lux) was found to reduce the oxidation of soybean oil (Lee & Min, 1988).

Yamauchi et al. (1993) studied the oxidative cleavage compounds of β-carotene which were also found to be formed after reaction with alkyl peroxides generated by 2,2'-azobis, 2,4-dimethylvaleronitrile (AMVN). They also showed that these β-carotene oxidation products were also found during the peroxyl radical-initiated peroxidation of methyl linoleate and its auto-oxidation in the bulk phase (Yamauchi et al., 1998). The products formed were formyl or cyclic ether containing groups at the position of the carbon–carbon double bonds. They also obtained similar oxidation products of canthaxanthin when treated either with peroxyl radicals generated by thermolysis of AMVN in benzene or when it reacted as an antioxidant during the peroxidation of methyl linoleate initiated by AMVN in bulk phase (Yamauchi & Kato, 1998). They concluded that these oxidized products were formed from the decomposition of oxygenated products which themselves were formed by the trapping of lipid peroxyl radicals by β-carotene or canthaxanthin. The thermal degradation of all-E-β-carotene, 9-Z-β-carotene, lycopene, and lutein was studied in an oil model system, safflower seed oil, at 75°C, 85˚C, and 95˚C. The data were fitted to a first-order kinetics for all carotenoids. Heating β-carotene at several temperatures was found to form 13-*Z*-carotene in higher amounts, followed by 9-*Z*-β-carotene. Several degradation products were formed during lycopene heating and lutein heating; however, they were not identified (Henry et al., 1998).

Recently Achir et al. (2010) studied the kinetics and degradation of all-*E*-β-carotene and all-*E*-lutein in palm olein and Vegetaline ® (at 120 to 180 ˚C). The variation in *E* and *Z* isomers of carotenoids were investigated using HPLC-DAD. They found that

initial all-*E*-β-carotene and all-*E*-lutein degradation rates increased with temperature in both oils. *E*-lutein was found to have more thermal resistance to degradation than all-*E*-β-carotene. They identified 13- *Z*- and 9-*Z*-β-carotene, and 13-*Z*, 9-*Z*, 13'-*Z*, and 9'-*Z*-lutein. They found that *E*/*Z* isomerization of carotenoids involved in the degradation reactions at rates that increased with temperature. The degradation rates were found to be lower in Vegetaline than in palm olein. Karabulut (2010) studied the effects of α-tocopherol, β-carotene and ascorbyl palmitate added to butter oil triacylglycerols. The fortified triacylglycerols were oxidized in an oven at 60 ˚C. α-Tocopherol was found to be the most effective, at the concentration of 50 μg/g. A better stability was achieved with a combination of all these antioxidants. However, the author reported a pro-oxidant effect of β-carotene and ascorbyl palmitate, when used individually or in binary combination.

Thus carotenoids were found to prevent the oxidation of lipids/TAGs during processing and during long-term storage, and can be used as a food additive. As a food additive, carotenoids are commonly dissolved in a lipid/oil because of their solubility therein. As lipids oxidize, β-carotene oxidation proceeds via a complicated co-oxidation mechanism, accompanying the lipid oxidation or vice versa (Takahashi et al., 2003). Such a phenomenon was also studied in lipid solution or in aqueous solution catalyzed by enzymes (Grosch & Laskawy, 1979), or in food systems in relation to carotenoid oxidation (Perez-Galvez & Minguez-Mosquera, 2001). However, the formation and interactions of oxidation products of carotenoids and lipids (triacylglycerols) is still a subject of great debate, and thus the main purpose of this dissertation.

1.2 Triacylglycerols

Triacylglycerols (TAGs) are the most important and abundant form of natural lipids in plants and animals. They possess significant nutritional values for humans. They are stored in plants by the oil seeds, in which the triacylglycerols also provide energy for their growth. These seeds represent the importance in term of commercial crops. Triacylglycerols are ideally suited to this storage function because of the highly reduced state of their fatty acids. Triacylglycerols have high energy contents i.e. about 37 kJ/g, compared with 17 kJ/g for protein and 16 kJ/g for carbohydrate, including glycogens - a storage form of energy in mammals (Brindley, 1991).

Chemically glycerol is esterified with three fatty acids of one, two or three different kinds forming a triacylglycerol. Each individual fatty acid is located on different carbons of glycerol (Figure 1.7). The word '*tri-acyl-glycerol*' echoes the presence of three esterified acyl groups attached to the glycerol backbone, is formally a replaced form of triglyceride. The fatty acid (FA) position at the carbon 1, 2 and 3 is designated by sn-1, sn-2, and sn-3 positions, where 'sn' is the abbreviation of stereo-specific numbering (Hirschmann, 1960). In plant oils fatty acids are distributed typically in non-random form and each position has a characteristic FA pattern in particular natural fats and oils. The fatty acid at sn-2 position has gained importance in terms of characterization of TAG origin and also for structure elucidation. While fatty acids at the sn-1 and sn-3 are digested first by the lipases enzymes yielding sn-2 mono-acylglycerols (Bernback et al., 1990). Thus the triacylglycerols profile may reflect the nutritional and metabolic history of each cell as its anticipated energy storage requirement (Han & Gross, 2001).

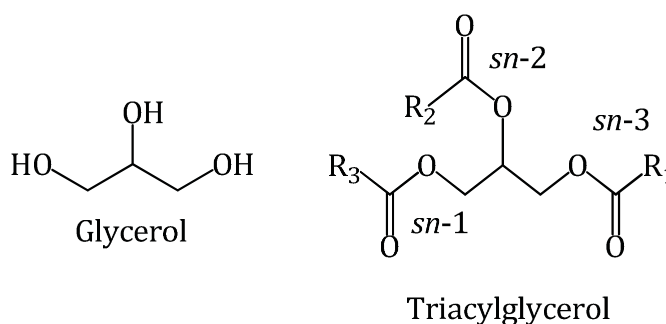


Figure 1.7. Structure of glycerol and triacylglycerols. R_1, R_2 and R_3 represent fatty acids.

The specific locations of each acyl chain are important in terms of chemical as well as physical properties like viscosity, pour point, melting point, heat of fusion, solubility, crystal structure and polymorphism (Foubert et al., 2007). The regio-isomer compositions, i.e. the positional distribution of fatty acid between the sn-2 and sn- 1/3 positions of TAGs have been shown to have biological effects like in infant nutrition, lipid absorption and metabolism and atherogenesis in humans (Carneilli et al., 1995; Kubow, 1996; Broun et al., 1999).

Fatty acids attached to the glycerol are either saturated or unsaturated fatty acid. The saturated fatty acids family begins with methanoic acid with a molecular formula of HCOOH. The hydrogen in HCOOH is replaced with methyl group(s) forming short to long chain fatty acids. Methanoic, ethanoic, and propanoic acids are not present in natural fats. Including butyric acid, they are water soluble, and are often omitted from the lipids. However, these acids are found in non-esterified form in many food products. More than 1000 fatty acids are known, but only 20 or less are present in significant amounts in the oils and fats of commercial importance (Table 1.1). The most common acids are saturated and unsaturated C16 and C18. Below C16 are usually characterized as short or medium chain fatty acids, while above C18 are known as long-chain acids. Most of the vegetable oils contain fatty acids with chain lengths between C16 and C22. The dominant are saturated or unsaturated C18 fatty acids in most plant oils. Palm kernel and coconut are the sources of medium-chain fatty acids and are referred to as lauric oils. In contrast to plant oils, animal fats have a wider range of chain length mostly saturated.

Table 1.1. Typical fatty acids composition (%) of some important vegetable oils.

Oil Type	Fatty acid (%)				
	16:0	*18:0*	*18:1*	*18:2*	*18:3*
Corn	10-17	1.6-3.3	25-42	39-61	0.7-1.3
Olive	7.5-20	0.5-5	55-83	3.5-21	0-1.5
Palm	40-46	4-7	36-41	9-12	10-13
Rapeseed [a]	3.3-6	1-2.5	52-67	16-25	6-14
Sunflower	2.7-6	3-5	80-87	4-9	0-1.0

[a] High erucic acid varieties.

In most natural triacylglycerols a random distribution of fatty acids is not common on the glycerol backbone. Generally in plant oils, unsaturated acids predominate at the sn-2 position and with more saturated acids at sn-1 and sn-3. The distribution of fatty acids at the sn-1 and sn-3 positions is often similar, although not identical. In the case of animal fats, the type of fatty acid predominating at the sn-2 position is more variable; for example, palmitate may be selectively present as well as unsaturated acids. Edible oils that are rich in one fatty acid contain much monoacid triacylglycerol, for example, olive, sunflower, corn and rapeseed oils containing OOO and LLL respectively. Table 1.2 shows a typical composition of TAGs in various edible oils.

Table 1.2: Typical triacylglycerol composition of some plant edible oils.

TAG	Relative Composition (%)[c]			
	Sunflower oil [a]	Corn oil [a]	Olive oil [a]	Rapeseed oil [b]
LLnLn	1.6	--	--	□ 0.5
LLLn	1.4	--	--	2.7
LLL	19.7	1.2	0.1	1.1
OLnL	--	--	--	5.7
OLL	27.8	14.6	0.9	8.0
PLL	9.9	0.6	0.3	1.1
OOL	18.0	18.1	11.6	23.5
PLO	12.4	13.3	2.2	5.4
PLP	0.2	--	--	0.7
OOO	4.2	17.0	60.7	16.8
POO	4.2	18.0	19.8	4.3
POP	0.2	1.3	0.6	0.8
GOO	--	5.3	--	1.3
OOS	0.1	8.1	3.2	1.2
SOP	--	0.9	0.2	--
AOO	--	0.4	--	--

[a] (Cunha & Oliveira, 2006), [b] (Holcapek et al., 2003), [c] May also include DAGs and MAGs.

The melting behavior and other chemical properties of triacylglycerols are thus reflected by the fatty acid composition. For example, triacylglycerols rich in long-chain and saturated acids possess high melting points, while those rich in polyunsaturated acids have lower melting points. However, the possibility that the fatty acids can be distributed in different molecular species with different melting points, complicate the situation. Thus, oils with similar fatty acid composition may have different solid fat content, polymorphic forms, and melting behavior as a result of a different triacylglycerol

composition. Mono- (MAG) and diacylglycerols (DAGs) are usually not present significantly in good quality oils. High levels may be found in badly stored seeds, resulting from the activity of lipolytic enzymes. They can also be produced industrially by partial hydrolysis or glycerolysis of triacylglycerols for use as food grade emulsifiers (Scrimgeour, 2005).

1.2.1 Triacylglycerols Oxidation

1.2.1.1 Formation of Fatty Acid Hydroperoxides

The fatty acids in the triacylglycerols are usually oxidized by a well known mechanism called free radical oxidation as proposed previously by Farmer (1945). This theory involves an attack of oxygen at the allylic position with the formation of unsaturated hydroperoxides. These hydroperoxides also take part in the auto-oxidation and thus initiate chain reaction (Gardner, 1987). Thus it is established that auto-oxidation of polyunsaturated fatty acid (PUFA) occurs as a chain reaction, which involves three phases namely initiation, propagation, and termination as shown in Figure 1.8 (Bolland, 1946; Bateman & Gee 1951; Bateman et al., 1951). By the action of initiators unsaturated fatty acid form a carbon centered alkyl radical (L*) and a peroxy radical (LOO*) (Kamal-Eldin et al., 2003).

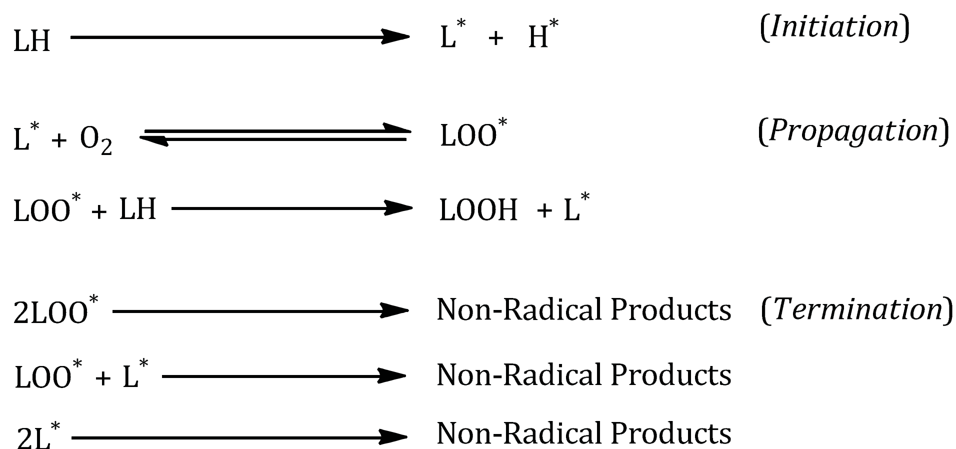


Figure 1.8: Free radical chain reactions during the auto-oxidation of lipids (free radical).*

These radicals than propagate to form hydroperoxides (LOOH) as primary oxidation products (Frankel, 1980; Frankel, 1991; Choe & Min, 2006). The newly formed alkyl radical thus starts another chain reaction. Different fatty acids shows different

susceptibility to auto-oxidation based on the dissociation energies of C-H bonds present in the fatty acid. As a general rule, the removal of hydrogen is much easier in the presence of a double bond in the fatty acid than a singly bonded fatty acid (saturated fatty acid). Experiments showed that the oxidation of the polyunsaturated fatty acids is dependent on the presence of the number of allylic methylene groups present in the fatty acids (Cosgrove et al., 1987; Wagner et al., 1994). Thus the oxidation of mono-unsaturated fatty acids like oleic acid is less pronounced than that of the poly-unsaturated fatty acids (linoleic, linolenic acids etc). This was confirmed by Gardner (1989), who showed that the bond dissociation energy of a C-H bond of the bis-allylic methylene (75 kcal/mol) is less than that of the mono-allylic methylene (88 kcal/mol).

Figure 1.9 shows the radical oxidation of methyl linolenate. It has been observed that the hydrogen is abstracted by radical librating initiators such as heat, light, metals and irradiation from the two allylic methylene groups on carbon-11 and 14 to form two pentadienyl radicals. The addition of oxygen resulted into the formation of four peroxy radicals, which lead to the formation of the corresponding conjugated dienoic hydroperoxides such as 9-, 12-, 13-, and 16-hydroperoxides (Frankel, 1991, Choe & Min, 2006). These hydroperoxides may be present in *Z* or *E* forms. The probabilities of formation of external 9-, and 16-hydroperoxides are significantly higher than the formation of 12-, and 13-hydroperoxides (Frankel et al., 1977; Chan & Levette, 1977).

About 95% of the primary oxidation of linoleate occurs due to abstraction of the bis-allylic hydrogen and the resulting isomerization of one of the double bonds. The minor amounts of non-conjugated 8-, 10-, 12-, and 14-hydroperoxides can also be obtained due to the abstraction of allylic hydrogen's (Haslbeck and Grosch 1983, Haslbeck et al., 1983, Schieberle and Grosch 1981). Smaller amounts of the bis-allylic 11-hydroperoxide are also formed in the presence of α-tocopherol as was shown by Brash (2000). Chan and Levett (1977) isolated four conjugated dienes hydroperoxides from the auto-oxidation of methyl linoleate. They showed that those hydroperoxide isomers are formed from both positional as well as geometrical isomerization of double bonds, which requires the opening of these double bonds and rotation of the resultant single bonds. The β-fragmentation of oxygen from the *E/Z* hydroperoxy radicals followed by bond rotation leads to the formation of *E/E* hydroperoxide isomers. The addition of oxygen

then leads to peroxyl radicals, which give 9- and 13-*E, E*- hydroperoxides, which are the thermodynamic products. (Tallman et al., 2001).

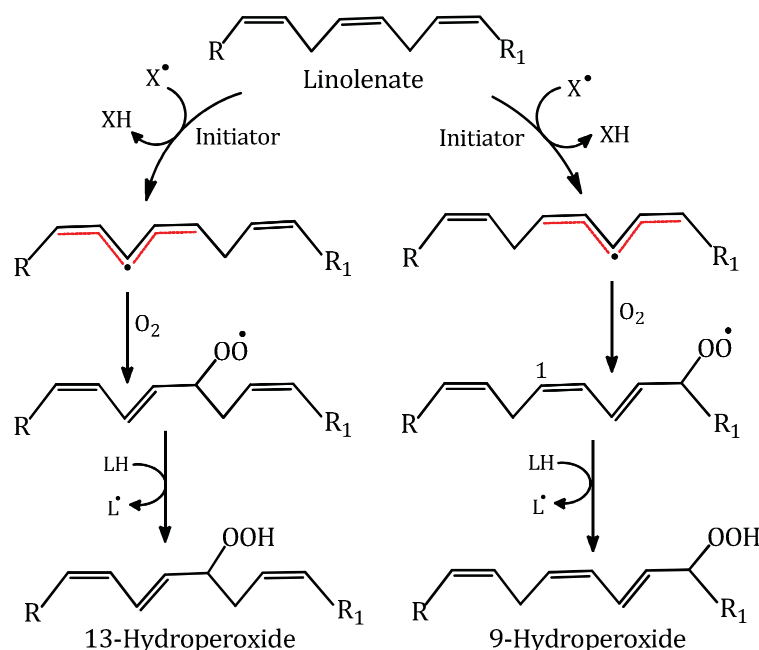


Figure 1.9: Free radical auto-oxidation of linolenic acid (Modified from Frankel, 1991). R= CH_3CH_2-; R_1= -$(CH_2)_7COOH$. E/Z isomers are not shown.

The linolenate peroxyl radical of 12- and 13-hydroperoxides also undergo rapid cyclization to form hydroperoxy-epidioxides by intra-molecular rearrangement (Coxon et al., 1981; Neff et al., 1981) as shown in Figure 1.10. It has been observed that this reaction is the major contributor of the smaller quantity of the 12- and 13-hydroperoxides as compared to 9- and 16-hydroperoxides (Frankel et al., 1977). Antioxidants donate hydrogen atoms to the free radicals resulting in non-radical products (Decker, 2002). Generally a compound which has a reduction potential lower than the free radical can only donate hydrogen, keeping in mind the kinetic favorability. Single electron reduction potentials of 1600, 1000 and 600 mV were observed for the fatty acids alkoxy, peroxy, and alkyl radicals respectively. While that of the antioxidants are 500 mV or less, so it is energetically favorable that antioxidants would react with these free radicals to form neutral species. Antioxidants such as α-tocopherol have been found to strongly inhibit the formation of hydroperoxy epidioxides. Peers et al. (1981) showed that 5% of α-tocopherol in methyl linolenate completely inhibits this cyclization.

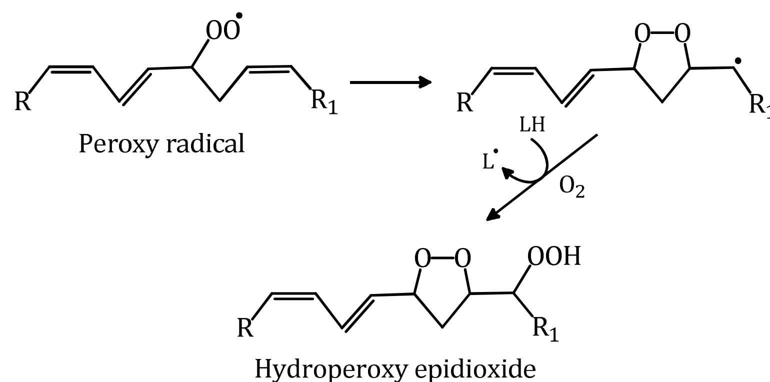


Figure 1.10. Formation of hydroperoxy epidioxide of linolenate.

Linoleic acid is also oxidized by the same free radical mechanism as shown figure 1.11. Two auto-oxidation products are formed which are 9- and 13-hydroperoxides. These hydroperoxides are formed in relative equal amounts (Frankel, 1985).

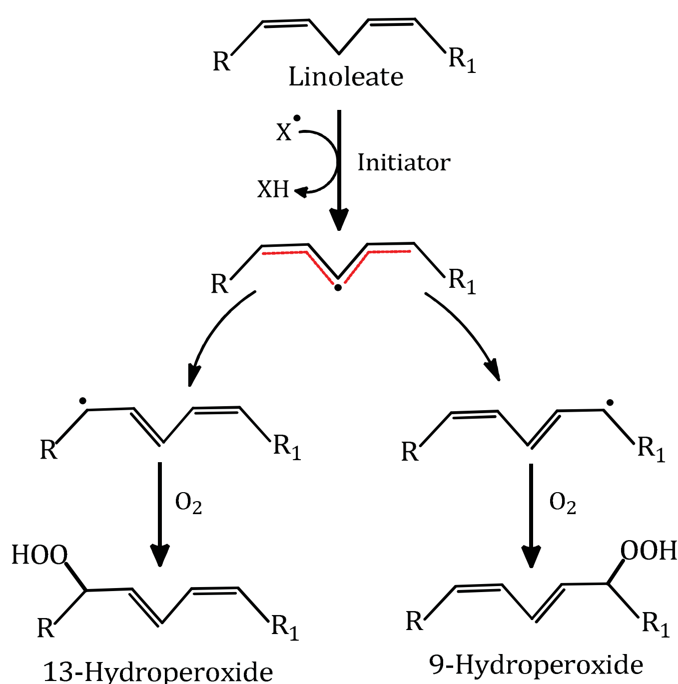


Figure 1.11: Formation of hydroperoxide in the auto-oxidation of linoleic acid (modified from Choe & Min, 2006). R= $CH_3(CH_2)_4$-; R_1= -$(CH_2)_7COOH$.

In oleic acid oxidation (Figure 1.12), the mechanism involves hydrogen atom abstraction at the carbon atoms 8 and 11 to give two allylic radicals. The oxygenation of these allylic radicals gives rise to four peroxyl radicals, which are 11-*Z*, 9-*E*, 8- *Z* and 10-*E*. It is important to note that the relative distribution of different hydroperoxide isomers is dependent on the hydrogen donating ability of these carbons on the fatty acid. It has

been found that formation of 11-*Z*, 9-*E*, 8-*Z* and 10-*E* hydroperoxides is highly favored in the presence of good hydrogen atom donors (Porter et al., 1995; Choe & Min, 2006).

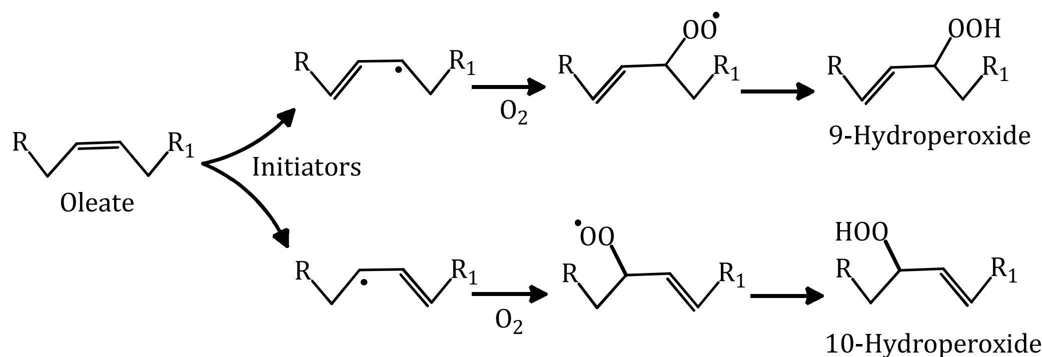


Figure 1.12: The formation of hydroperoxides of oleic acid (modified from Porter et al., 1995). R= $CH_3(CH_2)_6$-; R_1= -$(CH_2)_6$COOH. E/Z isomers are not shown.

1.2.1.2 Formation of Fatty acid Epoxides

The intra-molecular rearrangement of alkoxyl radicals to form epoxy-allylic radicals has been known for several years (Gardner, 1987; Hamberg, 1975). It was observed that the carbon-carbon bond rotation between the hydroperoxide groups and the double bond provides the least steric hindrance of the *E*-rotamer, thus *E*-isomers are predominant (Gardner, 1975). Epoxides have been identified as products of the auto-oxidation of methyl oleate (Lercker et al., 2003) and methyl linolenate (Neff et al., 1981). The epoxide formation was attributed to the rearrangement of alkoxyl radicals of these fatty acid methyl esters (Figure 1.13). The alkoxy radicals either form stable epoxides or epoxy-allylic radicals, which combined with other radicals form epoxy-hydroperoxides. The epoxy-allylic radicals can react with hydroxyl radicals to form an epoxy-hydroxides, which are also stable products. The epoxy-allylic radicals can also react with peroxyl radicals to form epoxy-ketones or epoxy-hydroxides (Gardner, 1989). It is worth mentioning that the reactivity of epoxy-allylic radical depends on many factors like catalyst, light, heat and presence and absence of other free radicals and antioxidants.

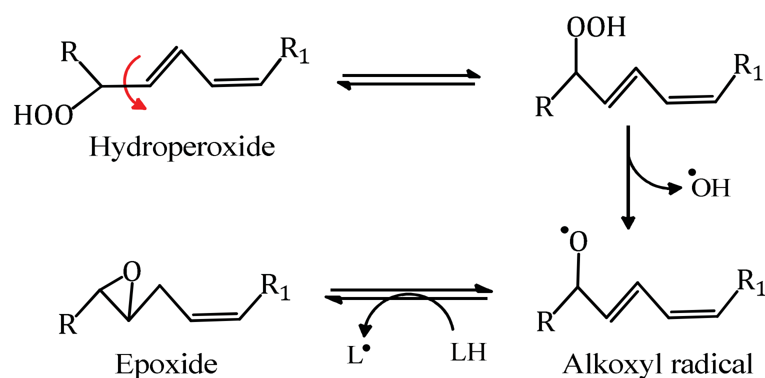


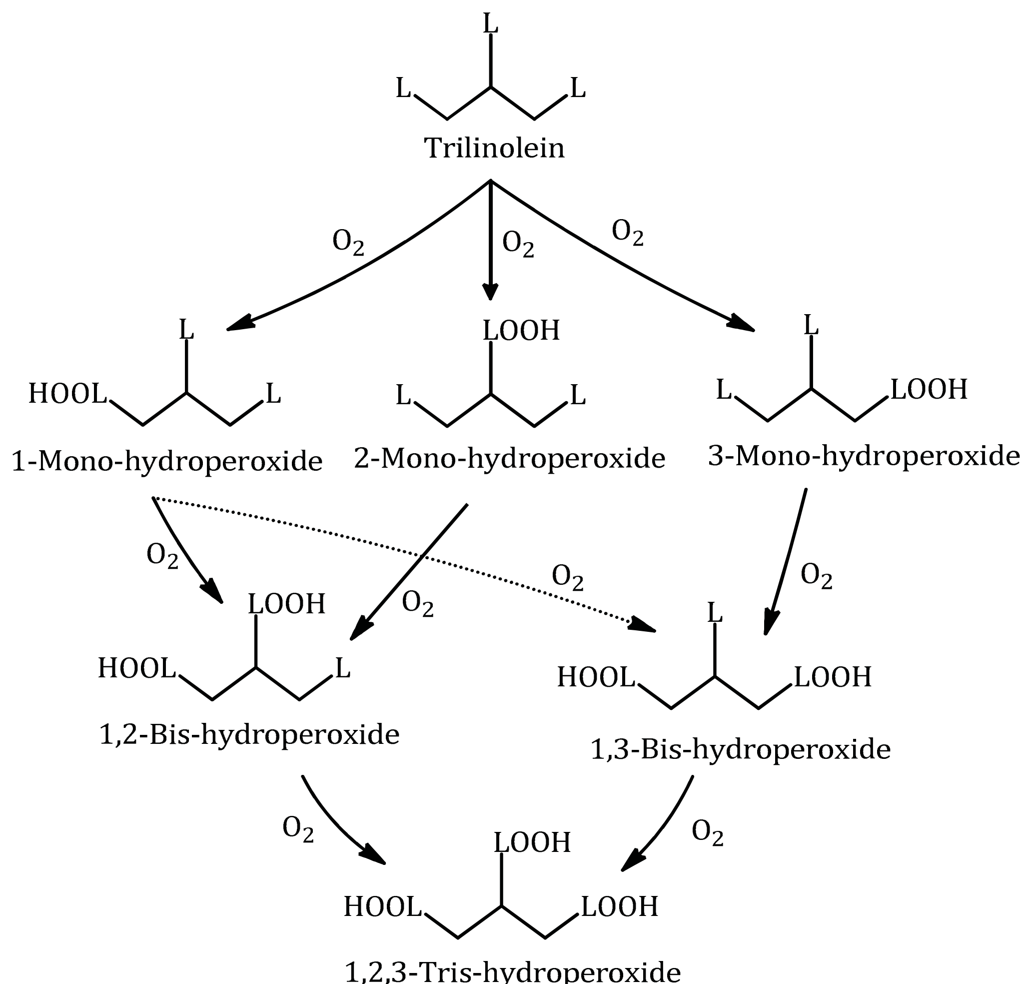
Figure 1.13: The formation of epoxides from hydroperoxides of linoleic acid (Gardner, 1989).

1.2.1.3 Formation of Triacylglycerols Hydroperoxides

The oxidation of triacylglycerols (TAGs) also follows the free radical reaction mechanism. However, steric hindrance, the level of unsaturation, presence of a pro-oxidant, and the exposure time play a critical role in the oxidation of TAGs. For this purpose model TAGs like trilinolein and trilinolenin have been oxidized to know the oxidative stability of vegetable oil TAGs (Frankel et al., 1990; Neff et al., 1990). TAGs were found to be oxidized by auto-oxidation. The oxidation products which are mono, bis and tris-hydroperoxides were formed by the sequential addition of oxygen to the trilinolein. It was found that the initial products of oxidation were mono-hydroperoxides, which further oxidized to form a mixture of 1,2-bis-hydroperoxides and 1,3-bis-hydroperoxides. These bis-hydroperoxides later form tris-hydroperoxides as shown in Figure 1.14. All these hydroperoxides were composed of their respective *Z-E and E-E* isomers. It has been found that during the oxidation of trilinolein no positional preference has been found between each of the sn-positions (Neff et al., 1990).

Similarly, trilinolenin is also oxidized by auto-oxidation. The products formed are 1, 2, or 3-mono-hydroperoxides, 1,2 and 1,3-bis-hydroperoxides and tris-hydroperoxides (Frankel et al., 1990). Other oxidation products formed were hydroperoxy-epidioxides by cyclization as shown in Figure 1.10. These cyclic hydroperoxides were the mixtures of 9 and 16-bis-hydroperoxy-epidioxides. High performance liquid chromatographic (HPLC) separation reveals that the ratio of the *E-Z*-16-linolenate hydroperoxides on sn-1,3 were higher relative to sn-2 of the corresponding *E-Z*-9-linolenate hydroperoxides. This suggests that a fatty acid at the sn-2 position of TAG is relatively more resistant to oxidation than at sn-1 & sn-3. Auto-oxidation of synthetic TAGs containing linolenic acid

and linoleic acid at different positions on the glycerol have been observed to form mono-hydroperoxides and hydroperoxy-epidioxides as the main products (Miyashita et al., 1990) as shown in Figure 1.14.



1.14: Mechanism of trilinolein auto-oxidation and formation of hydroperoxides.

These hydroperoxides are usually odorless and tasteless. However, further decomposition of these compounds produces an unpleasant odor and taste in lipid containing foods (Kamal-Eldin et al, 2003). These oxidized compounds and their decomposition products may have a negative impact on health regarding heart diseases and aging (Neff & Byrdwell, 1998). Jurek et al. (2004) showed that dietary lipid hydroperoxides are a key risk factor in colon carcinogenesis. These hydroperoxides produced from the dietary oils enhances growth of hepatocarcinoma (Rohr-Udilova, et al., 2008). Thus, studies have been conducted on the chemical characterization and utilization of antioxidants to prevent the formation or decomposition of these oxidized species. Discussion of the decomposition products is beyond the scope of this

dissertation, however, identification and characterization of the primary oxidized species as main goal of this dissertation will be discussed.

1.2.2 Mass Spectrometric Analysis of Triacylglycerols

Reversed phase high performance liquid chromatography (RP-HPLC) is one of the most widely employed techniques for the separation of the mixtures of TAGs. The separation on RP-HPLC is based on the chain length and degree of unsaturation (Ruiz-Gutierrez & Barron, 1995). Mobile phase composition also plays an essential role in the separation of TAGs. Both isocratic and gradient elution systems are used. Different detectors are used to identify the separated TAGs. For example, Refractive Index (RI) detectors have been used in most analyses using isocratic elution (Barron et al., 1988; Fabien et al., 1993; Moreda et al., 2003). Low sensitivity, poor separation of isocratic systems and low stability of the baseline signal were the major drawbacks of the RI detector. UV absorption at 210 nm provides a sensitive detection of non-oxidized TAGs. It can be used in both isocratic and gradient elution's (Nurmela & Satama, 1988; Barron & Santa-Maria, 1989). However, increasing the number of double bonds and conjugation of double bonds changes the absorption in the UV range, which is a major shortcoming. Recently the evaporative light scattering detector (ELSD) has been used for detection of TAGs. In the ELSD the solvent containing sample is nebulized in a heated tube using a pressurized gas (usually nitrogen or helium) and evaporates. The less and none-volatiles thus pass as an aerosol and reflect or refract a beam of light. The scattered light is than detected by a photodiode which represents the quantity of the separated analyte (Amaral et al., 2004; Van der Klift et al., 2008; Rombaut et al, 2009). The ELSD has a low background signal, good sensitivity, and a non-specific response and is compatible with a wide range of gradient solvent system.

Mass spectrometry is now a days a widely used technique together with the liquid chromatography. Currently, the two most popular atmospheric pressure ionization (API) interfaces are used for the analysis of TAGs, the atmospheric pressure chemical ionization (APCI) and electro-spray ionization (ESI). From the very beginning APCI-MS became increasingly popular for the identification of TAGs, while ESI-MS also finds a good place in analytical chemistry of TAGs. During the ionization, DAG ions ($M-RCOO^+$), and acyl ions ($RCOO^+$) are formed from TAGs. The intensity of DAG ions in the MS spectra is

very important for the structural elucidation of the corresponding TAG. However, the acyl ions or acylium ions formed by further loss of an oxygen atom (RCO^+) ion also serve as principal ions in structure identification. Table 1.3 showed different RCO^+ observed in the mass spectra of various vegetable oils.

Table 1.3: Common (RCO+) ions observed in mass spectra of various vegetable oils.

Trivial name	Abbreviation	Carbons: DB	(RCO^+) m/z
Palmitic	P	16:0	239
Palmitoleic	Po	16:1	237
Stearic	S	18:0	267
Oleic	O	18:1	265
Linoleic	L	18:2	263
Linolenic	Ln	18:3	261
Arachidic	A	20:0	295

1.2.2.1 APCI-Mass Spectrometric Analysis

Liquid chromatography coupled to mass spectrometry (LC-MS) for the analysis of TAGs has been used for the first time by Byrdwell and Emken (1995). They used an APCI interface on a Finnigan MAT single quadrupole mass spectrometer to characterize a mixture of synthetic TAGs with varying degrees of unsaturation. TAG standards were separated on a reversed-phase column, with a flow rate of 1 mL/min. In their spectra protonated molecules as base peaks $[M+H]^+$, and diacylglycerol (DAG) fragments $[M-RCOO]^+$ were the main ions detected. The APCI-MS mass spectra also exhibited DAG fragment ions that can determine the fatty acyl chains. Another method using HPLC/APCI-MS was applied to the natural mixtures of TAG in normal and genetically modified soybean oils (Neff & Byrdwell, 1995). They found that TAG having three or four sites of unsaturation produced either the protonated molecule or a DAG fragment ion as the base peak, depending on the distribution of the unsaturation in the molecule. The natural samples contain numerous pairs of TAGs having the same equivalent carbon number (ECN) and identical masses, but with different distributions of un-saturation in the fatty acyl chains. For example OOO and SOL, which both have an ECN of 48 and an isobaric molecular weight of 884.8. With less optimal chromatographic separation these TAGs could not be easily carried out. Both OOO and SOL was found to share a fragment at

m/z 603.5, which is isobaric to [OO]+ and [SL]+. However, in the spectrum of SOL the fragment at *m/z* 601.5 and 605.5, representing [OL]+ and [SO]+ respectively, while these are not present in the mass spectrum of OOO. Thus two isobaric TAGs could be easily distinguished by their DAGs fragment ions. The DAG fragment ions in mass spectra of individual TAG can allow us to readily deduce the identity of the TAG from its [DAG]+. Figure 1.15 showed a typical APCI-MS spectra of OOO and SOL showing the fragmentation of TAG into its fragments which helps to elucidate the exact chemical structure.

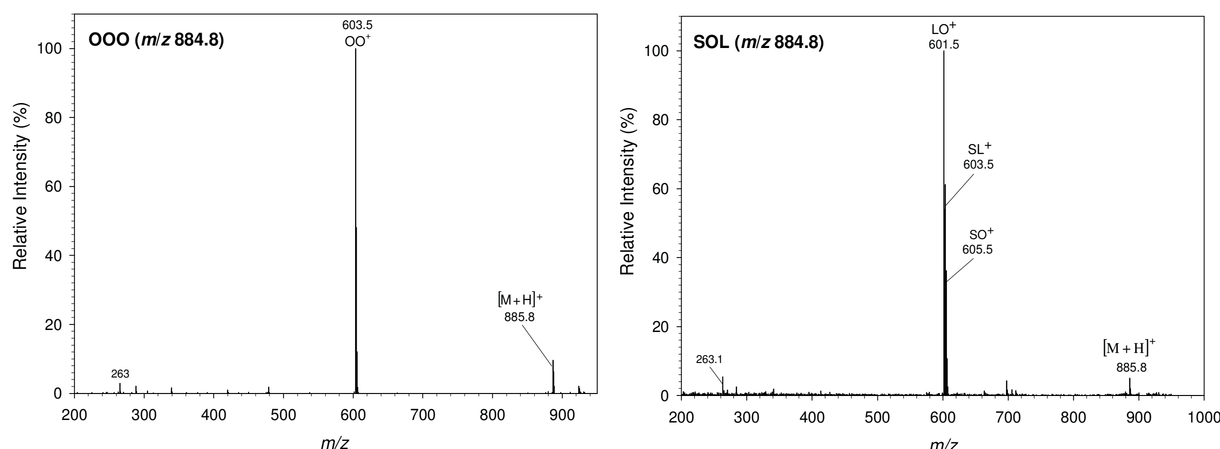


Figure 1.15: The APCI-MS spectra of OOO and SOL showing different DAGs fragments.

However, when a mixture of TAGs was separated using RP-HPLC and identified using APCI-MS, the minimal fragmentation in APCI produces intense diacylglycerol ions (Byrdwell and Emken, 1995; Byrdwell & Neff, 1999; Lin & Arcinas, 2008) and mostly less information on the molecular weight of the TAGs is observed. For the interpretation of APCI mass spectra, the lack of intact protonated molecules produced from some oxygen functional group containing TAGs is another shortcoming (Byrdwell & Neff, 2002). Because of the above reasons, recently Leskinen et al. (2007) proved that the LC-APCI-MS method is not applicable in the quantification of TAG regio-isomers. Contrary to this a recent work by Kofronova et al. (2009) showed that the HPLC provides excellent resolution, which allows the analysis of high-molecular-weight TAGs with the hyphenation with ACPI-MS is still used as a good tool for the identification of triacylglycerols.

1.2.2.2 ESI-Mass Spectrometric Analysis

Electro spray ionization mass spectrometry (ESI-MS) has also been used for the identification of TAGs. The first ever analysis of TAG by ESI-MS was carried out by Duffin et al. (1991). Sodium and ammonium adduct ions were obtained by the addition of sodium and ammonium acetate. They found that monoacylglycerols were more sensitive to give a higher response than diacylglycerols, which gave more response than triacylglycerols. Unsaturated TAGs produced more intense signals than saturated TAGs. However, they used a chromatographic separation, but the location of sites of unsaturation could not be determined from their mass spectra. Bond migration during fragmentation, were the major disadvantage. Myher and Kuksis (1995) and Sandra et al. (1997) also used ESI-MS for the analysis of TAGs.

Schuyl et al. (1998) used sodium acetate and ammonium acetate for silver-ion liquid chromatography coupled to ESI-MS of TAG analysis. Similarly, Cheng et al. (1998) employed ESI-MS/MS for the analysis of TAG. From their fragmentation patterns they were able to locate the positions of double bonds on the fatty acyl chains and the positions of the acyl chains on the glycerol backbone. Hsu and Turk (1999) identified lithiated adducts of standard TAGs by ESI-MS. The fragments in their MS/MS spectra of the lithiated adduct showed the correct position of fatty acid (FA) on the sn-2 position. Similarly, Han & Gross (2001) also reported the lithiated adducts of TAG using ESI-MS. They also reported identification of FAs on sn-2 position. Hvattum (2001) applied non-aqueous reversed-phase liquid chromatography with ESI-MS for the analysis of TAGs. Methanol and chloroform was used as solvent with ammonium formate and formic acid as adduct reagent. However, only ammonium adducts were used for identification of the molecular mass of the TAGs (Table 1.4). No molecular or protonated molecular ion with sufficient intensity was obtained. Marzilli et al. [2003] showed structural characterization of standard triacylglycerols using ESI-MS. They used ammonium acetate in the solvent system to obtain ammonium adducts, in addition to protonated molecular ions. Both ions were less intense; however, they did not study the TAGs in edible oils. More recently Hsu and Turk (2010) used ESI-MS for the structural elucidation of TAGs. Their method is useful in assignment of fatty acyl groups on the glycerol backbone and location of double bonds. However their mass spectra lack intense molecular ions to predict the exact chemical structure of TAGs.

Table 1.4: Different additives used for the identification of TAGs by ESI-MS.

Additive	Quantity	Adducts	Reference
Acetic acid	2%	$(M+H)^+$	Duffin et al., 1991
	0.001%	$(M+H)^+$	Hvattum, 2001
	0.1%	$(M+H)^+$	Ikeda et al., 2009
Ammonia	0.028%	$(M+NH_4)^+$	Ikeda et al., 2009
Ammonium acetate	10mM	$(M+NH_4)^+$	Duffin et al., 1991
	50mM	$(M+NH_4)^+$	Sandra et al., 1997
	0.5%	$(M+NH_4)^+$	Dermaux et al., 1999
	10mM	$(M+NH_4)^+$	Marzilli et al., 2003
	20mM	$(M+NH_4)^+$	Byrdwell & Neff, 2002
	10mM	$(M+NH_4)^+$	Gomez-Ariza et al., 2006
	10mM	$(M+NH_4)^+$	Cheng et al., 1998
Ammonium hydroxide	3%	$(M+NH_4)^+$	Kalo et al., 2003
Formic acid	0.5%	$(M+H)^+$	Segall et al., 2005
Lithium acetate	2mM	$(M+Li)^+$	Hsu & Turk, 1999
	100mM	$(M+Li)^+$	Lin & Arcinas, 2008
Lithium hydroxide	--	$(M+Li)^+$	Han & Gross, 2001
Silver nitrate	--	$(M+Ag)^+$	Sandra et al., 2002
Sodium acetate	10mM	$(M+Na)^+$	Duffin et al., 1991
	10mM	$(M+Na)^+$	Schuyl et al., 1998
	10mM	$(M+Na)^+$	Cheng et al., 1998

Wu et al. (2004) reported the use of FT-ICR-MS for analysis of TAGs in canola, olive, and soybean oils. A large set of distinct compounds were identified using negative ionization mode. In the positive ESI-MS mode the TAGs were identified by their characteristic $[DAG]^+$ and $[TAG+H]^+$ ions. Their technique can be used to detect the adulteration of olive oil. HPLC coupled to ESI-MS were used for the analysis of TAGs in oil from the *Syagrus coronata* (Segall et al., 2004). The authors found a substantial proportion of short to medium chain FA, such as caprylic, capric, lauric, and myristic acids in their oil. A dual parallel ESI-MS method was used to determine the TAGs composition of canola oil and of triolein oxidation products (Byrdwell & Neff, 2002). They employed two mass spectrometers with different interfaces i.e. ESI-MS and APCI-MS. Similar to Duffin et al. (1991) and Cheng et al. (1998), they also observed that unsaturated TAGs produced higher abundances than saturated TAGs by ESI-MS. Also from ESI-MS/MS mass spectra the positions of the fatty acyl chains on the glycerol

backbone can be determined from the $[DAG]^+$ fragment ion ratios. They also found that ESI-MS/MS mass spectra were more definitive and the overall results were more reproducible than APCI-MS mass spectra, which have also been shown previously to be useful for discriminating the positional isomers (Laakso & Voutilainen, 1996; Mottaram et al., 1997). It has been observed that in ESI-MS the higher response of the unsaturated TAGs is precisely opposite to the trend that has been reported extensively for APCI-MS for TAGs identification and quantification by APCI-MS. Thus ESI-MS was found to be much more sensitive for analysis of TAG, as their sodium or ammonium adducts, than is APCI-MS (Byrdwell, 2005).

Recently Lin & Arcinas (2008) reported the uses of ESI-MS for the analysis of TAGs in extra virgin olive oil. Lithium acetate was used to obtain corresponding lithiated ions. They showed that all fragments were lithiated. However, the lack of the molecular ions in their spectra is one of the main disadvantages. Thus for the characterization and identification of regio-specific TAGs, intense molecular and molecular adduct ions and other fragment ions are a useful tool to correctly identify each TAG in edible oils or fats. In addition, the abundance of diacylglycerols ions is also an important parameter for the exact location of each fatty acid.

1.2.3 LC-MS Analysis of Triacylglycerols Oxidation Products

As discussed above hydroperoxides, epoxides, hydroxides are among the major oxidized species of TAG. Liquid chromatography coupled to mass spectrometry (LC-MS) was successfully used for the analysis of synthetic isomers of TAG hydroperoxides (Endo et al., 1997). Sjovall et al. (1997) reported a successful HPLC-ESI-MS method for the analysis of peroxidized natural TAGs. They reported mono and di-hydroperoxides, hydroxides, epoxides, some aldehydes and other derivatives. Neff & Byrdwell (1998) studied the oxidation products from the auto-oxidation of standard TAGs (triolein, trilinolein and trilinolenin) using reversed phase HPLC coupled to APCI-MS. They oxidized TAGs samples in the dark at 50-60°C until the oxidation reached 30% of the original samples. Mono-hydroperoxides were the major oxidized products. There mass spectra showed useful fragments, like epoxide fragments, which were formed from hydroperoxides. An additional unsaturation was observed due to the loss of oxygen from the epoxides. Other oxidation products formed were stable epoxides, hydroxides,

epidioxides, and hydroperoxy-epidioxides. This was the first study to show the exact position of oxygenated species. Later on the same authors (Byrdwell & Neff, 1999) studied model triolein at frying temperatures. Triolein was heated at 190°C and the samples were analyzed using LC-APCI-MS. Triolein oxidation products include hydroperoxides, epoxides and a ketone (Figure 1.16). In addition of these products they are also identified triolein dimers and chain addition products.

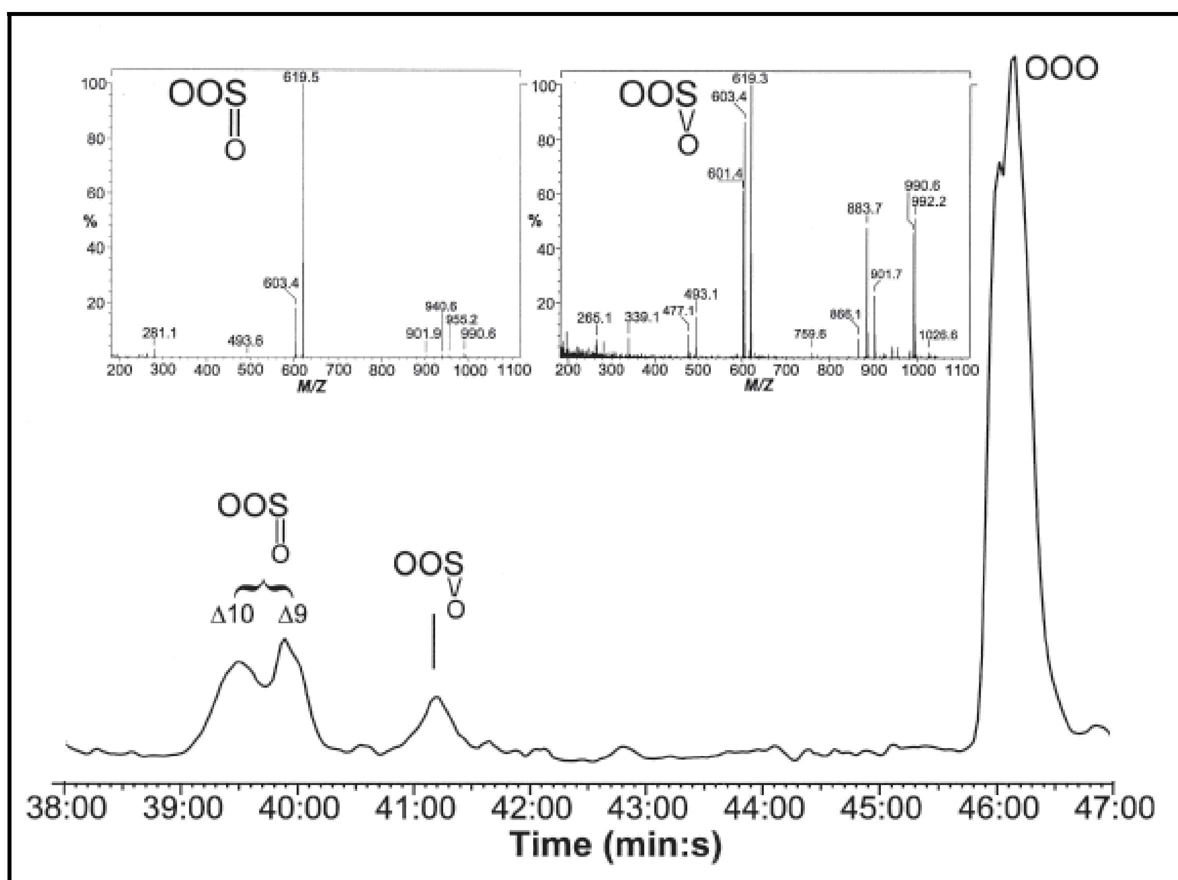


Figure 1.16: RIC chromatogram and mass spectra of the products of interesterification of keto-sterate with triolein (reproduced from Byrdwell & Neff, 1999).

Non-volatile lipid oxidation products of vegetable oils were analyzed using normal phase HPLC-MS (Steenhorst-Slikkerveer et al., 2000) using standard oxidized TAGs. The oxidized products in the oils were hydroperoxides, hydroxides, epoxides, and oxo-TAGs. However, the method is only suitable in the presence of available standards. Sjovall et al. (2001) separated *tert.*-butyl hydroperoxide oxidation products of synthetic unsaturated TAGs (SOS, OPP, SSL, and OOS) using reversed phase HPLC. Hydroperoxides, diepoxides and hydroxides were the major compounds of the oxidation mixture. These compounds were identified from their molecular weight by ESI-MS. However, mass spectra do not

provide information about DAGs fragments ions or acylium ions, which are essential for structural elucidation. In the same year, Byrdwell & Neff (2001) published their results on the auto-oxidation products of normal and genetically modified canola oils analyzed using LC-APCI-MS. The major auto-oxidation products were epoxides and hydroperoxides. They also observed neutral and polar chain shortened products. They found a decrease in the content of linoleic and linolenic acids during auto-oxidation. The same authors also reported for the first time the dual parallel ESI & APCI-MS for the analysis of TAGs and its oxidation products (Byrdwell & Neff, 2002). They found that ESI-MS of TAG oxidation products produce abundant ammonium adducts ions, than by APCI-MS.

Recently Lin (2009) studied the ratios of the regio-isomers of TAGs containing dihydroxy fatty acids in castor oils using HPLC and ESI-MS. The ratios were estimated by fragment ions formed from the loss of fatty acids at the sn-2 position in the ESI-MS spectra. Ikeda et al. (2009) reported an effective method for global analysis of TAGs molecular species from complex lipid mixtures of mouse liver and white adipose tissue (WAT) using reverse-phased HPLC coupled with electro spray ionization (ESI)-quadrupole/time of flight hybrid mass spectrometer (QTOF-MS). However, they did not show the molecular adducts in their mass spectra and also the location of oxidized species, which is essential for structural assignment and future properties.

Thus limited numbers of HPLC-MS methods are available, which could correctly identify the oxidized species of TAGs without prior extensive and laborious treatments. As mentioned above there is also the lack of HPLC-MS analysis of TAGs and its oxidized species in common edible oils such olive, corn, sunflower or rapeseed oils. The presence of antioxidants like carotenoids during the thermal treatment of TAG or edible oils is also an industrially important point of concern. Therefore we developed a one window method applicable for both TAGs composition and its oxidation products in model TAGs and edible oils.

1.3 Specific Objectives

Astaxanthin and β-carotene were added to high oleic triacylglycerols and edible oils such as corn oil, and olive oils and oxidized under strictly controlled conditions. The control samples and the oxidized samples were then analyzed to achieve the following objectives.

- ✓ To develop analytical methods (TLC & HPLC) suitable for characterization and identification of carotenoids and their oxidation products.
- ✓ To determine the degradation and oxidation products of carotenoids.
- ✓ To develop a reproducible HPLC-MS method for characterization of triacylglycerols.
- ✓ To use this method for the identification and structural characterization of oxidized triacylglycerols.
- ✓ To determine the interactions between carotenoids and triacylglycerols during thermal oxidation.
- ✓ To study the stability of normal and oxidized triacylglycerols in the presence of carotenoids.

2. RESULTS AND DISCUSSION

The methods developed and the results and detailed discussion of carotenoids and triacylglycerols interactions during thermal oxidation is presented in the form of four published and two submitted manuscripts. They are designated by letter I-VI in the conclusions. The published papers are reproduced with the kind permissions of the respective publishers.

I. **Alam Zeb** & M. Murkovic (2010). Thin-layer chromatographic analysis of carotenoids in plant and animal samples. *Journal of Planar Chromatography. 23* (2): 94-103.

II. **Alam Zeb** & M. Murkovic (2010). High-performance thin-layer chromatographic method for monitoring the thermal degradation of β-carotene in sunflower Oil. *Journal of Planar Chromatography*. *23* (1): 37-41.

III. **Alam Zeb** & M. Murkovic (2010). Analysis of triacylglycerols in refined edible oils by isocratic HPLC-ESI-MS. *European Journal of Lipid Science & Technology. 112* (8): 844-851.

IV. **Alam Zeb** & M. Murkovic (2010). Characterization of the effects of β-Carotene on the oxidation of triacylglycerols using HPLC-ESI-MS. *European Journal of Lipid Science & Technology. 112* (11): 1218-1228.

V. **Alam Zeb** & M. Murkovic (2010). Determination of β-carotene and triacylglycerols thermal oxidation in corn oil using LC-MS. (Submitted).

VI. **Alam Zeb** & M. Murkovic (2010). Carotenoids and triacylglycerols interactions during thermal oxidation of the refined olive oil. (Submitted).

Thin-Layer Chromatographic Analysis of Carotenoids in Plant and Animal Samples

Alam Zeb* and **Michael Murkovic**

Key Words

Carotenoids
Analysis
Food
Thin-layer chromatography
Separation techniques

Summary

Carotenoids are among the most widespread and important pigments in living organisms. They are found in common foods and vegetables. The characteristic pattern of alternating single and double bonds in the polyene backbone enables them to absorb excess energy from other molecules. The nature of the specific end groups on carotenoids may effect their polarity, thus solubility ranges from acetone to hexane. Because of this wide range of polarity, specific extraction and separation procedures are required. In these procedures use of planar chromatography in food analysis might seem a minor aspect of carotenoid analysis. This review describes available data on analysis of carotenoids by thin-layer chromatography (TLC). It has been found that petroleum ether, acetone, and hexane are the major mobile phases used for TLC. Thin-layer chromatography was found to have the potential to be the first choice for analysis of carotenoids in biological samples. The uses of other, orthogonal chromatographic methods, for example HPLC, spectroscopy (mass spectroscopy), scanning densitometry, and image analysis with TLC can enable precise analysis of carotenoids.

1 Introduction

The name carotenoids is derived from the fact that they are the major pigments in the carrot root, *Daucus carota* L. The carotenoids are certainly among the most widespread and important pigments in living organisms. Carotenoids are fat-soluble food components that are categorized as either xanthophylls (oxygen-containing carotenoids) or carotenes (hydrocarbon carotenoids) according to their chemical composition. Carotenoids are found in common foods of plant and animal origin [1]. Most xanthophylls are found in green leafy vegetables and nearly all carotenes are found in yellow vegetables [2]. The most important carotenoids are α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, zeaxanthin, violaxanthin, and neoxanthin. The first six carotenoids can normally be found in human plasma, an indication of their bioavailability.

Carotenoids play a crucial role in photosynthesis. They also occur in some non-photosynthetic bacteria, yeasts, and molds, where they may have a protective function against damage by light and oxygen. Although animals are incapable of synthesizing carotenoids, they incorporate carotenoids from their diet and these are accumulated in specific tissues. Within animals, carotenoids provide bright coloration, serve as antioxidants, and can be a source of vitamin A [3, 4]. Carotenoids are responsible for many colors, for example red, orange, and yellow of plant leaves, fruits, and flowers, and the colors of some birds, insects, fish, and crustaceans [5, 6]. Some familiar examples of carotenoid coloration are the oranges of carrots and citrus fruits, the reds of peppers and tomatoes, and the pinks of flamingoes and salmon [7]. More than 700 different carotenoids are known to occur naturally, and new carotenoids continue to be identified [8, 9].

Carotenoids are defined by their chemical structure. Most carotenoids are derived from a 40-carbon polyene chain, which can be regarded as the backbone of the molecule. With a few exceptions (phytofluene, ζ-carotene, and lycopene) the hydrocarbon chain is terminated by cyclic end-groups or complemented with oxygen-containing functional groups. The hydrocarbon carotenoids are known as carotenes, and oxygenated derivatives of these hydrocarbons are known as xanthophylls [5, 10]. A list of structures of important carotenoids is given in **Figure 1**. The principal carotenoid in carrots is β-carotene, a familiar carotene; lutein, the major yellow pigment of pumpkins, is a common xanthophyll [11]. The structure of a carotenoid ultimately determines its potential biological function(s) [12, 13]. The distinctive pattern of conjugated double bonds in the polyene backbone of carotenoids is what enables them to absorb excess energy from other molecules, and the nature of the specific end groups on carotenoids may affect their polarity. It has been shown that at least seven conjugated double bonds are needed for a carotenoid to have perceptible color [14], e.g. phytofluene con-

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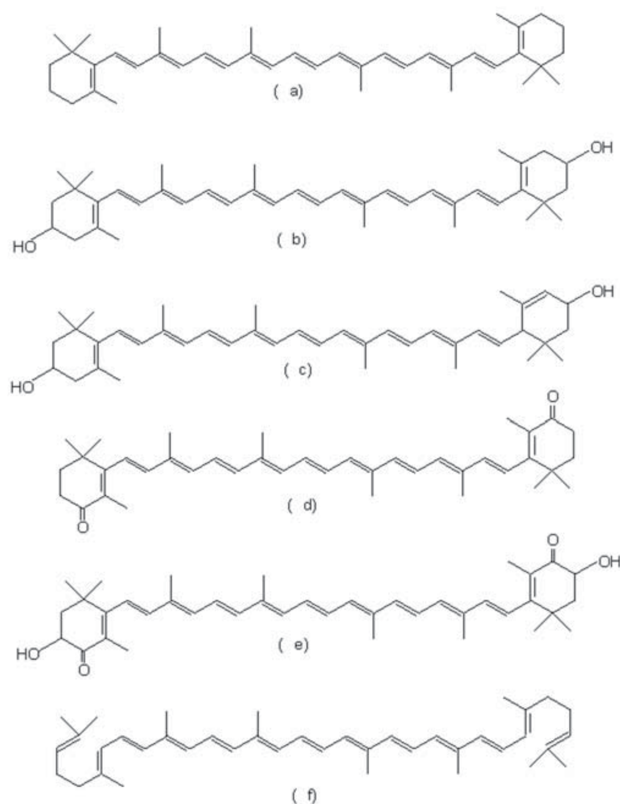

Figure 1

Structures of common carotenoids: (a) β-carotene, (b) lutein, (c) zeaxanthin, (d) canthaxanthin, (e) astaxanthin, and (f) lycopene.

tains five conjugated double bonds and is colorless. Some of the physicochemical properties of carotenoids are listed in **Table 1.**

1.1 Functions of Carotenoids

Carotenoids can serve several important functions in human beings. The most widely studied and well-understood nutritional role of carotenoids is their provitamin A activity [15–17]. Deficiency of vitamin A is a major cause of premature death in developing nations, particularly among children [18]. Retenoids originating from provitamin A have many vital systemic functions in humans. They can be synthesized within the body from some carotenoids, notably β-carotene.

Carotenoids also play a significant potential role in human health by acting as biological antioxidants, protecting cells and tissues from the damaging effects of free radicals and singlet oxygen [19–22]. Lycopene, the hydrocarbon carotenoid that gives tomatoes their red color, is particularly effective at quenching the destructive potential of singlet oxygen [23, 24]. Lutein and zeaxanthin (xanthophylls) are accumulated in the macula lutea in the eye [25, 26]. The presence of these two carotenoids reflects their close relationship with age-related macular degeneration (AMD). There is epidemiological evidence that lycopene reduces the incidence of prostate cancer [27]. Other health benefits of carotenoids that may be related to their anti-oxidative potential include enhancement of immune system function [28], protection from sunburn [29], and inhibition of the development of certain types of cancer [30–32].

Table 1

Physicochemical properties of some important carotenoids [14].

Carotenoid	Color	Structure	Solvent	Absorption coefficient Max [nm]	$A^{1\%}_{1cm}$
Phytofluene	Colorless	Acyclic	Petroleum ether	348	1350
			Hexane	348	1577
ζ-Carotene	Light Yellow	Acyclic	Hexane	400	2555
Lycopene	Red	Acyclic	Petroleum ether	470	3450
γ-Carotene	Red-orange	Monocyclic	Petroleum ether	462	3100
			Hexane	462	2760
β-Carotene	Orange	Bicyclic	Petroleum ether	450	2592
			Ethanol	450	2620
α-Carotene	Yellow	Bicyclic	Petroleum ether	444	2800
			Hexane	445	2710
β-Cryptoxanthin	Orange	Bicyclic	Petroleum ether	449	2386
			Hexane	450	2460
α-Cryptoxanthin	Orange	Bicyclic	Hexane	445	2636
Zeaxanthin	Yellow-orange	Bicyclic	Petroleum ether	449	2348
			Ethanol	450	2480
Lutein	Yellow	Bicyclic	Ethanol	445	2550
			Diethyl ether	445	2480
Astaxanthin	Red	Bicyclic	Hexane	470	2100
Violaxanthin	Red	Bicyclic	Ethanol	440	2550
			Acetone	442	2400

1.2 Occurrence of Carotenoids

The most important sources for carotenoids are plants. The composition and quantity of carotenoids in foods depends on the geographical origin, species, climate, soil condition and structure, fertilizers [33], and state of ripeness [34]. In plants the brilliant colors of the carotenoids mature after the chlorophyll content decreases, thus the carotenoids are responsible for the beautiful colors of most fruits, for example pineapple, oranges, lemons, grapefruit, tomatoes, paprika, and rose hips [2]. Sea buckthorn fruits are rich in carotenoid pigments located in membranes and the fleshy mesocarp. In general, the carotenoid composition of sea buckthorn berries comprises almost 20% β-carotene, 30% γ-carotene, 30% lycopene, and 15% xanthophylls [35, 36]. Pulp oil contains higher levels of β-carotene than seed oil, and *Hippophae salicifolia* seems to have the highest level of β-carotene in both pulp and seed oil among all species. The amount of β-carotene constitutes 15–55% of total carotenoids, depending on origin [37].

Carotenoids are present in nearly all vegetable oils, including corn, groundnut, soybean, rapeseed, linseed, olive, barley, sunflower, cottonseed, soybean, and red palm oils [33, 38–40]. The concentration of carotenoids in some of these vegetable oils is usually low, less than 100 ppm. Of the vegetable oils that are widely consumed, red palm oil and sea buckthorn oil contain the highest known concentrations of agriculturally derived carotenoids [41]. It has been established that carotenoids in these oils are the main source of antioxidants, which contribute to their excellent oxidative stability [42]. In fact, crude palm oil is the world's richest natural plant source of carotenes, in terms of retinol equivalents [43]. It contains about 15 to 300 times as many retinol equivalents as carrots, leafy green vegetables, and tomatoes. The concentration variation depends on several factors, for example species, variety, climate, origin, and ripeness. In addition to natural variation the choice of analytical method also contributes to variation in the carotenoid content of foods. The pigment must usually be extracted before analysis, sometimes from a complex matrix. Therefore, efficient extraction and analytical procedures are required.

2 Analysis of Carotenoids

2.1 Sampling

Samples for carotenoid analysis are obtained from different sources as described above. The sampling methods are however, different, depending on the type of sample. The objective of sampling is usually to secure a portion of the material that statically represents the type of analyte under investigation [44]. For investigation of carotenoids, sampling of food products may be achieved directly from commercial market or from fields. The possibility of variation may exist in both, however. The samples are stored in dim light or dark in a suitable storage medium at low temperature (–18°C), if possible under nitrogen or argon atmosphere.

2.2 Sample Preparation

The sample brought to the laboratory is typically large, and is transformed into a homogenous and small representative sample for further analysis [14]. Sample-preparation schemes depend on the type of food under investigation. Because a variety of factors affect the carotenoid composition of food, proper sample preparation is necessary [45]. Because of, mainly, oxidative instability of most of the carotenoids, the sample preparation is usually carried out in the absence of oxygen and in dimmed light, preferably in brown glassware [46]. Addition of small amounts of a variety of antioxidants, for example BHT or pyrogallol, or flushing with nitrogen, or argon can prevent oxidation of carotenoids.

2.3 Extraction of Carotenoids

Carotenoids are insoluble in water and soluble in organic solvents. Various organic solvents, for example acetone, tetrahydrofuran (THF), *n*-hexane, pentane, petroleum ether, methanol, and ethanol, and mixtures of these solvents can be used for the extraction of carotenoids, as described in detail by *Rodriguez-Bernaldo de Quiros* and *Costa* [47]. Different solvent systems are used for different samples. The same solvent system in the same or a different ratio can be used for a variety of foods, e.g. THF–methanol 1:1 (*v*/*v*) was used to extract carotenoids from wheat grain and pumkin by *Hentschel et al.* [48] and *Murkovic et al.* [49], respectively. Ethanol and hexane have been used successfully for analysis of carotenoids in fruits and vegetables, with good recovery and precision. The extraction method was found suitable for a wide range of sample matrices in carotenoid analyses [50]. The use of a potential reference material in interlaboratory studies (seventeen European labs) for analysis of carotenoids in a mixed vegetable material has been studied [51]. It was observed that the accuracy of the 'in-house' standard concentration may in some laboratories be a significant source of variation, particularly for lycopene.

Extraction of carotenoids must be performed very quickly, avoiding exposure to light, oxygen, high temperature, and prooxidant metals (iron, copper) in order to minimize auto-oxidation and isomerization [51, 52]. Different neutralizing agents can be used to buffer the acids librated during tissue degradation [53–55].

Different extraction procedures are used to isolate carotenoids, including simple solvent extraction (*Soxhlet*), lipid phase distribution [56], solid-phase extraction [57], accelerated solvent extraction [58], and supercritical-fluid extraction [59–62]. These methods have been compared [59, 63]. It was found that supercritical-fluid extraction was the best method for extraction of carotenoids [64].

2.4 Saponification

Saponification is an efficient procedure for removal of chlorophyll and most of the unwanted lipids, which are also the source of interference in chromatographic separation. It should be carried out to evaluate the presence of carotenol esters. *Rodriguez-Bernaldo de Quiros* and *Costa* [47] reviewed in detail the saponification conditions necessary for carotenoid analysis. Provitamin A carotenoids, for example α-carotene, β-carotene, γ-carotene, and β-cryptoxanthin, are stable during saponification [65, 66] whereas others are quantitatively destroyed [14, 67]. It is therefore suggested that saponification is avoided and alternative methods, for example direct solvent extraction, are used for analysis of carotenoids in food products [68].

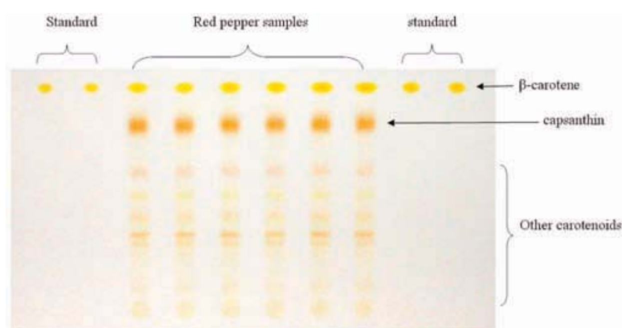

Figure 2

HPTLC plate showing the separation of carotenoids in red pepper developed with petroleum ether–hexane–acetone 2:1:1 (*v*/*v*).

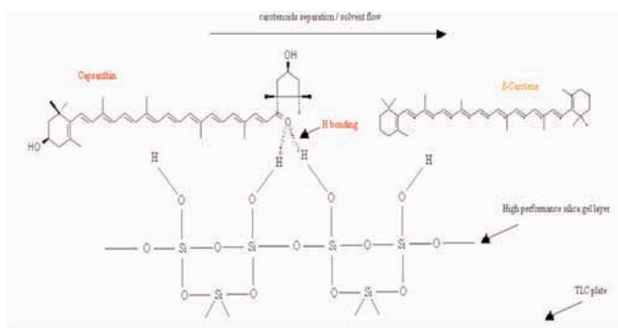

Figure 3

Schematic representation of the separation of oxygenated and non-oxygenated carotenoids on the surface of a TLC plate.

2.5 Chromatographic Analysis

Chromatography in descending, gravity-flow columns, referred to as open column chromatography (OCC), is useful for separating and purifying carotenoids to be used as standards for HPLC and other techniques. Separation of the carotenoid pigments is followed visually. A low pressure may also be useful at the top of the column (e.g. nitrogen gas); this technique is known as flash column chromatography. High-performance thin-layer chromatography (HPTLC), which is efficient for monitoring the progress of chemical tests for identification purposes, is now widely used for quantification of carotenoids in biological samples [69–73]. Several recent review articles [47, 74–80] describe the uses of all the chromatographic techniques used for analysis of carotenoids or related compounds. Less attention has been devoted to TLC, however. This article is limited to thin-layer chromatographic analysis of carotenoids in plant species and animal tissue.

3 Thin-Layer Chromatographic Analysis of Carotenoids

Although several new techniques have been used for analysis of carotenoids form various sources, literature describing the analysis of carotenoids by thin-layer chromatography in detail is limited [81–84]. Thin-layer chromatography is comparably easy to use and is employed for the separation and qualitative and quantitative analysis of carotenoids in foods and non-food products.

Thin-layer chromatography (TLC) is widely used for food and pharmaceutical analyses. TLC has been applied in food analyses to study composition, adulteration, contaminants, degraded and decomposed products of carbohydrates, proteins, lipids, and vitamins [84]. The technique is often used for separation and isolation of individual classes of molecules, because TLC is rapid, effective, and relatively inexpensive. Before use of silica it is necessary to neutralize its acidity to avoid epoxide–furanoxide rearrangement of carotenoids and chlorophyll pheophytinization [14, 33, 45, 85].

Early TLC methods used silica as stationary phase and a non-polar mobile phase for separation of carotenoids [79, 86–88]. Alumina and diatomaceous earth did not find application to the analysis of such pigments. Our observations (unpublished) showed that carotenoids extracted from red pepper could easily be separated on silica gel plates by use of petroleum ether–hexane–acetone 2:1:1 *(v/v)*, as shown in **Figure 2**. Non-oxygenated carotenoids, for example β-carotene, have higher R_F values than oxygenated capsanthin, possible because of the presence of hydrogen bonding in the case of capsanthin (**Figure 3**). *Davis* [89] described some important and practical aspects, for example choice of adsorbents, preparation and running of chromatograms, detection and elution of carotenoids by old TLC techniques. Progress in and commercialization of new stationary TLC phases (octyl, octadecyl, cyano, diol, and aminopropylsilica) has, however, vastly increased the scope of applications of TLC. The separation of color pigments of paprika has been performed on a substantial number of stationary phases and the results have been compared [88]. A recent review by *Poole* [90], showed some of the characteristics of different thin layers and mobile phases for a variety of TLC systems.

Although it might seem that planar chromatography is of minor importance in the analysis of carotenoids in food, it has been shown that thin-layer chromatography (TLC and HPTLC) is used for 20% of all pigment analysis in food, which is more than for other food group analyses [91].

3.1 TLC of Carotenoids from Microbial and Animal Sources

It is evident that a variety of microorganisms and small animals are also sources of carotenoids. Isolation, identification, quantification of these carotenoids is important for subsequent study of metabolism and its physiological importance for the particular species. In this case carotenoids are usually extracted with acetone [92], or a mixture of hexane with petroleum ether and ethanol [93]. After washing the petroleum ether extract with water the pigments are stored at low temperature (below –20°C). Saponification is carried out using methanolic KOH, with subsequent column chromatography. Thin-layer chromatography is carried out using different mobile phases, as listed in **Table 2**. It is clear from the table that HPTLC plates have rarely been used [94, 95].

For separation of the mixture of carotenoids petroleum ether–acetone 75:25 (*v*/*v*) and hexane–acetone 4:1 (*v*/*v*) [96] have been used. β-Carotene and lutein have been separated by use of petroleum–diethyl ether–acetic acid 80:20:1 *(v/v)* [97] and petroleum ether–acetonitrile–methanol 1:2:2 *(v/v)* has also been used. It is clear from Table 2 that petroleum ether and acetone are commonly used organic solvents for TLC or HPTLC.

Table 2

Analysis of carotenoids in microorganisms and animals by thin-layer chromatographic methods.

Sample	Carotenoids	Mobile phase (*v/v*)	Plate	Ref.
Bacterial cells	Keto-carotenoids	Acetone–petroleum ether (15:85)	TLC	92
		Methanol–benzene (3:97)	TLC	92
Spirulina platensis	Mixture of carotenoids	Petroleum ether–acetone (75:25)	TLC	93
Pomacea analiculata	Astaxanthin	Hexane–acetone (80:20)	HPTLC	94
Helisoma trivolvis	β-Carotene, lutein	Petroleum ether–diethyl ether–acetic acid (80:20:1)	TLC	97
Larval trematodes	β-Carotene, lutein	Petroleum ether–acetonitrile–methanol (1:2:2)	HPTLC	95
Serum, animal tissues	Retenoids and carotenoids mixture	Hexane–acetone (4:1)	TLC	96

Table 3

Analysis of carotenoids from plants sources.

Sample	Carotenoids	Mobile phase (*v/v*)	Plate	Ref.
Plants tissues	Carotenoids isomers	*p*-Methyl anisole–petroleum ether (1.5:98.5)	TLC	101
		Acetone–petroleum ether (1.2:98.8)		
Algal cultures	Fucoxanthin	Methanol–water (9:1)	HPTLC	112
Plant extract	Mixture of carotenoids	Chloroform–hexane–methanol (20:70:05)	HPTLC	110
Potamogeton crispus	neoxanthin, violaxanthin, lutein and β-carotene	Hexane–ethyl acetate–acetone–methanol (27:4:2:2)	TLC	102
Dandelion	Lutein epoxide	Diethyl ether–petroleum ether (3:1)	TLC	103
Spinach leaves	β-Carotene	Petroleum ether–acetone (7:3)	HPTLC	104
Spinach leaves	Crotocin	Chloroform–methanol–acetic acid (1:0.1:0.013)	HPTLC	111
Spinach	Violaxanthin, neoxanthin	Methanol–acetone–hexane (1:29:70)	TLC	105
Carrots	β-Carotene	Ethyl acetate–light petroleum (3:17)	TLC	125
β-Carotene	Degraded products	Acetone–petroleum ether (4:95)	TLC	106
Vitamin mixtures	Vitamin A	Petroleum ether–chloroform–acetone (50:10:17)	TLC	107
Olive oil	β-Carotene, lutein, violaxanthin, and neoxanthin	Petroleum ether–acetone–diethylamine (10:4:1)	TLC	100, 108
Edible oil	Astaxanthin	Acetone–hexane (–)	TLC	109

3.2 TLC of Carotenoids from Plant Sources

The most favorable TLC systems for separation of oxygenated carotenoids [98], Cu (II) complexes of chlorophylls, and allomerization products of pheophytins *a* and *b* [99] have been described. TLC of 13 carotenoids on silica gel, MgO–Kieselguhr (1:1, *w/w*), and NaOH-impregnated silica gel plates using light petroleum, diethyl ether, and light petroleum–acetone 9:1 as mobile phases and visualization under UV light was carried out to identify the pigments in yellow passion fruit [100]. In most cases petroleum ether with acetone was used as major organic mobile phase [101–109]. Hexane was found to be the second major mobile phase used in TLC of carotenoids from plant sources [102, 105, 109, 110]. In analyses of carotenoids in plants or foods, HPTLC plate usage was also smaller [104, 110–112] compared with simple or preparative TLC plates (**Table 3**). A similar mobile phase (petroleum ether–acetone) in a different ratio has been used for analysis of carotenoid isomers, β-carotene, oxidized carotenoids, vitamin A, and carotenoid mixtures [101, 104, 106–109]. Spinach leaves were used as standard for the identification of violaxanthin and neoxanthin in different biological samples [104, 105, 111]. Use of TLC for isolation and identification of carotenoids in edible oils has received little attention [108, 109].

3.3 Normal-Phase TLC Analysis of Carotenoids

In normal-phase thin-layer chromatography, the stationary phases are polar and mobile phases are usually non-polar or of low polarity. Thin-layer chromatography of carotenoid on silica layers with petroleum ether containing *tert*-butanol or *tert*-pentanol resulted in improved separation of oxygenated carotenoids compared with acetone–petroleum ether. The effects of the presence of oxygenated substituents on retention of the individual carotenoids were different for the *tert*-butanol and acetone systems [113]. Carotenoids were extracted from *Capsicum annum* L. using acetone–petroleum ether (1:1, *v/v*). Separation of the carotenoids was performed on silica gel as stationary phase; the highest R_F values were obtained for carotenes, followed by oxygenated carotenes, as listed in **Table 4** [114]. It was also sug-

Table 4

Separation of carotenoids from *Capsicum annum* L. by TLC on silica with acetone–petroleum ether 1:1 as mobile phase [114].

Carotenoids	R_F
β-Carotene	0.98
Cryptoxanthin	0.64
Zeaxanthin	0.58
Antheraxanthin	0.42
Violaxanthin	0.35
Capsanthin	0.29
Capsorubin	0.23
Neoxanthin	0.18

Table 5

Separation of tomato carotenoids by use of different adsorbents and mobile phases [115].

Adsorbent	Mobile phase	hR_F value[a)]	
	n-Hexane–acetone (*v*/*v*)	β-Carotene	Lycopene
MgO–HSC	80:20	85	10
MgO–cellulose	80:20	83	7
MgO–silica	70:30	70	17
HSC–cellulose	100:0	80	45
HSC–silica	100:0	60	30
Cellulose	100:0	83	20
Cellulose–silica	100:0	62	19
Silica	100:0	88	38

[a)] $hR_F = R_F \times 100$

gested that the best separations could be achieved by use of adsorption TLC on alumina with hexane–chloroform mixtures as mobile phases [82].

Carotenoids from different types of tomato have been separated by use of a variety of adsorbents and mobile phases. It has been revealed that the most suitable TLC system for separation and identification was the combination of a MgO–hyflosupercel–cellulose 10:9:1 (*w/w*) layer with *n*-hexane–isopropanol–methanol 100:2:0.2 (*v/v*) as mobile phase. β-Carotene and a small amount of lycopene were identified In yellow tomatoes (**Table 5**), and phytoene, phytofluene, β, ζ, and γ-carotene, neurosporene, and all-*E*-lycopene were identified in red tomatoes [115]. Similarly, rapid separation of citrus carotenoids has been performed by successive thin-layer chromatographic separations on two different adsorbents. First, use of silica gel with acetone–petroleum ether 30:70 (*v/v*) as mobile phase enabled preliminary fractionation into groups of different polarity (hydrocarbons, mono-ols, diols, and polyols). Further separation of each group into individual carotenoids was achieved by re-chromatography on MgO–Kieselguhr 1:1 (*w/w*) with the same mobile phase [116]. The pigment composition of the marine alga *Codium fragile* has been quantitatively determined by use of $MgO–CaSO_4$ 1:4 (*w/w*) TLC plates with 4% (*v/v*) *n*-propanol in petroleum ether as mobile phase. The alga was found to contain α and ε-carotenes, siphonoxanthin, siphonein, neoxanthin, and violaxanthin. Most of the carotenoids were found to be present at low concentrations, but siphonoxanthin and its ester siphonein accounted for as much as 60% of the total carotenoids present [117].

3.4 Reversed-Phase TLC Analysis of Carotenoids

Most of the work performed on analysis of carotenoids is based on the normal-phase TLC, with limited use of reversed-phase analysis. Reversed-phase TLC (RP-TLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight-chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. In this case, R_F values are lower for molecules which are less polar, whereas polar molecules elute more readily. Thus retention time can be increased by adding a polar solvent to the mobile phase, and retention time can be reduced by adding a more hydrophobic solvent. Mobile phases for reversed-phase TLC consist of various mixtures of hexane, carbon tetrachloride, chloroform, acetonitrile, acetone, tetrahydrofuran, pyridine, acetic acid, methanol, and 1-propanol [82].

Hayashi et al. [118, 119] developed two thin-layer chromatographic methods for analysis of food colorants in tomato, orange, and marigold. The extracted colors, containing lycopene, β-cryptoxanthin, and lutein, were analyzed by reversed-phase TLC on RP-18 $F_{254}S$ with acetonitrile–acetone–hexane 11:7:2 (*v/v*) and acetone–water 9:1 (*v/v*) as mobile phases. It was found that reversed-phase TLC is a useful technique for separation of carotenoids in foods. Silica gel, aluminum oxide, diatomaceous earth, polyamide, cyano, diol, and amino plates have been tested for their ability to separate the color pigments of six chili powders of different origin by both adsorption and reversed-phase thin-layer chromatography [120]. The best separation of colored pigments was achieved on impregnated diatomaceous earth layers with acetone–water 17:3 (*v/v*) as mobile phase. Similarly, crocin and crocetin were extracted from gardenia yellow and separated by reversed-phase TLC on RP-18 $F_{254}S$ with acetonitrile–tetrahydrofuran–oxalic acid 7:8:7 (*v/v*) as mobile phase. It was found that the separation and the spectra obtained were not affected by coexisting substances in foods [121].

3.5 TLC Analysis of Carotenoids with Scanning Densitometry

Different scanning densitometers and software are available in the market. Some valuable software (e.g. CAMAG, Shimadzu) directly monitors and analyzes the TLC plate whereas other software (Just TLC, UN-Scan-IT) can analyze images obtained by use of a camera after plate development. The latter are products less expensive and easier to use and to interpret in comparison with expensive HPLC. The application of scanning densitometry in carotenoid analysis is not well studied. The chlorophyll and carotenoid content of in Tuo cha were analyzed by HPTLC by *Zhong-xi et al.* [122]. Scanning densitometry was performed with a CAMAG TLC scanner. A typical densitogram (CAMAG TLC Scanner, winCATS software) of the carotenoids profile of paprika is given in **Figure 4** [123]. In another study, thin-layer chromatography on 20 cm × 20 cm aluminum foil plates precoated with silica gel 60 F_{254} (Merck, Germany) was performed

for analysis of carotenoid profiles in grapes, musts, and fortified wines. The mobile phase used was acetone–hexane 3:7 (*v*/*v*) and a pre-run with a 2.5% (*w*/*v*) solution of citric acid in methanol. Qualitative analysis of TLC plates was performed using an imaging densitometer (model Q5-700; Bio-Rad, USA) [124]. It was found that densitometry is a useful technique for analysis of carotenoids. Similarly, the carotenoid composition of extracts of the fruits of *Rosa canina* were assessed by TLC and HPLC and the results compared. The extracts were separated on silica plates in two steps. The first involved the use of 15% acetone in petroleum ether and the second 100% petroleum ether. The chromatograms were analyzed using a Shimadzu CS-9000 dual wavelength flying spot scanner. Both chromatographic analyses revealed β-carotene, lycopene, β-cryptoxanthin, rubixanthin, zeaxanthin, and lutein as major carotenoids [125].

An analytical method for the food colors turmeric oleoresin, gardenia yellow, and annatto extract in foods has been established using reversed-phase thin-layer chromatography with scanning densitometry. The method involves clean-up of the colors with a C_{18} cartridge, separation of the colors by reversed-phase TLC on C_{18} plates with acetonitrile–tetrahydrofuran–oxalic acid 7:8:7 (*v*/*v*) as mobile phase, and measurement of the visible absorption spectra of the colors, using scanning densitometry, without isolation of the colors. Eighty-nine commercial foods were analyzed, and the chromatographic behavior and spectra of the colors were investigated. It was found that the spots always gave the same R_F values and spectra as the standards, with good reproducibility. The method was considered useful for rapid analysis of turmeric oleoresin, gardenia yellow, and annatto extract (including water-soluble annatto) in food [126], and scanning densitometry was found to be a useful method for quantification of carotenoids.

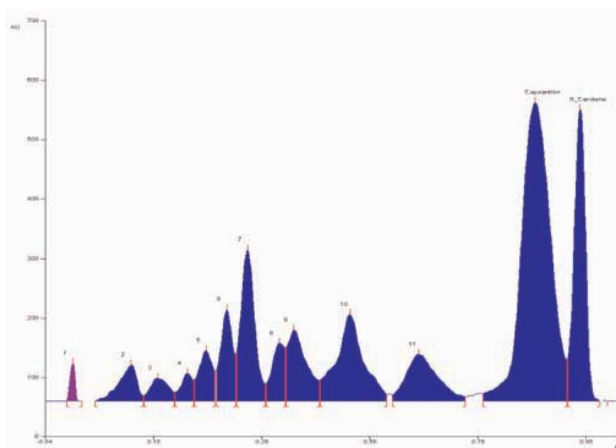

Figure 4

Typical HPTLC densitogram showing separation of carotenoids from paprika [123].

4 Advantages of TLC in Carotenoid Analysis

Because carotenoids can be oxidized or degraded if exposed to intense light or heat, or stored for a long time, rapid analysis and appropriate storage conditions are required. The following points thus make TLC a useful and reliable tool for analysis of carotenoids.

– Carotenoids can be analyzed quantitatively and qualitatively [127] in a very short time.

– A large number of samples can be measured in a single run, which is not possible in other chromatographic techniques.

– The analyses are usually accurate and simple, and less time consuming than for other chromatographic techniques.

– Introduction of high-performance thin-layer plates has increased the efficiency of separation.

– Sensitivity is high for most of the carotenoids.

5 Conclusion and Future Studies

Most separations of carotenoids have been achieved by silica by use of a non-polar or low-polarity mobile phase, and acyclic, monocyclic, and bicyclic carotenoids are clearly separated. For more complex extracts containing a larger number of hydrocarbons or degradation products, overlap of bands becomes a problem, however. For such samples another type of plate, for example MgO, or mixtures of adsorbents can be used. Because different carotenoids have different retention on the same TLC plate, a combination of both is recommended. *Schiedt* [128] has used four different layer systems with different R_F values. Similarly, a single mobile phase is rarely used for mixtures of carotenoids.

Carotenoids oxidation on the surface of the TLC plate and inaccurate analysis are matters of concern in carotenoid analysis. The former can be eliminated by use of low temperature, protection of the plate, and short analysis time; the latter can be removed to highest degree by use of co-chromatography, for example HPLC [82, 129] or spectroscopy [130, 131], diode-array scanning densitometry [132], and image-analysis software [133, 134].

The literature describing the role of TLC in carotenoid analysis is still limited, and much work is necessary. In this regards we have selected some topics for which research on thin-layer chromatography of carotenoids is required:

– study of more carotenoids from animal sources and their complete banding pattern;

– study of the huge amounts of carotenoids present in edible oils, their oxidation, and their reactions;

– isolation of derived products of carotenoids as standard materials for liquid chromatography; and

– selection of best mobile phase for TLC which is more widely applicable to all biological samples.

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High-Performance Thin-Layer Chromatographic Method for Monitoring the Thermal Degradation of β-Carotene in Sunflower Oil

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Key Words

HPTLC
β-Carotene
Degradation
Sunflower oil

Summary

Carotenoids are important pigments found in foods and biological samples. Among carotenoids, β-carotene is the major carotenoid present in vegetable oils. It plays an important role in the thermal stability of the vegetable oil. We established a simple, precise, specific, sensitive, repeatable, and accurate HPTLC method for the analysis of β-carotene in fortified vegetable oils and assessment of its degradation. Analysis was performed on silica gel HPTLC plates with petroleum ether–hexane–acetone 2:3:1 (*v*/*v*) as mobile phase and densitometric detection. The R_F of β-carotene was 0.91 and regression analysis showed response was a linear function quantification of amount of β-carotene in the range 100–600 ng (r^2 = 0.99991). The limits of detection and quantification were 0.11 and 0.37 ng, respectively. The thermal degradation (1–5 h at 100°C) of β-carotene in fortified sunflower oil was studied. It was observed that this HPTLC method could be used for efficient analysis and monitoring of the degradation of β-carotene in edible oils.

1 Introduction

β-Carotene is one of the most widely studied and well-understood nutritional carotenoid. It acts as a provitamin A [1, 2] and is important in human health because it acts as a biological antioxidant, protecting cells and tissues from the damaging effects of free radicals and singlet oxygen [3, 4]; it is also a potential anti-carcinogen [5]. In plants, β-carotene is present in nearly all yellow fruits and vegetables, for example apricots, mangos, carrots, palm fruit, tomatoes, and many other plants. Generally carotenoids also occur in some non-photosynthetic bacteria, yeasts, and molds, where they may have a protective function against damage by light and oxygen. In contrast, animals have no ability to synthesize carotenoids; thus they incorporate carotenoids from their diet.

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Carotenoids are also present in nearly all vegetable oils, including corn, groundnut, soybean, rapeseed, linseed, olive, barley, sunflower, cottonseed, sea buckthorn, red palm oil, and soybean oils [6–8]. However, during refining the carotenoids are decomposed or reduced to very low levels (~0.1 μg g^{-1} in sunflower oil) [9, 10]. Thus addition of synthetic antioxidants is beneficial for nutritional purposes; literature on the degradation of synthetic carotenoids such as β-carotene in edible oils is limited, however [11, 12].

High-performance liquid chromatography (HPLC), either reversed-phase [13–15] or normal-phase [16, 17], is usually used to measure the carotenoid content. Although HPLC is currently the preferred method for carotenoid analysis, it is still subject to several sources of error. It is also expensive to purchase, and maintenance charges are high, so not all quality-control departments of the food industry or of food institutions can afford HPLC systems. Thus, high-performance thin-layer chromatography (HPTLC) can be an alternative in food analysis. In carotenoid analyses the use of TLC is usually limited to purification and separation of individual carotenoids before subsequent determination by HPLC, or for qualitative analysis in foods [18–20]. There is no quantitative HPTLC method for analysis of carotenoids, especially β-carotene, in vegetable oils. The purpose of the work discussed in this paper was to develop a rapid and accurate HPTLC method for quantitative analysis of β-carotene in sunflower oil, as an example, and to use the method to monitor the thermal degradation in a β-carotene–sunflower oil model system.

2 Experimental

2.1 Materials

(All-*E*)-β-carotene (97.0%) was from Sigma Aldrich, USA. Other chemicals and reagents were of analytical grade. Sunflower oil (Spar Austria) was purchased at a local market in Graz, Austria.

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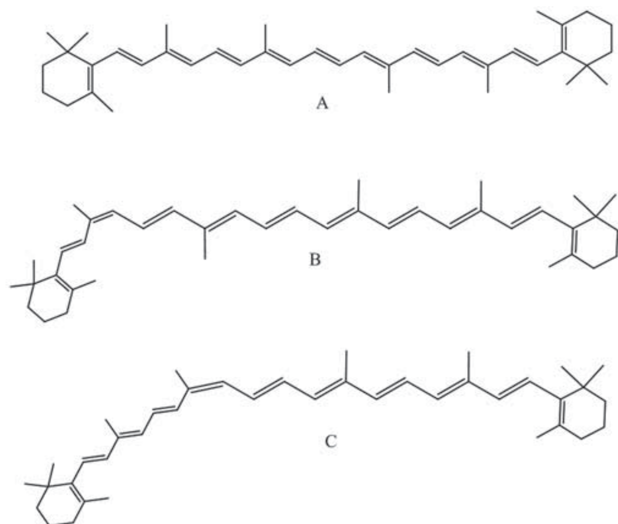

Figure 1

Structural formulae of β-carotene: (A) (all-*E*)-β-carotene, (B) (9-*Z*)-β-carotene, and (C) (13-*Z*)-β-carotene.

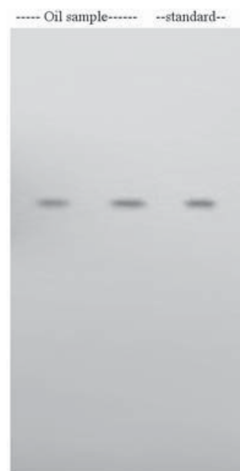

Figure 2

Silica gel HPTLC plate showing the separation of β-carotene using petroleum–hexane–acetone 2:3:1 (*v*/*v*) as mobile phase.

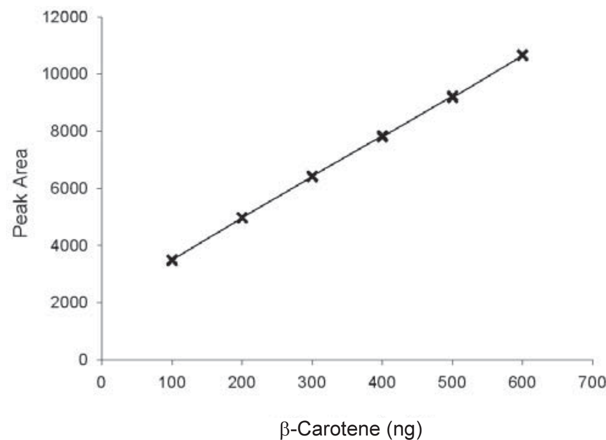

Figure 3

Correlation function for peak area (six-point calibration, twofold analysis) for β-carotene (Y = 2114.704 + 14.238X, r = 0.99991, RSD = ±0.53%).

2.2 Preparation of Standard and Sample

A standard solution (100 ng μL^{-1}) of β-carotene was prepared in hexane. BHT (0.01 mL) was added as antioxidant. The standard solution was stored at –20°C when not in use. The UV–visible spectrum was recorded using a Cary 50 UV-visible spectrophotometer. (All-*E*)-β-carotene (5 ± 0.1 mg) dissolved in acetone was added to sunflower oil (50 ± 0.0050 g) to furnish a β-carotene concentration of 100 $\mu g\ g^{-1}$. The sample was sonicated for 1 min and then kept for 24 h under nitrogen before sealing airtight in a glass bottle. Acetone was evaporated from the β-carotene-fortified oil with nitrogen. All preparations were carried out under nitrogen environment and in the absence of light or air.

2.3 Thermal Degradation of β-Carotene

From the stock of β-carotene-fortified sunflower oil, 5 g (±0.0050 g) was placed in a reaction vessel and oxidized using a Rancimat 679 (Metrohm, Switzerland). Air flow rate was 20 L h^{-1} and the temperature of the heating blocks was 100°C. The samples were heated for 1 to 5h. SF1, SF2, SF3, SF4, and SF5 denote fortified sunflower oil oxidized for 1, 2, 3, 4, and 5 h in the Rancimat.

2.4 Extraction of β-Carotene

β-Carotene was extracted using the method described by *Minguez-Mosquera et al*. [21], with some modifications. The fortified sunflower oil (10 ± 0.0050 g) was directly dissolved in *N*,*N*-dimethylformamide (DMF) then extracted with hexane (8 × 50 mL) in a separating funnel. Chlorophylls, chlorophyll derivatives, and xanthophylls were retained in the DMF phase, and were discarded. The hexane extracts contained lipids and β-carotene. The hexane phases were combined and concentrated to 50 mL with nitrogen. Carotene was extracted from oxidized oil (1 g) by use of the same method and solvent ratio. The extracted β-carotene was kept at –20°C until analysis on the same day. The identity of the extracted β-carotene from the oxidized oils was confirmed by use of a Cary 50 UV–visible spectrophotometer.

2.5 High-Performance Thin-Layer Chromatography

High-performance thin-layer chromatography was performed on 20 cm × 10 cm aluminum-backed silica gel 60 HPTLC plates (Merck, Germany). The plates were pre-washed with 100% methanol and activated at 100°C for 5 min. Samples and standards were applied to the plates as bands 4 mm wide by means of a CAMAG (Muttenz, Switzerland) automatic sampler III. The plates were then developed with petroleum ether–hexane–acetone 2:1:1 (*v*/*v*) in a pre-saturated CAMAG twin trough chamber (20 × 10 cm^2). Ascending development was performed to a distance of 70 mm from the point of application under ambient conditions. After development, plates were dried in air (1 min) and with a nitrogen flush to avoid oxidation of β-carotene. Densitometric scanning was performed on a CAMAG TLC Scanner III controlled by winCATS (v. 1.4.3) software. Other conditions were slit dimensions 6 mm × 0.90 mm, scanning speed 100 mm s^{-1}, and wavelength 450 nm. Sample amounts were determined from the intensity of diffusely reflect-

ed light. Time from sample application to scanning was 19 min, under nitrogen and reduced daylight.

2.6 Quantification of β-Carotene

In accordance with International Conference on Harmonization (ICH) guidelines [22], the calibration plot was established by use of six standard solutions in the concentration range 100–600 ng. Standard solutions (1 µL) were applied to the HPTLC plate to furnish final amounts of 100, 200, 300, 400, 500, and 600 ng per band, in duplicate. Evaluation was based on the linear regression of amount of standard against peak area. Quantitative degradation of β-carotene was measured. Results are expressed as means from three independent experiments. The data were analyzed statistically by one way analysis of variance (ANOVA) using XLSTAT (Addinsoft, v. 7.5.2). Means were compared and significance was accepted at the 5% level ($P < 0.05$).

2.7 Method Validation

The method was validated in accordance with ICH guidelines [22], for precision, repeatability, and accuracy. Instrumental precision was checked by scanning of the same band of β-carotene (600 ng) seven times; the result is presented as *CV* [%]. Variability was assessed by analyzing aliquots of standard solutions of β-carotene (100, 300, and 600 ng) on the same day and on different days. Repeatability was assessed by analyzing a band containing 600 ng β-carotene seven times; the result is presented as *CV* [%]. The limits of detection (LOD) and quantification (LOQ) were calculated from the calibration plot for the standard compounds. The specificity of the method was ascertained by analyzing β-carotene standards and samples of equivalent concentration. The identity of the bands was confirmed by comparing retention factors (R_F) and spectra of the bands from the sample with those from the standard.

3 Results and Discussion

3.1 Quantification of β-Carotene

In this study, we quantified β-carotene pre-spiked in sunflower oil. The concentration of the carotene in the oil sample was 100 µg g^{-1}. (All-*E*)-β-carotene and some of its geometrical isomers (**Figure 1**) were measured as total β-carotene. HPTLC on silica gel plates with petroleum ether–hexane–acetone 2:1:1 (*v*/*v*) as mobile phase (**Figure 2**) was found to enable highly efficient quantification of β-carotene. The extracted and quantified amount of β-carotene in the pre-spiked sample was 92.7 µg g^{-1}, which is indicative of the high accuracy of the method. Quantification was performed using a six-point best-fit standard calibration plot as shown in **Figure 3**. Linear regression with a correlation coefficient of 0.99991 was achieved. The regression equation was:

$$Y = 2114.704 + 14.238X$$

The final concentration of β-carotene was measured by use of the formula β-Carotene [µg g^{-1}] = $(C \times V \times D)/(W \times A)$, where C is the concentration calculated from the calibration plot, V is the final volume from sample preparation, D is the dilution factor (if any), W is the weight of the sample, and A is the amount of sample applied to the plate.

Table 1

Degradation of β-carotene in sunflower oil (sample weight 5 g) at 100°C with aeration at 20 L h^{-1}.

Sample #	Treatment time [h]	Amount of β-carotene [µg g^{-1}][a)]
SF0	0	92.7[b)]
SF1	1	1.62[c)]
SF2	2	0.638[d)]
SF3	3	0.660[d)]
SF4	4	0.0590[e)]
SF5	5	0.0580[e)]

[a)]Values are means from duplicate readings; different superscript letters (b–e) in the same column represent significance at $P < 0.05$

3.2 Degradation of β-Carotene in Sunflower oil

β-Carotene is one of most effective antioxidants, because of its ability to reduce excited singlet oxygen back down to its less reactive triplet state. It provides stability to vegetable oils [23]. We have developed a rapid and accurate HPTLC method for monitoring the degradation of β-carotene in sunflower oil. In sunflower oil fortified with 100 µg g^{-1} β-carotene the β-carotene is significantly degraded in the first hour of its thermal treatment (**Table 1**). Further degradation or reduction was not significant for the next two hours. However, it has been shown to decrease significantly ($P < 0.05$) after 3 h. We observed that under similar conditions β-carotene is more thermally stable in unsaturated triglyceride mixtures, for example triolein, than in vegetable oils [24]. Thus we can assume that the thermal oxidation of β-carotene is more favored in the presence of other pro-oxidants (for example chlorophyll) present in vegetable oils. *Anguelova* and *Warthesen* [25] showed that β-carotene is degraded faster in methyl linoleate at higher temperature. The rapid degradation of β-carotene in lipid systems can also be related to the higher reactivity of the β-carotene radical adduct.

It has also been observed that (all-*E*)-β-carotene was converted to its *Z* isomer, as shown by recording the UV–visible absorption spectrum (**Figure 4**). The appearance of a new band near 340 nm in the spectrum is characteristics of the *Z* isomer of β-carotene [26]. *Warner* and *Frankel* [27] found that at levels ≥20 ppm, β-carotene contributed to poor flavor and color in soybean oil, whereas 5 to 10 ppm β-carotene reduced photosensitized oxidation of the oil without reducing oil quality.

3.3 Specificity of the Method

The specificity of the method was ascertained by comparing the peak purity of the spectrum and the R_F values of standard and sample using a Camag TLC scanner. The peak purity was correlated at $r^2 = 0.999$ at the start–middle and middle–end of the spectrum (**Figure 5**). The R_F value of the β-carotene from the oil samples and the standard was 0.91, as shown in the **Figure 6**.

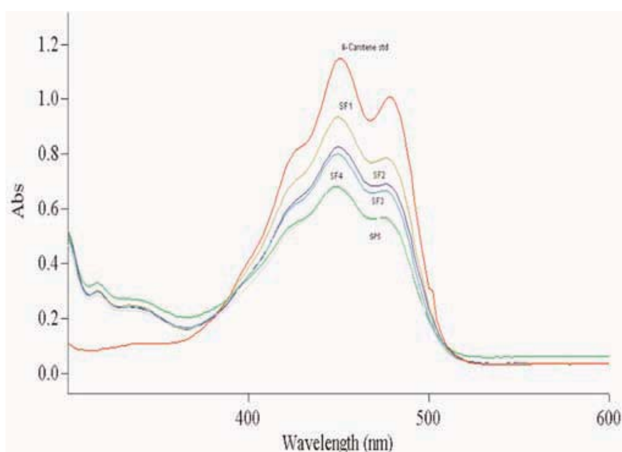

Figure 4

Isomerization of standard (all-*E*)-β-carotene to its *Z* isomers in sunflower oil. SF1 to SF5 denote fortified sunflower oil oxidized for 1 to 5 h.

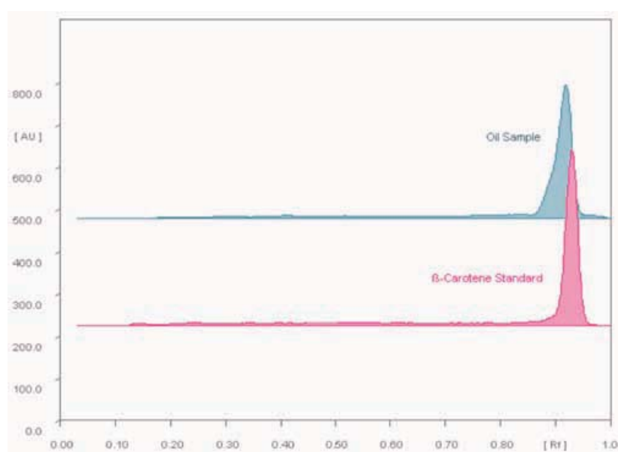

Figure 6

Comparison of the R_F values of β-carotene from oil sample and from a standard.

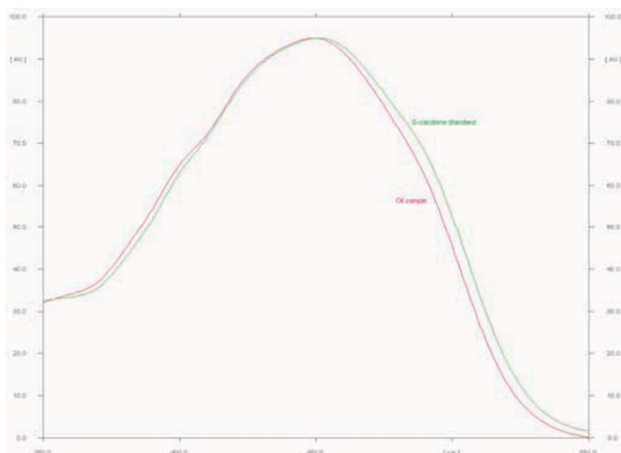

Figure 5

Comparison of the spectra of β-carotene from oil sample and from a standard, both measured on an HPTLC plate (data resolution = 10 nm per step).

3.4 Precision

Instrumental precision was based on the peak area measured by replicate ($n = 7$) scanning of a 600-ng band. Results showed precision was high ($CV = 0.07\%$) for the ATS III scanner. Variability, as intra-day and inter-day precision, was assessed by replicate ($n = 5$) analysis of standard solution of β-carotene (100, 300, 600 ng) applied in triplicate on the same day and on different days. Average intra-day precision and inter-day precision were $CV = 0.54\%$ and $CV = 0.50\%$, respectively (**Table 2**). This means no significant variation was observed during intra and inter-day analysis.

3.5 Repeatability

The repeatability of the method was assessed by replicate ($n = 7$) measurement of the peak areas of bands containing 600 ng (the largest amount of standard analyzed) on an HPTLC plate. The coefficient of variation ($CV = 0.83\%$) indicated no significant change was observed during repeated analysis of the β-carotene.

Table 2

Intra-day and inter-day precision of HPTLC method ($n = 5$).

Amount of β-carotene [ng per band]	Intra-day precision Peak area	*CV* [%]	Inter-day precision Peak area	*CV* [%]
100	5212.3	1.05	5200.75	0.86
	5182.8		5245.05	
	5289.3		5290.85	
300	8602.6	0.08	8691.45	0.27
	8600.0		8737.00	
	8614.3		8703.45	
600	10707.0	0.49	10875.60	0.38
	10713.9		10885.85	
	10801.8		10952.75	
Average precision		0.54		0.50

3.6 Limits of Detection (LOD) and Quantification (LOQ)

The LOD and LOQ were defined as the amounts for which the signal-to-noise ratios were 3 and 10, based on the standard deviation of the calibration plot. The LOD represents the lowest concentrations of β-carotene that can be detected, whereas the LOQ represents the lowest concentrations of β-carotene that can be quantified with acceptable precision and accuracy. The LOD and LOQ were 0.11 and 0.37 ng, indicative of the high sensitivity of our method for quantification of β-carotene in vegetable oils.

4 Conclusion

We have established an HPTLC method for monitoring the degradation of β-carotene in fortified vegetable oils. The method was found to be simple, precise, specific, sensitive, repeatable, and accurate and can be used for quantification of β-carotene in vegetable oils. This method is a good alternative to HPLC methods. It can be also used in routine analysis of food and biological formulations containing β-carotene.

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Research Article

Analysis of triacylglycerols in refined edible oils by isocratic HPLC-ESI-MS

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A simple, fast and reproducible reversed-phase high performance liquid chromatography (HPLC) method coupled to electrospray ionization mass spectrometry (ESI-MS) for the analysis of triacylglycerols (TAGs) species in the commercial edible oils has been developed. The TAGs species were separated using isocratic 18% isopropanol in methanol and a Phenomenex C18 column. The ESI-MS conditions were optimized using flow injection analysis of standard TAG. Fifteen, fourteen, and sixteen TAGs were separated and identified in corn oil, rapeseed oil, and sunflower oil, respectively. The presence of intense protonated molecular ($M + H^+$), ammonium ($M + NH_4^+$), and sodium ($M + Na^+$) adducts ions and their respective diacylglycerols ions in the ESI-MS spectra showed correct identification of TAGs. Some minor potassium adducts ($M + K^+$) were also found. In addition, the identity of the fatty acid, position of each fatty acid, and the location of the double bond in the fatty acid moiety were explained. It was found that this isocratic method is useful for fast screening and identification of triacylglycerols in lipids.

Keywords: triacylglycerols / HPLC-ESI-MS / corn oil / rapeseed oil / sunflower oil

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1 Introduction

Edible oils are a complex mixture of triacylglycerols (TAGs). They are composed of a glycerol backbone esterified with saturated, unsaturated fatty acids or mixture of both. Thus structural characterization of TAG is required to know the exact chain length of the acyls group, degrees of unsaturation, and locations of double bonds [1]. Regio-specific locations of each acyl chain are important in terms of chemical as well as physical properties like viscosity, pour point, melting point, heat of fusion, solubility, crystal structure, and polymorphism [2]. It also affects the human absorption from different foods and is thus an important parameter for food industries.

Reversed phase HPLC is the most extensively used separation technique for the analysis of TAG in oils and fats [3]. Several detection systems were used for the identification and characterization of TAGs including ultraviolet (UV), refractive index [4], evaporative light scattering detector (ELSD), [5, 6, 2] and mass spectrometry [7, 8]. TAGs regio-isomers have been analyzed in vegetable oils using atmospheric chemical ionization (APCI) mass spectrometry [9, 3, 6, 7]. Positional distribution of fatty acyl chains of TAG has been analyzed in palm oil, cocoa butter, beef, pork, and chicken fats using HPLC-APCI-MS. Individual regio-isomers were identified and quantified by linear calibration against the standards [7]. However, when a mixture of TAG was separated using RP-HPLC and identified using APCI-MS, the minimal fragmentation in APCI produced intense diacylglycerols ions [8, 10] and in general less information about molecular weight of the TAG is provided. Also during interpretation of APCI mass spectra, the lack of intact protonated molecules produced from some oxygen functional group containing TAGs is another shortcoming [11]. Because of the above reasons, Leskinen *et al.* [12] have recently proved that the LC-APCI-MS method is not applicable in the quantification of TAG regio-isomers. Contrary to this, a recent work showed that the HPLC-APCI-MS method provides excellent chromatographic resolution and allows the analysis of high-molecular-weight TAG and can still be used as a good tool for the identification of TAGs [13].

Electrospray ionization mass spectrometry (ESI-MS) has also been used for the identification of TAGs [14–16]. Hsu and Turk [1] studied the lithiated adducts of TAGs using ESI-MS. They distinguish between isomeric TAGs species. Also the double bond position on each fatty acyl chain

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Abbreviation: ESI-MS, electrospray ionization mass spectrometry; **ELSD,** evaporative light scattering detector; **TAGs,** triacylglycerols

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has been located. However, the lack of molecular ions and less intense lithium molecular adducts ions were the major discrepancies. Similarly, Hvattum [17] studied the identification of standard TAGs using LC-ESI-MS. Methanol and chloroform were used as solvent with ammonium formate and formic acid as adduct reagent. However, only ammonium adducts were used for identification of molecular mass of the TAG. No molecular or protonated molecular ion with sufficient intensity was obtained. Marzilli *et al.* [18] showed structural characterization of standard TAGs using ESI-MS. They used ammonium acetate in the solvent system to obtain ammonium adducts, in addition to protonated molecular ions. Both ions were less intense; however, they did not study the TAGs in edible oils. Recently Lin and Arcinas [19] reported the uses of ESI-MS for the analysis of TAGs in extra virgin olive oil. Lithium acetate was used to obtain corresponding lithiated ions. They showed that all fragmentations formed lithiated adducts. However, the lack of the molecular ions in their spectra is one of the main disadvantages. Thus for the characterization and identification of regio-specific TAGs, intense molecular and molecular adduct ions and other fragment ions are useful tool to correctly identify each TAG in edible oils or fats. In addition, the abundance of diacylglycerols ions is also an important parameter for the exact location of each fatty acid. It is based on the fact that 1,2-diacylglycerols ions would be prominent than 1,3-diacylglycerols, because of the steric hindrance at the sn-2 position.

Here we present a simple, fast, and reproducible isocratic RP-HPLC-ESI-MS method for the identification of regio-specific TAGs in commercial edible oils like corn, rapeseed and sunflower oils. The fatty acids (FAs) in each TAG and the regio-isomerism of these FAs were determined from their mass spectra on the basis of specific fragmentation and relative abundances.

2 Materials and methods

2.1 Materials

Isopropanol and methanol were HPLC grade from Baker B.V (The Netherlands). Ammonium/sodium acetate and TAGs mixture were from Sigma Aldrich (Germany). All other chemicals and reagents were of ACS grade from Sigma Aldrich USA. Corn (CO), rapeseed (RPO), and sunflower oils (SFO) were purchased at a local market in Graz, Austria.

2.2 Sample preparation

The oil samples (50 mg $\pm$ 0.5 mg) were dissolved in 2 mL of acetone. HPLC solvent (18% isopropanol in methanol) of 2 mL were added. Acetone was used, because of its effects in improving the selectivity of TAG pairs or groups with the same partition number (PN) and complete dissolution of oils. The samples were then directly injected in to the HPLC system.

2.3 Liquid chromatography-mass spectrometry

Liquid chromatography was carried out using an Agilent HP 1100 system equipped with vacuum degasser, quaternary pump, auto sampler, temperature-controlled column oven, LC coupled to ESI-MS (Agilent, Waldbronn, Germany). For the separation a Phenomenex C18 (150 mm \times 3 mm) (Germany) column was used. The analytes were eluted using an isocratic solvent system consists of 18% isopropanol in methanol (0.1% acetic acid). Ammonium acetate (0.05%) and sodium acetate (0.001%) were added to the solvent system. The flow rate was maintained at 0.6 mL/min. The separation time was 35 min. Mass spectrometric conditions were optimized using flow injection analysis of standard TAGs. The optimized conditions are positive ESI mode, fragmentor potential 150 V, drying gas temperature 350 °C, capillary voltage 4000 V. The ESI-MS spectra were obtained at *m/z* range of 200 to 1000. Reproducibility of the percent peak area and retention time was determined using a mixture of standard TAGs, five times at day 1 and day 2 and were expressed as %CV.

3 Results and discussion

3.1 Optimization of HPLC and ESI-MS conditions

A reversed phase C18 column was used for the separation of TAGs in edible oils. Different organic solvents (acetonitrile, propionitrile, acetone, methanol, isopropanol, and dichloromethane) and their mixtures were tried for the separation of TAGs. For optimization of HPLC, only the ELSD detector was used, as it was easy to optimize and a better baseline was obtained in short time. We found 18% isopropanol in methanol to be a better solvent system with good separation. The background noises in chromatograms and subsequent MS spectra are low using isopropanol in methanol. The other solvent systems were rejected on the basis of toxicity [20], separation potential, and high cost. Thus, this method was simple and in short time (35 min) a separation of most of the important TAGs has been achieved and could be used for fast screening of TAGs identification of other edible oils and fats.

Mass spectrometry conditions were optimized using standard triacylglycerol (tripalmitin). Generally, if mass spectrometry is carried out using non-aqueous solvent system, the intensity of the total ion current is relatively low. Therefore either ammonium acetate or sodium acetate has been added to the solvent in order to increase the ionization efficiency. The addition of these ions to the eluent did not influence the retention time which was observed by other groups as well [11, 21]. We found that sodium acetate formed more intense adducts at a very low concentration. The addition of these additives also resulted in the better peak shape and

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information of the molecular masses (from the molecular ions/molecular adducts ions) were correctly obtained. A fragmentor voltage of 150 was selected based on the more information about the fragments and consequent structure of TAG. Sjovall *et al.* [14] used a fragmentation voltage of more than 100 V for the identification of natural triacylglycerol peroxide using reversed phase HPLC coupled to electrospray mass spectrometry. We found that the ammonium and sodium molecular adducts were the intense ions in ESI-MS at fragmentation potential of 150 V. The potassium adducts were rare with lower intensity.

A remarkable reproducibility of the percent peak area and retention time has been achieved and was expressed as %CV. The reproducibilities (Fig. 1) for the five selected standard TAGs (2-LLL, 4-OLL, 7-OLO, 9-OOO and 12-OSO) at day 1 were 2.4%, 3.6%, 2.3%, 1.1% and 1.7%, while it was 2.4%, 4.1%, 3.0%, 1.3%, and 3.4% at day 2 measurements. The reproducibility of retention time was 1% at both days measurements for all five peaks.

3.2 Fatty acid composition in TAGs

Fatty acids are esterified with glycerol forming TAGs of different molecular masses. Fragmentation plays an important role in the identification of the fatty acid moiety on the glycerol backbone. Fragment ions that reflect the mass of each fatty acid substituent are observed at *m/z* values corresponding to the loss of a fragment from the TAG forming diacylglycerols (M-RCOO$^+$ or M-RCO$^+$). For example in the spectra of LLL (Fig. 2A), the loss of *m/z* 281.2 is expecting to be a RCOO$^+$ ion, but it is *m/z* 263.3, which is an acylium ion (RCO$^+$). It is formed from further fragmentation of RCOO$^+$ with the loss of water molecule (RCOO$^+$ -H_2O). Hsu and Turk [1] found that in addition to lithiated RCOO$^+$, RCO$^+$ and (RCO$^+$ -18) are also formed during fragmentation. The *m/z* of diacylglycerol fragment (LL$^+$) was 599.5, while the protonated molecular ions *m/z* 879.7. So the loss of an acylium ion at *m/z* 263.3 confirmed that the fatty acid is linolenoyl ion (L$^+$). Similarly in the ESI-MS spectrum of POO, the m/z 289.3, represent a lithiated product of oleic acid (Fig. 2B). Thus the mass of each fatty acid substituent may be reflected by a lithiated product (RCOOH + Li)$^+$, an acylum ion RCO$^+$ and (RCO$^+$ -18)$^+$ in ESI-MS spectra of TAGs. Since the ammonium acetate added to the solvent system contains 0.005% lithium as mentioned on label, therefore some rare lithiated products can be expected. The formation of lithiated products also depends on the reactivity of each ion toward the lithium and the applied fragmentation potential.

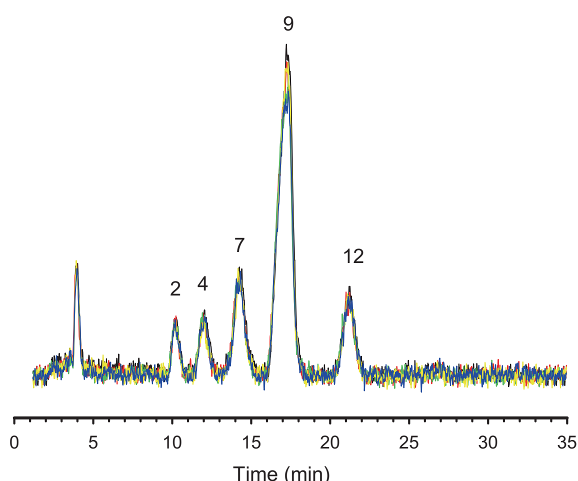

Figure 1. Separation reproducibility using five consecutive injections of standard mixture of TAGs (2-LLL, 4-OLL, 7-OLO, 9-OOO and 12-OSO).

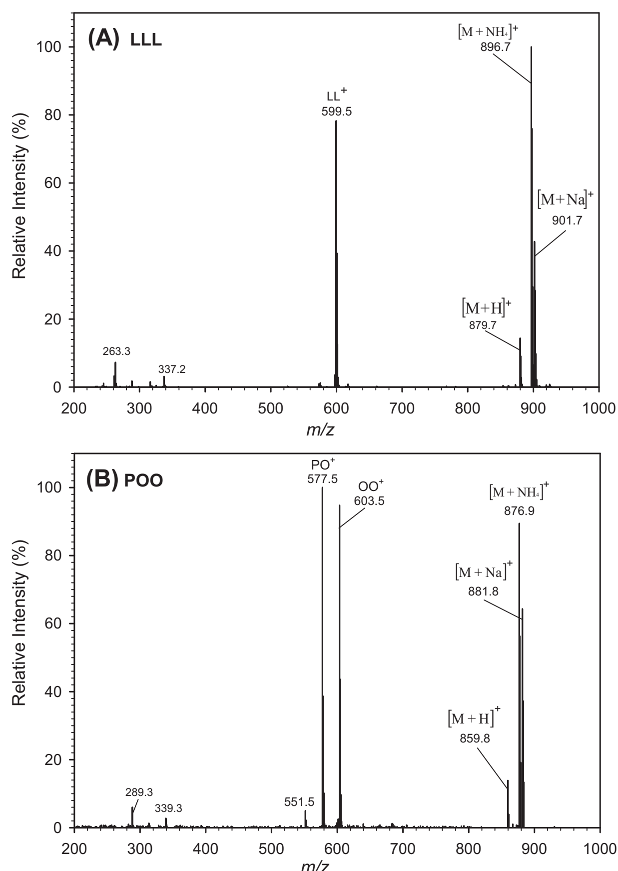

Figure 2. The type of fatty acid as shown by ESI-MS spectra of (A) LLL and (B) POO.

3.3 Position of fatty acid in TAGs

The regio-specific determination of the fatty acid location on the glycerol backbone plays an important role in the separation of the mixture of TAGs. Taking into account a few generally accepted considerations, TAGs positional isomers

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were identified. It includes, that less abundant fatty acids are often esterified on sn-3 position, and the absence of palmitic acid in the sn-2 position in plant TAGs [3]. It is also based on intensity of diacylglycerols and the fact that α and β-diacylglycerol fragments are energetically more favored. However, the sn-1 and sn-3 position are indistinguishable in MS spectrum [22, 12], also there is no official recommendation for designation of sn-1/3 position. In the ESI-MS spectrum of OLnL (Fig. 3A), the *m/z* 879.7, 896.7 and 901.7 corresponds to the protonated molecular ion, and molecular adducts of ammonium and sodium respectively. The diacylglycerols fragments at *m/z* 597.5, 599.5 and 601.5 correspond to LLn^+ , OLn^+ , and OL^+ respectively. The intensity of OLn^+ is higher than others, while LLn^+ has higher intensity than OL^+ , which means that Ln is at sn-2 position and O and L bound at sn-1 and sn-3 position. In this spectra the *m/z* 261.3, 263.3 and 265.3 are RCO^+ ions reflecting O, L and Ln fatty acids. Similarly, in the ESI-MS spectrum of PLO (Fig. 3B), the *m/z* 857.8, 874.8, and 879.8 corresponds to the protonated molecular ion ($M + H^+$), molecular adducts of ammonium ($M + NH_4^+$), and sodium ($M + Na^+$), respectively. The ions at *m/z* 575.5, 577.5 and 601.5 correspond to PL^+, PO^+, and LO^+, respectively, are the diacylglycerols fragments. The high intensity of PL^+ and LO^+ showed that the palmitic (C16:0) is esterified on sn-1 and oleic acid (C18:1) on sn-3 position.

3.4 Position of double bonds in the fatty acids

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 a single bond. In addition to the diacylglycerols fragments ions of *m/z* 577.5, 579.5, and 605.5 of PO^+ , PS^+ , and SO^+ , respectively, there is another fragment ion of *m/z* 699.7 in ESI-MS spectra of POS (Fig. 4A). The mechanism of formation of this fragment ion has been presented at Fig. 5. Since saturated acyl groups (palmitic and stearic) on sn-1 and sn-3 are energetically more resistant to cleavage than an unsaturated acyl group, a neutral loss of C_9H_{20} molecule may occurs at the double bond position. Further fragmentation occurs at the loss of carbonyl oxygen atoms, resulting in to the formation of a double bond on

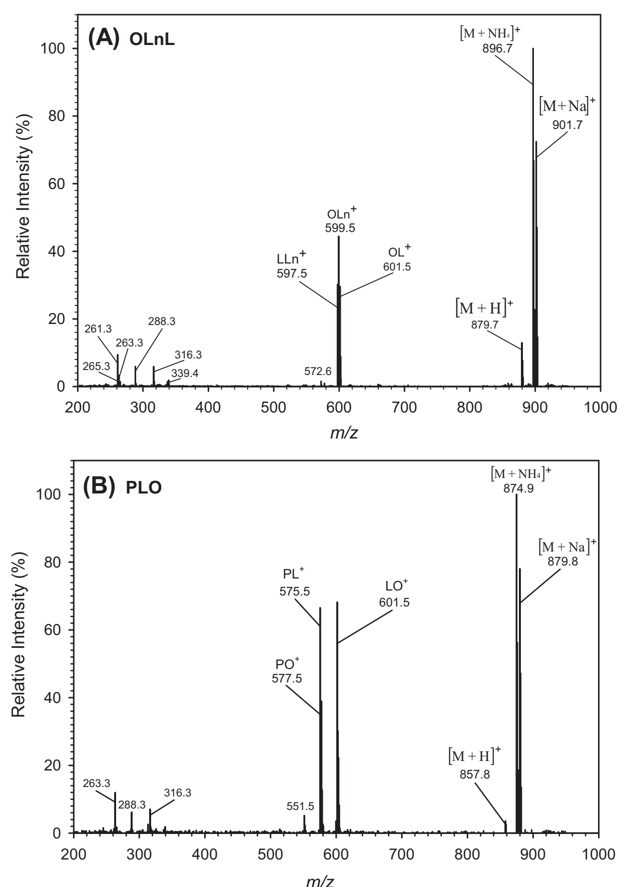

Figure 3. The regio-specific determination of position of fatty acid in TAG using ESI-MS spectra. (A) ESI-MS spectra of OLnL (B) ESI-MS spectra of PLO.

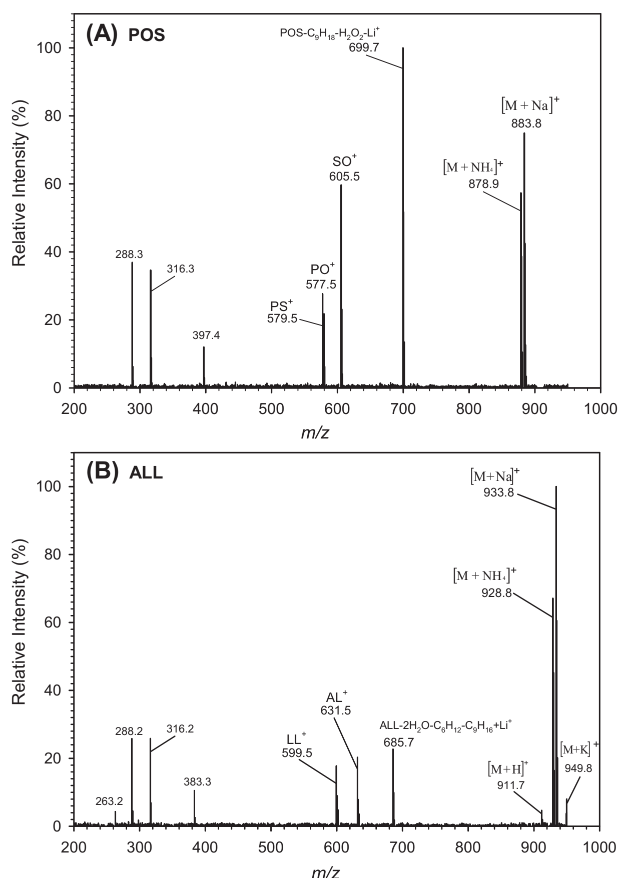

Figure 4. Possible fragmentation in ESI-MS spectra showing the position of double bond in fatty acid of TAGs. (A) ESI-MS spectra of POS (B) ESI-MS spectra of ALL.

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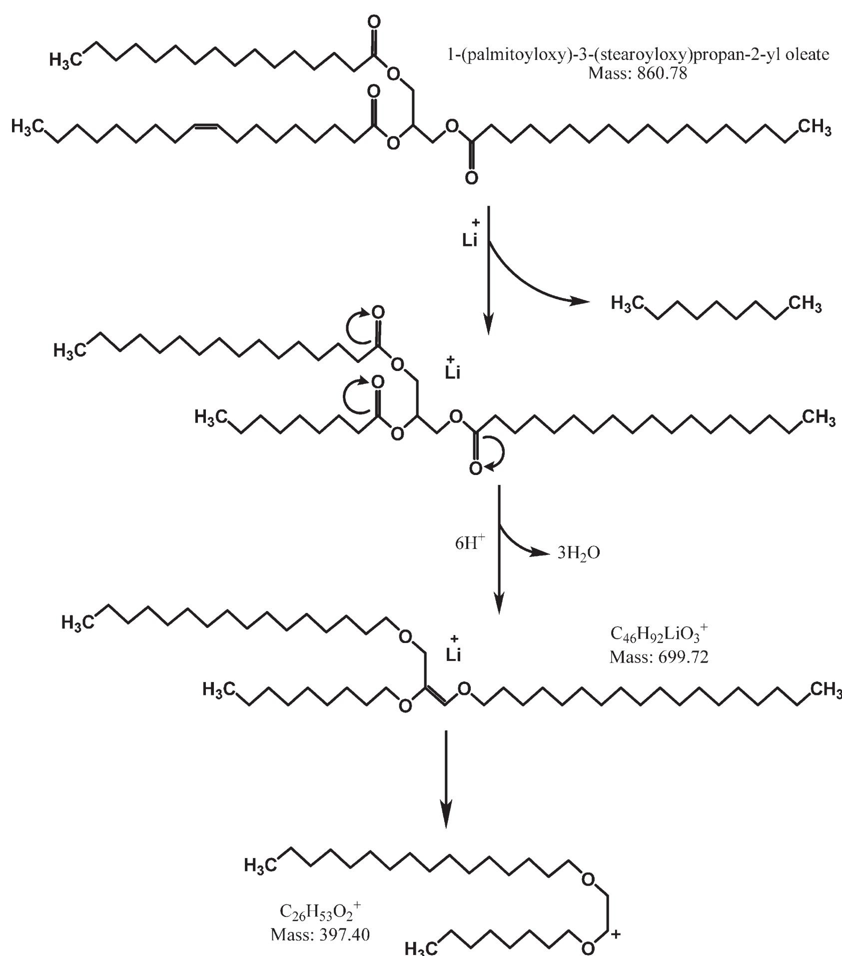

Figure 5. Hypothetical fragmentation reaction showing the position of double bonds of fatty acid in TAGs.

glycerol between carbon 2 & 3 forming a lithiated adduct of *m/z* 699.7. An impurity of lithium in the solvent system might be the reason of the formation of lithiated adducts. Hsu & Turk, [1] showed that at high fragmentation potential a trace amount of lithium is enough for the formation of lithiated adducts. The proposed double bond at carbon 2 & 3 position was confirmed by the presence of an ion of *m/z* 397.4, which may formed by further loss of $C_{19}H_{38}O^+$ and a terminal carbon atom. The presence of acetic acid may increase the chances of protonation and formation of neutral molecule. Another example of similar fragmentation pathway has been found in ALL as shown in Fig. 4B. We found that the cleavage at the double bond on C9 is more favored on the acyl group at sn-3 than at C12. While on the acyl group at sn-2 position the cleavage might occurs at the double bond on C12 carbon, because sn-2 position is usually more resistant than sn-3. Thus an ion of *m/z* 685.7 is formed, which explain the exact position of double bond on both acyl groups. An ion of *m/z* 383.3 is the resultant fragment ion from the *m/z* 685.7 ion. Hsu & Turk, [1] used source collisionally activated dissociation-MS experiments with LLL yielded a tandem spectrum of dilithiated adduct of L, which shows that the 9 and 12 position of double bond. They did not explain why the cleavage occur other than double bond. The fragmentation in our MS spectrum is different from their results in respect of very clear cleavage/fragmentation on the double bond position.

3.5 Triacylglycerols in edible oil

TAGs were separated using isocratic RP-HPLC. Different ratios of methanol and isopropanol were used (results not shown). We found better separation with 18% isopropanol in methanol with 0.1% acetic acid. A mixture of standard TAGs was separated in order to confirm the identity of each TAG in the edible oils (Fig. 6A). They are peak 2, 4, 7, 9, and 12, corresponds to LLL, OLL, OLO, OOO and OSO. Comparison of the retention time and mass spectra of standard mixture of TAGs with the edible oils TAGS was carried out in order to correctly identify each TAG.

Table 1 shows three types of TAGs i.e made of either one kind of fatty acid (LLL, OOO etc); two kinds of fatty acids (PLL, PLP etc) and three different kinds of fatty acids (POS, PLO etc). TAGs of the first kind has usually one DAG

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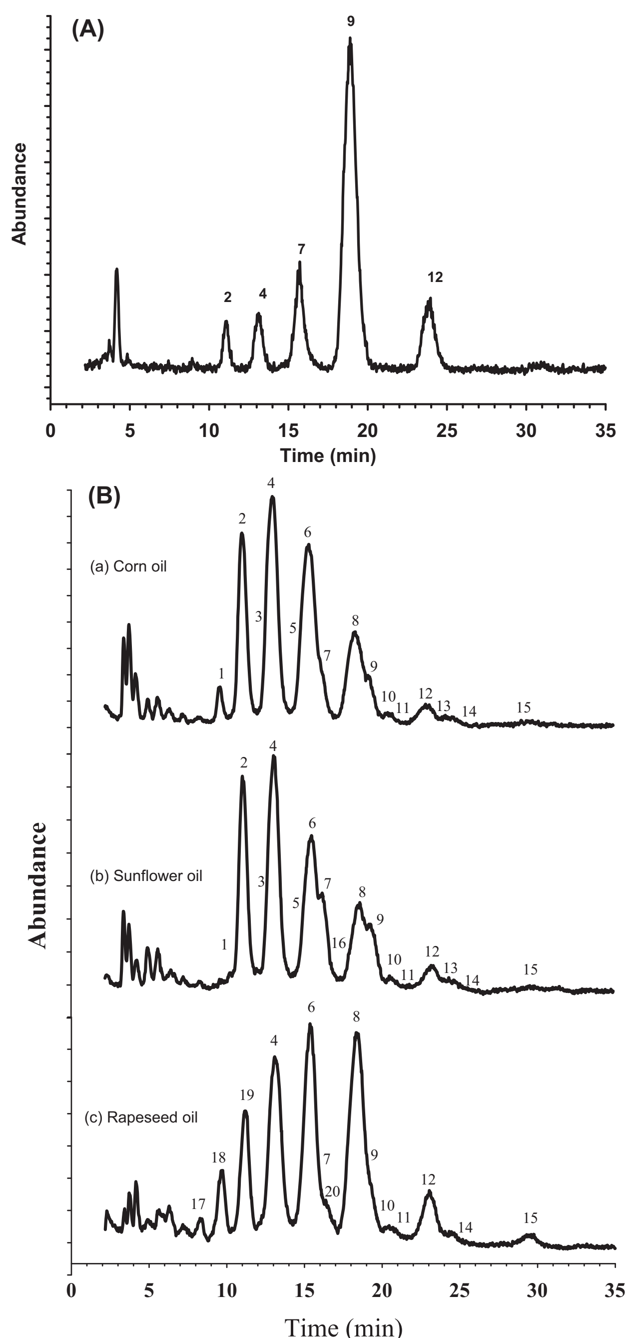

Figure 6. HPLC-ESI-MS chromatograms of edible oil. (A) Corn oil (B) Sunflower oil (C) Corn oil. The peak identification is explained in Table 1. The numbering represents identification only.

fragment ion, *i.e.* LL (*m/z* 599) for LLL. Two DAG fragments ions were observed for TAGs of the second kind such as LL (*m/z* 599) and LLn (*m/z* 597) for LLLn (*m/z* 877). Similarly third kind reveal three DAG fragment ions, *i.e.* OLLn (*m/z* 879) has three fragment ions LL (*m/z* 599), LLn (*m/z* 597), and OL (*m/z* 601).

The total ion ESI-chromatograms of corn, rapeseed, and sunflower oils are shown in Fig. 6B. Co-elutions are observed for some TAGs. The earlier studies reported on average of 15-25 TAGs, while in our study in corn oil 15 TAGs were identified (Fig. 6A). Van der Klift [6] identified 44 TAGs using two-dimensional liquid chromatography with UV, ELSD, and MS detection. However they did not identified AOO. The possible reason may be the variation in composition due to variety, origin, environmental conditions, and methods of extract of corn oil. The details of all TAGs found in corn oil with chromatographic characteristics and MS identification are shown in Table 1. The major TAGS were LLLn, LLL, OLL, OLO, SLL, POO, OOO, and OSO. The minor includes AOO, ALO, POS, SOL/SLO, ALL, PLO, PLP, and PLL. Some peaks were results of co-elution of up to four TAGs, *i.e.* two TAGS (POS & OSO, PLO & PLP) and four TAGs (SLO/SOL, OOO & POO). Holcapek *et al.*, [23] indicated that the pairs SLL/OLO and POO/SLO were co-eluting. However, in our separation both SOL and SLO co-eluted at a retention time of 19.3 and 19.6, respectively. The main reason may be the use of different solvent systems and columns. Although the co-elution did not affect the possibility of positive identification of TAG, it was achieved by using the extracted ion chromatograms. Proton ($M + H^+$), ammonium ($M + NH_4^+$), and sodium ($M + Na^+$) adducts ions were the major ions in the ESI-MS spectra. In all cases the diacylglycerols fragments or molecular adducts were of the high intensity making the spectra easy to interpret.

In rapeseed oil a total of 14 TAGs were identified (Fig. 6B). The major TAGs were LLnLn, OLnLn, OLLn, OLL, OLO, POO, OOO, OSO, and AOO. The minor TAGs includes ALO, POS, SOL/SLO, ALL, PLO, and GLL. Co-eluting peaks were ALO & OSO, ALL & SOL/SLO, POO & GLL, and OLO & PLO. Rapeseed oil was found to contain more linolenic acid TAGs than corn and sunflower oils. Further it was confirmed by fatty acids determination using gas chromatography. Results of gas chromatography (results not shown here) are in accordance with the current method.

In sunflower oil a total of 16 TAGs were identified as shown in Fig. 6B. The major TAGs were LLLn, LLL, OLL, OLO, SLL, OOO, SOL/SLO, and OSO. The minor TAGs includes AOO, POS, ALL, POO, PLL, and PLP. Co-eluting peaks were POS & OSO, SOL/SLO, POO & OOO, and OLO & SLL. Sandra *et al.* [24] found 15 TAGs in sunflower oil using silver ion packed column supercritical fluid chromatography coupled to APCI-MS. Acetonitrile and isopropanol (6/4, v/v) were used as solven system. Due to the differences in ionization mode and other parameters, they did not report some important TAGs like AOO, ALL, and LLLn. Sunflower oil was found to contain more linoleic acid TAGs than other studied oils.

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Table 1. Peak identification from electrospray ionization mass spectrometry of triacylglycerols in edible oils. A common number represent the same TAG present in the oils. Retention time difference is negligible in comparison to each peak.

Peak #	Ret. time (min)	TAG	$M + H^+$	$M + NH_4^+$	$M + Na^+$	DG fragments (*m*/*z*)		
1	9.6	LLLn	877	894	899	LL 599	LLn 597	–
2	11.0	LLL	879	896	901	LL 599	–	–
3	12.2	PLL	855	873	878	LS 599	PL 575	–
4	13.6	OLL	881	898	903	LL 599	LO 601	–
5	14.5	PLP	831	849	854	PP 551	PL 575	
6	15.2	PLO	857	875	880	LO 601	PL 575	PO 577
7	16.3	OLO	883	900	905	OL 601	LL 599	–
8	18.0	POO	859	877	882	PO577	LL 603	–
9	18.6	OOO	885	902	907	OO 603	–	–
10	19.3	SOL	885	902	907	SO 605	SL 603	LO 601
10	19.6	SLO	885	902	907	SO 605	SL 603	LO 601
11	20.3	ALL	911	919	924	AL 631	LL 599	–
12	22.8	OSO	887	904	909	OO 603	OS 605	–
13	23.2	POS	861	879	884	SO 605	PS 579	PO 577
14	24.7	ALO	913	931	936	LO 601	AL 631	AO 633
15	30	AOO	912	933	937	AO 633	OO 603	–
16	16.8	SLL	883	900	905	SL 603	LL 599	–
17	8.5	LLnLn	875	892	897	LnL 597	LnLn 595	–
18	9.9	OLnLn	877	894	899	LnO 599	LnLn 595	–
19	11.5	OLnL	879	896	901	LLn 597	LnO 599	OL 601
20	16.9	GLL	909	926	931	GL 629	LL 599	–

4 Conclusions

A novel method incorporating reversed-phase HPLC method coupled to ESI-MS for analysis of TAG species in edible oils has been developed. The TAG species were separated using reversed phase isocratic 18% isopropanol in methanol. In edible oils, the numbers of identified TAG were between 14 and 16. The presence of intense proton ($M + H^+$), ammonium ($M + NH_4^+$), and sodium ($M + Na^+$) adduct ions and their respective diacylglycerols ions in the ESI-MS spectra allowed correct identification of TAG. Therefore no MS-MS is required. Potassium adducts ($M + K^+$) were less abundant. In addition, the method provides information on identity of the fatty acid, position of each fatty acid, and the location of the double bond in the fatty acid moiety.

The authors have declared no conflict of interest.

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Research Article

Characterization of the effects of β-carotene on the thermal oxidation of triacylglycerols using HPLC-ESI-MS

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RP HPLC method coupled to ESI-MS was used for the analysis and characterization of the oxidation of model triacylglycerols (TAGs) in presence of β-carotene. β-Carotene was added to the TAGs and oxidized in the Rancimat at 110°C. The samples were separated isocratically using a mixture of isopropanol with methanol and a Phenomenex C18 column. β-Carotene degradation was measured using high performance TLC. We found that β-carotene plays an important role during the thermal degradation of high oleic acid model TAGs. Half of the β-carotene was degraded before 3 h of thermal treatment. β-Carotene significantly increases the peroxide value of the TAGs after the third hour, suggesting a pro-oxidant action. However, different TAGs show different activity toward thermal treatment and β-carotene. The LLL was found to be less stable, OLL and OLO were stable till 10 and 12 h respectively, while POO, OOO, and OSO were the stable TAGs till 14 h. In TAGs, replacing linoleic acid by oleic acid, the stability of the corresponding TAG was found to increase by 2 h. A new class of oxidized TAGs was reported for the first time, together with previously reported species. The proposed mechanism of formation and identification of the newly identified species have been explained. Among the oxidized species of TAGs, mono-hydroperoxides, bis-hydroperoxides, epoxy-epidioxides, and epoxides were the major compounds identified.

Keywords: β-Carotene oxidation / Free radical reaction / HPLC-ESI-MS / Oxidized triacylglycerols / Thermal stability / Triacylglycerols

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1 Introduction

Triacylglycerols (TAGs) are important components of edible oils in terms of nutrition and stability. Unsaturated TAGs, especially with polyunsaturated fatty acids, are highly susceptible toward heat. This is an important point of concern of the food chemists and food manufacturers. Thermal stress was found to have significant influence on the nutritional and sensory quality of the TAGs containing lipids [1]. One of the most important reactions occurring during the oxidation of unsaturated TAG is the free radical-mediated oxidation, which leads primarily to the formation of conjugated fatty acid hydroperoxides. Usually these hydroperoxides are more stable than free radical species. However, they are still weak oxidizing agents and decompose to peroxyl and alkoxyl radicals [2]. These free radical compounds form a wide variety of secondary oxidation products, resulting in to the rancidity of oils or fats. These non-volatile decomposition products gradually accumulate in the oil and are absorbed by the fried foods, and finally ingested [3].

These oxidation products thus contribute toward potent off-flavors and have direct impact on the quality of many food products [4, 5]. Normal phase HPLC-MS (HPLC-MS) was used to study the non-volatile oxidized species of TAGs [6], which include epoxy-TAG, oxo-TAG, hydroperoxy, and hydroxyl-TAGs. However, the study lacks information on the correct position of these oxygen containing functional groups which is necessary for the correct assignment of the chemical structure. The oxidized species of *tert*-butyl hydroperoxide oxidation products of unsaturated TAGs have been studied using RP HPLC coupled to ESI-MS [7]. Ammonium molecular adducts were the major ions for the identification of the oxidized species. There was no clear structural

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Abbreviations: TAG, triacylglycerol; **HPTLC,** high performance TLC; **POV,** peroxide value; **EIC,** extracted ion chromatogram; **P,** palmitic; **S,** stearic; **A,** arachidic; **G,** gadoleic; **Ln,** linolenic; **L,** linoleic; **O,** oleic

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identification of oxidized species. Neff and Byrdwell [8] analyzed the oxidation products from the auto-oxidation of three standard TAGs (triolein, trilinolein, and trilinolenin) using RP HPLC coupled with atmospheric chemical ionization MS (APCI-MS). Mono-hydroperoxides were among the major oxidized TAGs. Other species were epoxides, hydroperoxy-epidioxides, and hydroxides of TAGs. The structure of each oxidized species was correctly identified. The method was further used to study other products formed from triolein oxidation [9]. The products included were hydroperoxides, epoxides, and a ketone. Other products shown were formed by either shortening of an acyl chain on the intact triolein or dimerization of triolein. However, the effect of temperature on the stability of each TAG, the effects of any antioxidants on the formation of oxidized products; and thermal oxidation products formed from the mixed TAGs were not studied.

In order to increase stability of TAGs, different antioxidants are used. As a food additive, β-carotene as antioxidants and colorant is commonly dissolved in a lipid/oil. As lipids oxidize, β-carotene oxidation proceeds via a complicated co-oxidation mechanism, accompanying the lipid oxidation or *vice versa* [10]. To the best of our knowledge there is lack of literature regarding the effect of β-carotene on the thermal oxidation and the formation of TAGs oxidation species. For this purpose, we used our recently developed isocratic HPLC coupled to ESI mass spectrometric method for characterization of TAGs species in edible oils [11]. The work presented here is the application and extension of that method with the following aims: (i) to know the effects of β-carotene on TAGs oxidation and (ii) to identify oxidation products of model TAGs.

2 Materials and methods

2.1 Materials

Ammonium acetate and TAGs mixtures were from Sigma–Aldrich (Germany). All other chemicals and reagents were of ACS grade from Sigma–Aldrich USA and as reported [11].

2.2 Sample preparation and Rancimat oxidation

All-*E*-β-carotene dissolved in acetone was added to the TAGs mixture to furnish a β-carotene concentration of 300 μg/g ± 0.5 μg. The sample was sonicated for 1 min and then kept for 1 h under nitrogen before sealing airtight in a glass bottle. Acetone was evaporated using nitrogen. From the stock of β-carotene fortified TAGs, 4 g (±0.0050 g) were added to the reaction vessel and oxidized using the Rancimat Metrohm model 679 (Metrohm AG, Switzerland). The air flow rate of 20 L/h and the temperature of the heating block were set to 110°C. The samples were heated for 1–14 h. The oxidized samples were stored at −20°C. All preparations were carried out under nitrogen environment and in the daylight. A control sample of TAGs without β-carotene was also oxidized under similar conditions.

2.3 Quantification of β-carotene

The oxidized TAGs sample (100 ± 0.5 mg) containing β-carotene was mixed with 4 mL of acetone, vortexed for 10 s and kept at −70°C for 3 h. TAGs were crystallized at −70°C and β-carotene was separated by rapid sampling. The extract was spotted on the high performance TLC (HPTLC) plate and measured using densitometric scanning performed on a Camag TLC Scanner III controlled by winCATS (v. 1.4.3) software [12].

2.4 Liquid chromatography-mass spectrometry

A sample of 40 ± 0.5 mg of the oxidized TAGs (with β-carotene and control) was mixed with 1 mL of acetone and 3 mL of HPLC solvent. LC was carried out using an Agilent HP 1100 system coupled to ESI-MS (Agilent, Waldbronn, Germany). The samples were separated using a Phenomenex C18 (150 × 3 mm^2) (Germany) column and eluted using an isocratic solvent system consists of 18% isopropanol in methanol (0.1% acetic acid). Other conditions of LC and MS were the same as reported in our previous work [11].

2.5 Peroxide value

The peroxide value of each sample was determined using AOCS official method (method Cd 8b-90) [13] and expressed as meq O_2/kg of fat.

2.6 Statistical analysis

All samples were measured in triplicate or otherwise mentioned. Data were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison method at $\alpha = 0.01$ or $\alpha = 0.05$ using SigmaPlot for windows version 11.0 (Systat Software, Inc, 2008).

3 Results and discussion

During heating of β-carotene rich edible oils such as red palm oil and sea buckthorn oil, that contain more than 500 μg/g of β-carotene [14–17], or carotenoids rich foods in edible oils the thermo-chemical effects of carotenoids on TAGs stability and the formation of oxidized compounds are an important issue during daily cooking. In order to mimic such a system we added 300 μg/g of all-*E*-β-carotene to the TAGs model system to study the effect of relatively high concentrations of β-carotene on the TAGs oxidation during thermal treatment. The temperature of 110°C for thermal oxidation in the Rancimat was selected as best temperature for studying both the oxidation of β-carotene as well as TAGs. Earlier studies

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also revealed that the temperature of 110°C in the Rancimat for the oxidation of oils or fats is optimal [18]. Similar studies were also carried out using lower temperatures like 90 and 100°C. A very slow degradation of β-carotene was observed at these temperatures; however, there was no significant degradation or change in the chemistry of TAGs.

3.1 β-Carotene degradation

All-*E*-β-carotene was quantified using a fast HPTLC method [12], using a standard calibration curve in the range of 100 to 600 ng (Fig. 1A). The regression equation was:

$$Y = 2806.52 + 13.067X$$

The concentration of total β-carotene was measured using the formula β-carotene (μg/g) = $(C \times V \times D)/(W \times A)$, where C is the concentration calculated from the calibration plot, V is the final volume from sample preparation, D is the dilution factor (if any), W is the weight of the sample, and A is the amount of sample applied to the plate. LOD for β-carotene was 0.21 ng, while LOQ was 0.65 ng. The concentration reported here is the sum of all stereoisomers of β-carotene, as this HPTLC method does not separate individual isomers. Figure 1B shows that the β-carotene is stable during the first hour of thermal treatment. A significant degradation was observed at the third hour of thermal treatment. About half of the β-carotene was degraded during this treatment. Further degradation was significant till 8 h, showing that concentration of β-carotene decreased as a function of heating time. There were still traces of β-carotene present after 10 h; however, they could not be quantified since the concentrations were below the LOQ. Achir *et al.* [19] reported a similar trend in the oxidation of all-*E*-β-carotene for palm olein and Vegetaline at four different temperatures (120–180°C). However, the studies did not show an effect of lower temperatures on β-carotene and its effects on TAGs stability as well as the formation of oxidized species. Previously we reported [12] that β-carotene was significantly degraded during the first hour of thermal treatment in the Rancimat. The presence of high levels of polyunsaturated TAGs and the presence of other pro-oxidants were the main reasons for a higher oxidation of carotene. Takahashi *et al.* [20], proposed a novel kinetic model for the co-oxidation of β-carotene in oleic acid. The reaction mechanism consisted of the oxidation of carotene, the oxidation of oleic acid, and the cross-reaction of β-carotene with oleic acid. However, our present results show that β-carotene starts degrading before the high oleic TAGs as shown by the peroxide values. Therefore it is suggested that β-carotene or its products act as pro-oxidant toward high oleic TAGs. It was assumed in the earlier studies [21] that during thermal treatment, part of all-*E*-β-carotene is converted to its isomers, while rest takes part in the oxidative degradation reactions, producing oxidized species and cleavage products. This is in accordance with our results showing a significant initial degradation and the oxidized or degraded or the remaining β-carotene may take part in pro-oxidation.

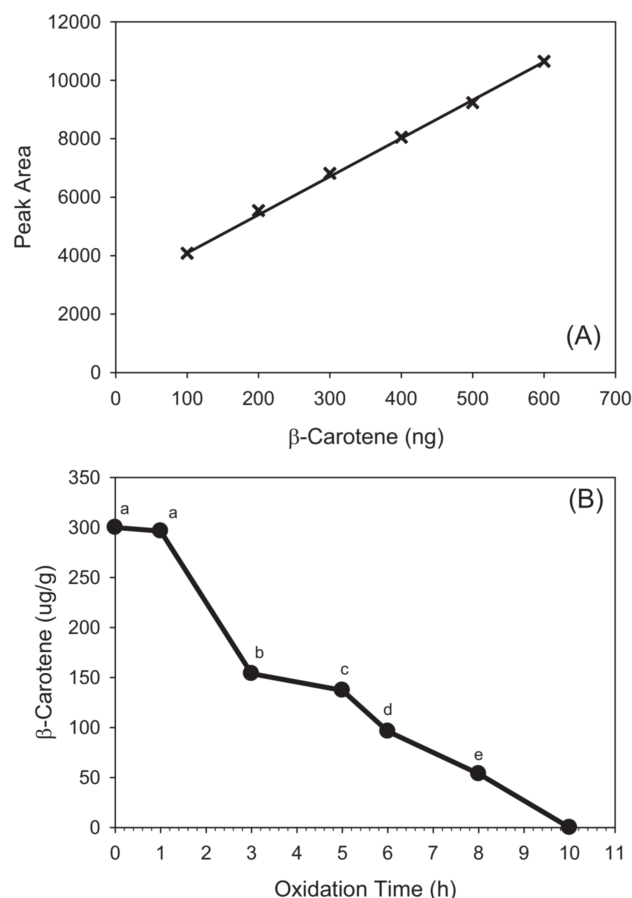

Figure 1. (A) Correlation function for peak area (six-point calibration, two-fold analysis) for **β**-carotene ($Y = 2806.529 + 13.067X$, $r = 0.9999$, RSD = ±0.84). (B) **β**-Carotene degradation in the Rancimat at 110°C. Different letters are significant difference ($p<0.05$).

3.2 Effect of β-carotene on the peroxide value

Hydroperoxides are formed during the initial stages of the thermal oxidation of the TAGs. Thus the peroxide value is used to measure the oxidation level. Figure 2 shows the effect of β-carotene on the peroxide value of model TAGs. It has been found that there was no significant effect of β-carotene till 3 h. There was a steady increase in the peroxide value till 8 h in the control sample. A significant ($p<0.05$) change in the POV was observed after 8 h of thermal treatment. Further treatment till 14 h shows significant production of hydroperoxides in both control and β-carotene treated sample. The results showed that β-carotene induced oxidation of TAGs resulting in the formation of more peroxides than in the control samples oxidized under the same conditions. The

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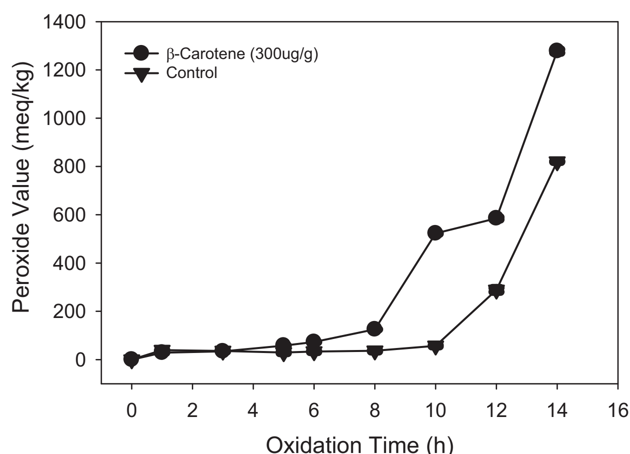

Figure 2. Effects of β-carotene on the peroxide value (meq/kg of O_2) of TAGs model system.

longer oxidation time and increased oxygen presence (20 L/h) also cause the high POV than normal cooking. The results are in agreement with Heinonen *et al.* [22], who showed that a low concentration (20 μg/g) of β-carotene acts as pro-oxidant in oil-in-water emulsions by increasing the peroxide values, hexanal and 2-pentanal formation.

3.3 Effect of β-carotene on the stability of triacylglycerols

The TAGs model system consists of LLL, OLL, OLO, POO, OOO, OSO, and AOO corresponding to the peaks 1–7 (Fig. 3A). A very small fraction of non-oxidized diacylglycerols was also present. The control as well as the β-carotene fortified samples was oxidized in the Rancimat for 1–14 h in order to get the initial as well as secondary oxidation products in a high amount. The small peaks eluted earlier in the control sample are diacylglycerols (not oxidized). These peaks disappear as the sample is oxidized in the Rancimat. All TAGs were stable till 8 h in the presence and absence of β-carotene (Fig. 3B). In the TAGs model oxidized for 10 h in the Rancimat, two new peaks (8 and 9) appeared (Fig. 3C). It has been found that these peaks are produced more in the presence of β-carotene showing the pro-oxidative effect. Similarly, at 12 h of thermal oxidation, we were able to identify a total of nine new peaks (peaks 8–16) (Fig. 3D). A significant protective effect of β-carotene degradation products was found in the stability of peak 3 (OLO) and the newly formed peak 16. This effect is reversed when looking at the formation of these new oxidation products (peaks 8–15). At 14 h of thermal treatment peaks 4–7 are stable in the presence of oxidized products of β-carotene (Fig. 3E). However, the amounts of other oxidized species increase in the presence of β-carotene. These results show a combined antioxidant and pro-oxidant effect of β-carotene. But the sum of both effects reflects that the pro-oxidant action of β-carotene is prominent during thermal oxidation at 110°C in the Rancimat. Different TAGs were found to have different activities toward heating in the presence of β-carotene. Similar results were obtained by Karabulut [23], who showed that at 60°C, β-carotene had a pro-oxidative effect on the butter oil TAGs at the concentration of 50 μg/g. The addition of tocopherols was found to reverse the effect of β-carotene.

Figure 4A shows the oxidation and degradation of peak 1 (LLL) at 110°C in the Rancimat. The degradation is very slow till 8 h of thermal treatment. However, a complete loss of LLL has been observed at 10 h. There was no difference in the reaction in presence or absence of β-carotene. Similarly, the peak 2 (OLL) was stable till 10 h of thermal treatment (Fig. 4B). Thus replacing a single linoleic acid moiety by oleic acid increased the stability for two more hours. However a small pro-oxidant effect of β-carotene was observed at 10 h of thermal treatment in OLL. This pro-oxidant effect may result into the complete loss of OLL at 12 h. Figure 4C shows the effects of β-carotene during thermal oxidation of OLO. The thermal oxidation of peak 3 (OLO) was not significant till 10 h. However, there was a significant change at 12 h. The presence of second oleic acid group increases the stability of OLO by 2 h as compared to OLL. Complete loss has been observed in OLO at 14 h of thermal oxidation. Peak 4 and 5 representing POO and OOO (co-eluting peaks) were the major stable TAGs (Fig. 4D). There was no significant effect of β-carotene till 10 h. A significant loss (about 50%) of these TAGs has been observed at 14 h of thermal treatment. At 14 h, these TAGs showed more stability in the presence of degraded/oxidized products of β-carotene. A similar trend was also observed in peak 6 (OSO), which shows a good stability in the degraded or oxidized environment (Fig. 4E).

3.4 Formation of oxidized triacylglycerols

The major pathways regarding the TAGs oxidation includes initiation, propagation, and termination. For this purpose standard TAGs or their mixtures were oxidized and the mechanisms of products formation have been reported [1–3, 10]. For example, model trilinolein and trilinolenin were used to study the oxidation of oil TAGs [24]. The initial oxidation products were found to be hydroperoxides. In our work, we have identified some mono- and bis-hydroperoxides and epoxides. The formation of TAGs hydroperoxides by free radical reaction has been reported by several authors. In addition to this we also identified epoxides and epoxy-epidioxides. As an example we present here a simplified mechanism of the formation of epoxy-epidioxide of triolein (Fig. 5). Triolein bis-hydroperoxide is formed from the free radical reactions of oxygen. The sn-1 and sn-3 position is believed to be more prone to hydroperoxidation. The attack of free radicals on triolein bis-hydroperoxides may result in the formation of epoxy hydroperoxy radicals, hydroxides and a non-radical product. Triolein epoxy-epidioxide (peak 12) is

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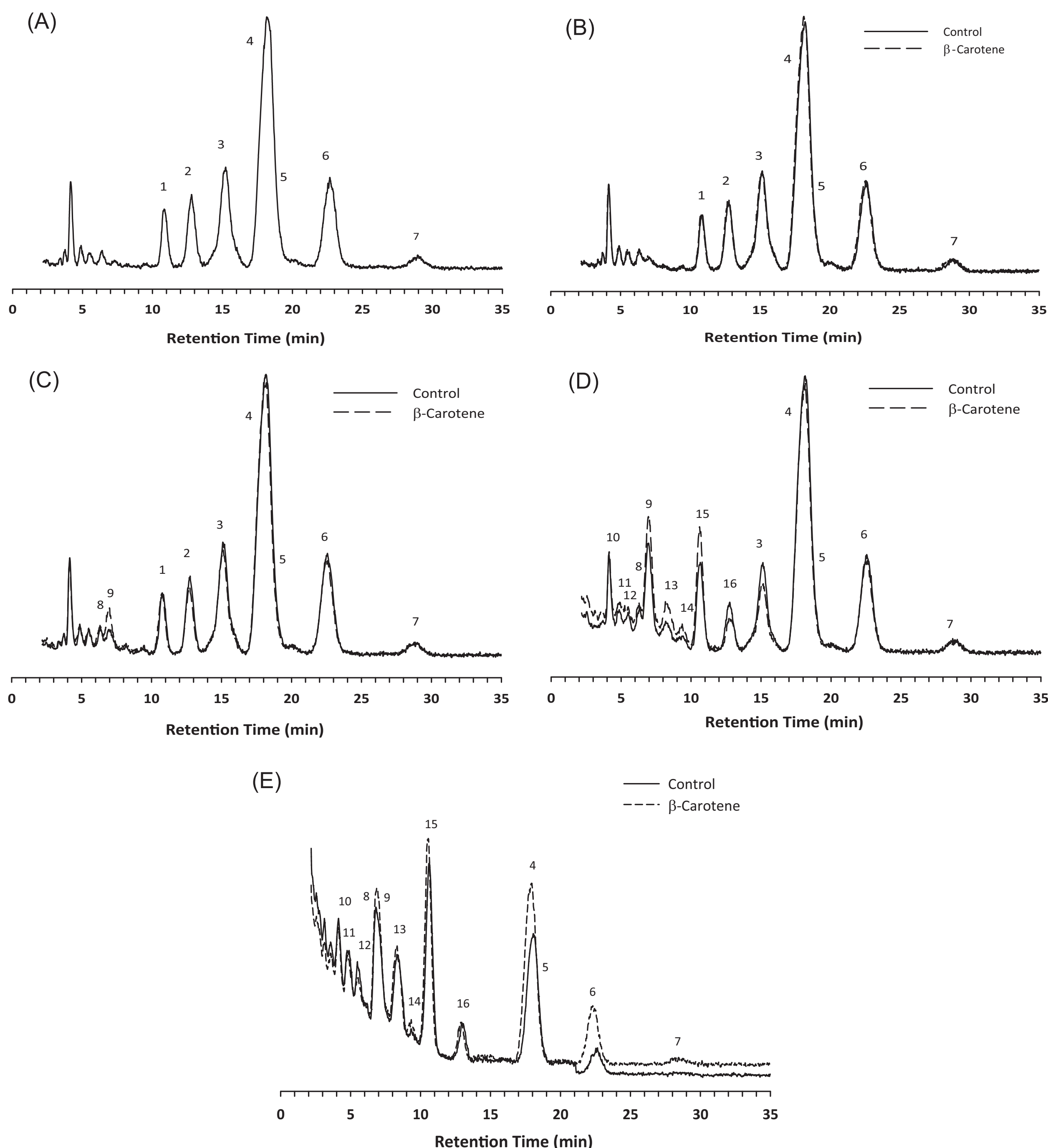

Figure 3. Comparative stability of TAGs in the presence and absence of **β**-carotene. (A) ESI-MS chromatograms of the model TAGs at 8 h of oxidation. (B) ESI-MS chromatograms of the model TAGs at 10 h of oxidation. (C) ESI-MS chromatograms of the model TAGs at 12 h of oxidation. (D) ESI-MS chromatograms of the model TAGs at 14 h of oxidation. The identity of each peak number is discussed in the text.

formed from the rearrangement of a free radical electron. The mechanism of formation of hydroperoxy-epidioxide has been reported by several authors [25–27], however, we reported here for the first time the formation and characterization of epoxy-epidioxide species.

3.5 Identification of oxidized triacylglycerols

The isocratic method developed was very useful for the characterization and identification of oxidized TAGs in edible oils or fats. Previous work on the characterization

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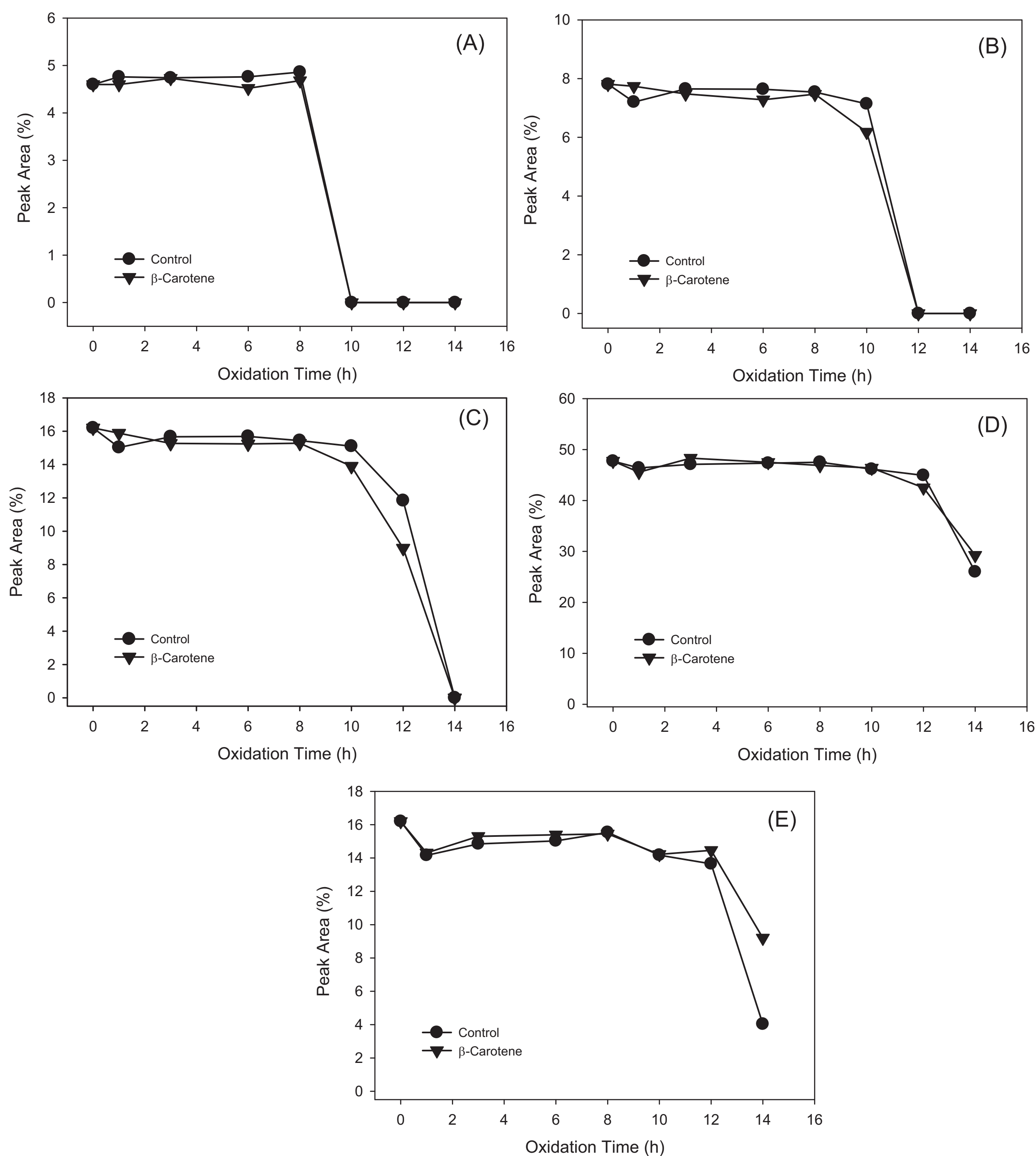

Figure 4. Effects of β-carotene on the stability of individual TAG. (A) LLL, (B) OLL, (C) OLO, (D) POO/OOO, and (E) OSO.

and identification was based on oxidation of standard fatty acids [1–2, 25] using GC-MS [27] or other simple analytical techniques. These techniques were laborious, time consuming and the exact structure elucidation was not possible. Modern LC coupled to MS made the identification of oxidized TAG much easier [6, 7, 28]. However, none of them showed the position of oxygen functional groups on TAGs, Very few reports [8, 9, 29] are available on the correct identification of individual standard TAGs oxidized species with APCI-MS. Here we report the oxidation of model TAGs mixtures using ESI-MS. We found that each class of oxidation products yielded characteristic mass spectra, which

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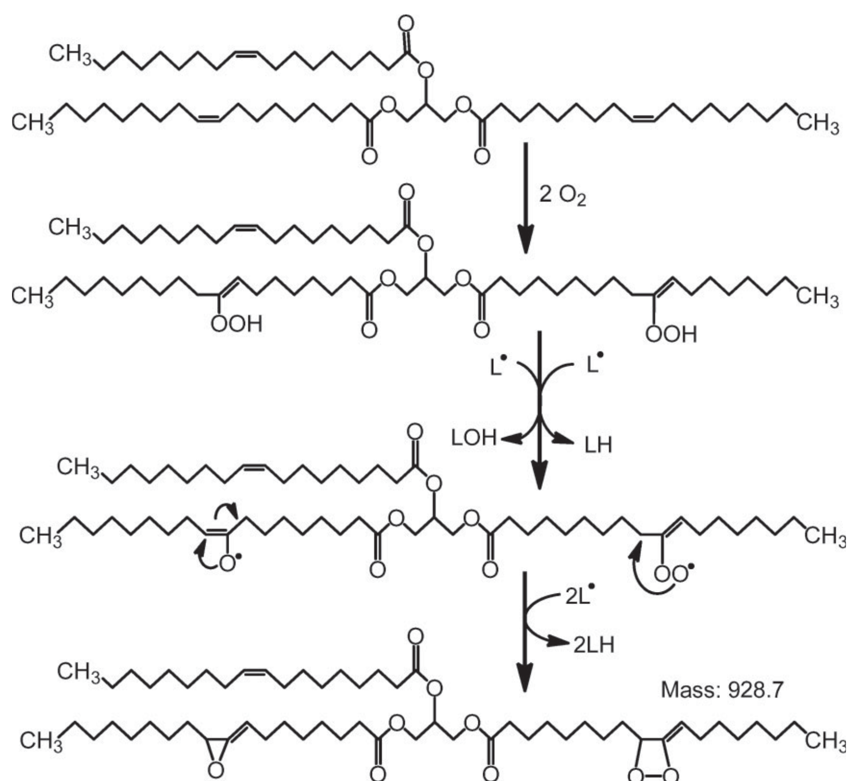

Figure 5. Formation of triolein epoxy-epidioxide. The pathway follows the free radical reaction mechanism.

were differentiable on basis of their relative proportions of different fragments produced from relatively similar fragmentation pathways.

Figure 6A shows the extracted ion chromatogram (EIC) and the respective mass spectra of triolein mono-hydroperoxide (peak 13). We observed that a primary fragment was formed due to the loss of an outer –OH group, resulting in the formation of a stable epoxide. The *m*/*z* 899.8 represents $[M\text{-}H_2O]^+$. Such fragments were the prominent one. The fragments at *m*/*z* 921.8 and 936.8 represent the sodium adducts $[M\text{-}H_2O + 23]^+$ and potassium adducts $[M\text{-}H_2O + 23]^+$ of the corresponding epoxide. We were also able to get a relatively small amount of the protonated molecular ion of mono-hydroperoxide. The *m*/*z* 917.8 corresponds to $[M + H]^+$. The fragment ions of *m*/*z* 603.5 and 617.5 are dioleoyl glycerol and mono-epoxy dioleoyl glycerol ions, respectively. The presence of molecular and its adducts ions and the intense epoxy dioleoyl glycerol ions indicate the stability of these ions for further fragmentation. Therefore we were not able to get any hydrocarbon fragment (or a fragment with a neutral loss equivalent to hydrocarbons) which could explain the position of epoxide or the double bond. So we assumed the possibility of C-9 and C-10, which is already reported in literature [1–3]. Figure 6B shows the EIC and the respective mass spectra of OLL bis-hydroperoxide (peak 10). Two pathways are possible: the loss of an outer –OH to form a stable epoxide or a complete loss of –OOH group forming an additional double bond as proposed previously by Neff and Byrdwell [8]. The *m*/*z* of 913.7 corresponds to the loss of two –OH groups from the parent molecule forming a stable bis-epoxide. We found a loss of unsaturation near the epoxy group during the formation of the bis-epoxide ion. The *m*/*z* of 897.7 represents the loss of an epoxide oxygen resulting in a double bond formation at the site of epoxide. The *m*/*z* of 937.8 was found to be the sodium adduct of the diepoxide fragment ion. The site of unsaturation can be observed by the fragments at *m*/*z* 601.5 and 615.5 which correspond to the isobaric linoleoyl oleoyl glycerol $[LO]^+$ ion and an epoxy dioleoyl glycerol $[LO_{ep}]^+$ respectively. The diepoxy DAG fragment was also observed at *m*/*z* 631.5. The fragment ions at *m*/*z* 467.2, 479.3, and 497.4 are formed from the epoxy-diacylglycerol designating Δ7, Δ8, and Δ9 respectively. These fragments ions may easily explain the position of an epoxide ring on the fatty acid backbone. The details will be presented later. A similar spectrum (Fig. 6C) is obtained from triolein bis-hydroperoxide (peak 11). The EIC shows an intense sharp peak of *m*/*z* 881.7 formed from the loss of two –OOH groups. The intense diacylglycerols, epoxy-

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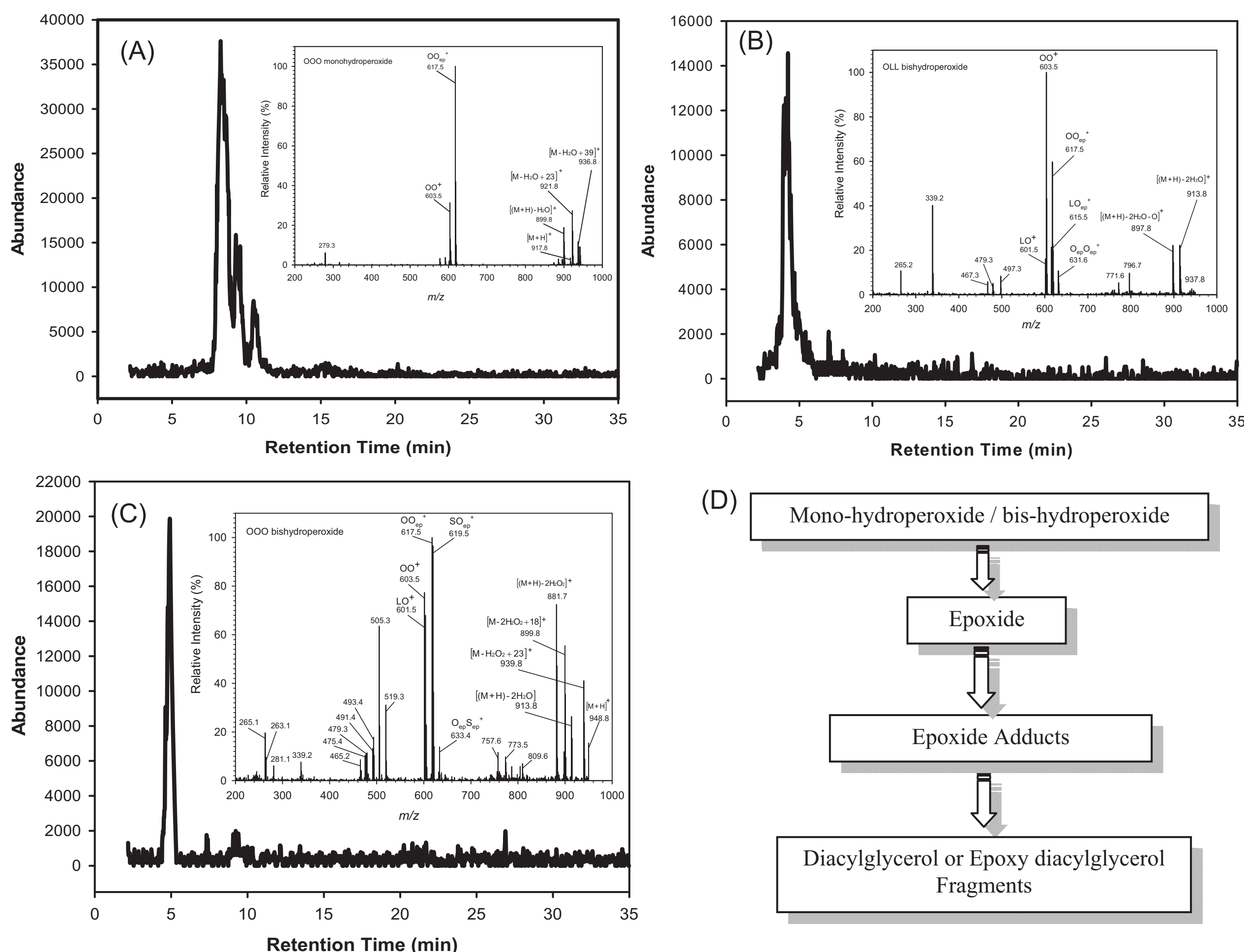

Figure 6. EIC and ESI-MS spectra of hydroperoxides. (A) OOO-mono-hydroperoxide (*m*/*z* 921.8), (B) OLL-bis-hydroperoxide (*m*/*z* 913.8), (C) OOO-bis-hydroperoxide (*m*/*z* 881.8), (D) A typical fragmentation mechanism of hydroperoxides.

diacylglycerols ions and the fragments formed from the loss of hydrocarbons are more prominent explain the correct structure of the compounds and the possible isomers. Figure 6D shows the fragmentation pathway of mono- and bis-hydroperoxide. Briefly, hydroperoxides forms a stable epoxide. Epoxides than forms adducts and also further fragmented to diacylglycerol or epoxy-diacylglycerol fragment. Thus the mono- and bis-hydroperoxides follow the same fragmentation mechanism as explained later in this manuscript for epoxy-epidioxides. From the sizes of the bis-hydroperoxides peaks (peak 10 and 11), it clear that these were produced in lower amounts than mono-hydroperoxide (peak 13).

Figure 7A shows the EIC and corresponding mass spectra of triolein epoxy-epidioxide (peak 12). The ESI-MS spectra shows a *m*/*z* of 913.8 corresponding to the loss of one oxygen from the epidioxide group forming an another epoxide between carbon 9 and 10. The fragment ions at *m*/*z* 883.7 and 897.8 correspond to $[(M + H)\text{-}3H_2O]^+$ and $[(M + H)\text{-}H_2O_2]^+$, respectively. We also found the molecular adducts of triolein diepoxides ion, they are *m*/*z* 932.8 and *m*/*z* 937.8, which are the ammonium and sodium adducts, respectively. It has been previously reported that traces of sodium in the solvent system may result in sodium adducts [18]. The diacylglycerols fragments, which play an important role in the elucidation of the chemical structures of TAGs were also formed. The *m*/*z* 617.4 and *m*/*z* 631.5 are the epoxy diacylglycerols fragments formed from the triolein diepoxide (*m*/*z* 913.7) molecular ion. The fragments *m*/*z* 633.5 and 647.5 were believed to form from the parent molecule, showing the presence of an epidioxide functional moiety. The proposed mechanism is shown in Fig. 7B. The fragment ions at *m*/*z* 493.4 and 519.4 are formed by the loss of $[C_9H_{16}O]^+$ and $[C_8H_{16}]^+$ from the diepoxide ion (*m*/*z* 631.5). This explains that the epoxide was between C9 and C10. If this epoxide was at C8 and C9, a fragment of *m*/*z* 477.4 and 507.4 would have been formed, which was not the case. Figure 7C shows a similar ESI-MS spectrum of an OOS epoxy-epidioxide (peak 8). The mechanism of formation and fragmentation

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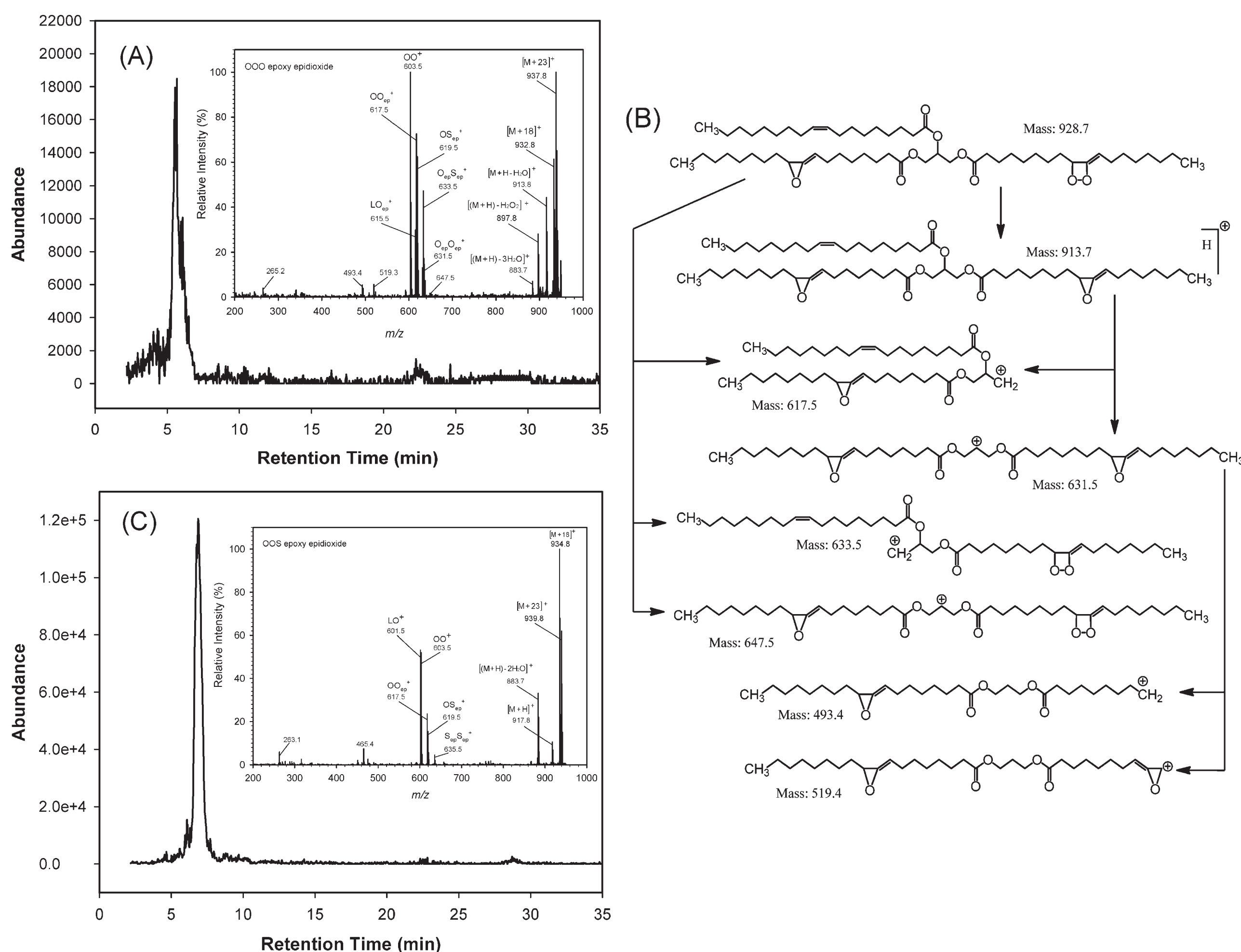

Figure 7. EIC and ESI-MS spectra of epoxy-epidioxide, (A) OOO-epoxy-epidioxide (*m/z* 932.8), (B) Proposed fragmentation mechanism for the identification of OOO-epoxy-epidioxide, (C) OOS-epoxy-epidioxide.

of this compound was the same as explained above. These epoxy-epidioxides were formed more than any of the oxidized TAGs species as can been seen from Fig. 3.

Mono-epoxides were also among the oxidized TAGs species at 12 and 14 h of thermal oxidations in the Rancimat at 110°C. Figure 8A shows the EIC and ESI-MS spectra of triolein mono-epoxide (peak 14). We observed that epoxides were the more stable in ESI mass spectrometer. In the case of triolein mono-epoxide, the *m/z* 899.8 was identified as protonated molecular ion, while *m/z* 916.8, 921.8, and 939.8 were the ammonium, sodium, and potassium molecular adducts. A loss of the epoxide may result in the formation of an additional double bond. This unsaturation was confirmed by the fragment of *m/z* 601.5, which is isobaric to LO. Similar spectra (Fig. 8B) were also observed for the OOS mono-epoxide (peak 15). The loss of epoxide oxygen, proton, sodium, and ammonium adducts were obtained, which are important in elucidation of chemical structure. The position of epoxide was between C8 and C9, as shown by the *m/z* 477.4 and 493.4, respectively. We also observed the SOS mono-epoxide (peak 16) and OLO mono-epoxide (peak 9) in our previous [30] and present work. Epoxides of all types were found to produce high abundances of the adduct ions like sodium, potassium and ammonium than non-cyclic oxidized products of TAGs. That means that TAGs containing more oxygen atoms form more stable adducts with positive cations such as NH_4^+, Na^+, and K^+. Previously it was observed that traces of sodium and potassium present in the eluents could possibly form the respective adducts [31].

The elution order was (i) bis-hydroperoxides (ii) epoxy-epidioxides (iii) mono-hydroperoxides, and (iv) mono-epoxides. This reflects that oxidized TAGs were separated on the basis of polarity as well as equivalent carbon number (ECN). The presence of more than two fragment ions (formed from the epoxy diacylglycerols) can be attributed to the different isomers of the same compounds with different positions of the oxidized functional group. We were unable to interpret

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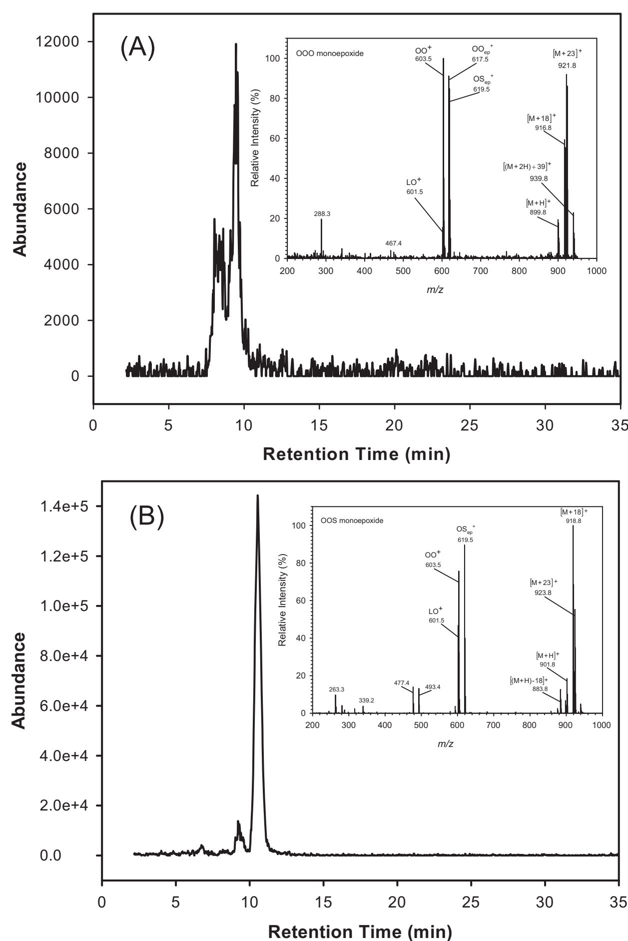

Figure 8. EIC and ESI-MS spectra of epoxides, (A) OOO-mono-epoxide (*m/z* 916.8), (B) OOS-mono-epoxide (*m/z* 923.8).

the complex mass spectra from the substances eluting before 4 min. They may be a result from chain shortened species as reported earlier.

4 Conclusion

During the thermal degradation of high oleic acid model TAGs, β-carotene was found to play an important role. Half of the β-carotene was degraded within 3 h of thermal treatment. β-Carotene increases significantly the peroxide value of TAGs after 3 h, which suggests that β-carotene or its degradation products act as a pro-oxidant. However, different TAGs show different activities during thermal treatment and β-carotene. The trilinolein (LLL) was found unstable, while POO, OOO, and OSO were the stable TAGs. The OLL is stable till 10 h, while the stability of OLO reached 12 h. We noted that by replacing a linoleic acid residue by oleic acid in the TAG, the stability of the corresponding TAG would increase by 2 h. We also reported new oxidized species together with already reported ones. The mechanism of formation and the identification of the newly identified species have been explained. Among the oxidized species of TAGs, mono-hydroperoxides, bis-hydroperoxides, epoxy-epidioxide, and epoxides were the major compounds identified. We found the isocratic HPLC-ESI-MS a useful method for the identification and characterization of TAGs and its oxidized species.

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The authors have declared no conflict of interest.

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Title

Determination of Thermal Oxidation and Oxidation Products of β-Carotene and Triacylglycerols in Corn Oil using LC-MS

Running title

Thermal Oxidation of β-Carotene and Triacylglycerols

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Abbreviations

TAGs, triacylglycerols; ESI-MS, electrospray ionization mass spectrometry; POV, peroxide value; EIC, extracted ion chromatogram. P, palmitic; S, stearic; A, arachidic; G, gadoleic; Ln, linolenic; L, linoleic; O, oleic; ep, epoxide.

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Abstract

A reversed phase HPLC-DAD coupled to APCI-MS method is reported for the determination of the degradation of β-carotene and characterization of the oxidation products in corn oil. Corn oil containing β-carotene was oxidized in the Rancimat at 110 °C from 1 to 14 h. A significant degradation of β-carotene was observed in corn oil during accelerated thermal oxidation. We have identified a total of eight different oxidized products of β-carotene, which includes 8′-apo-β-carotenal, 6′-apo-β-carotenal, 5,6-epoxy-8′-apo-β-carotenal, β-carotene-2,2′-dione, 13-*Z*-5,6,5′,6′-diepoxy-β-carotene, all-*E*-5,8-epoxy-β-carotene, all-*E*-5,6-epoxy-β-carotene, and 15-*Z*-5,6-epoxy-β-carotene. Corn oil triacylglycerols (TAGs) oxidation products were also identified using isocratic HPLC-ESI-MS. We found that β-carotene promoted oxidation of TAGs especially at longer exposure times. For the first time we have identified two new classes of oxidized TAGs in corn oil, which were epidioxy bis-hydroperoxides and hydroxy bis-hydroperoxides. Other oxidation products of TAGs were mono-epoxides, mono-hydroperoxides, and epoxy hydroperoxides. It has been found that complementing the HPLC-DAD method for β-carotene with ESI-MS method for TAGs oxidation provides a comprehensive set of analytical tools to characterize carotenoids and triacylglycerols oxidation and degradation in edible oils.

Key words

β-Carotene, HPLC-DAD, oxidation products, corn oil triacylglycerols , oxidized triacylglycerols, HPLC-ESI-MS, oxidation, free radical reaction.

Introduction

β-Carotene (β,β-carotene) is one of the most important and widely studied carotenoid. It is a strongly red-orange colored organic compound and a good source of vitamin A. It serves as biological antioxidant and is helpful in maintaining human health. In food industries it is used as a colorant and also as a source of pro-vitamin A [1]. Generally during food processing and thermal treatment, β-carotene is oxidized at different temperatures. Carotenoids degradation in food or similar model systems is highly complex phenomena, however, some authors proposed a first order kinetic model for the degradation [2-4]. Borsarelli and Mercadante [5] showed a simplified mechanism of overall changes occurring in carotenoids during heating. Most of the β-carotene oxidation was studied in organic solutions [6-9]. The complete range of oxidized products formed was identified using HPLC-DAD and HPLC-MS. Moreover, most of these systems rarely represent the real food matrix, especially the oxidation of carotenoids in oils or triacylglycerols.

Studies of Hunter & Krackenberger [10] showed the oxidization of β-carotene at 50 °C in benzene and in peanut oil. They found that the rate of the oxidation process was much higher in benzene than in the oil. They attributed this fact to the presence of a natural antioxidant in the peanut oil. Yamauchi et al. [11] studied the oxidative cleavage compounds of β-carotene, which were found to be formed after reaction with alkyl peroxides generated by 2,2′-azobis (2,4-dimethylvaleronitrile, AMVN). They also showed that the oxidized β-carotene compounds were also found during the peroxyl radical-initiated peroxidation of methyl linoleate and its auto-oxidation in the bulk phase [12]. The products formed were formyl or cyclic ether containing groups in the chain of carbon–carbon double bonds. The thermal degradation of all-*E*-β-carotene, 9-*Z*-β-carotene, lycopene, and lutein was studied in an oil model system, safflower seed oil, at 75 °C, 85 °C, and 95 °C. Heating β-carotene at several temperatures was found to form 13-*Z*-carotene in higher amounts, followed by 9-*Z*-β-carotene. Several degradation products were formed during lycopene heating and lutein heating; however, they were not identified [13]. Recently Achir et al. [14] studied the kinetics and degradation of all-*E*-β-carotene and all-*E*-lutein in palm olein and Vegetaline (at 120 to 180 °C) using HPLC-DAD. They found that initial all-*E*-β-carotene and all-*E*-lutein degradation rates increased with temperature in both oils. Karabulut [15] studied the oxidation of fortified

triacylglycerols (α-tocopherol, β-carotene and ascorbyl palmitate) in an oven at 60 °C. α-Tocopherol was found to be the most effective antioxidant, while a pro-oxidant effect of β-carotene and ascorbyl palmitate was observed. However, in none of these studies the oxidized products of carotenoids and lipids have been available. Recently we reported the pro-oxidant action of β-carotene in model TAGs [16]. The β-carotene was found to degrade much earlier than TAGs and the correlation of β-carotene and different TAGs stability was presented. Similar to the previous studies we were not able to show what oxidation products of β-carotene were formed during such oil or TAGs thermal oxidation. A one window method is therefore required to both quantify the amount of β-carotene and identify its degradation products. The formation of oxidation products of β-carotene in lipids or oils under relatively similar conditions has not been reported so far and thus is the subject of great interest, and the main purpose of this paper.

Experimental

Reagents and Materials

Acetone and methanol were purchased from Mallinckrodt Baker (The Netherlands), *tert*-butyl methyl ether (MTBE) was from Merck (Darmstadt, Germany). The deionized water was obtained from a Milli-Q water purification system (Millipore AS, Bedford, MA, USA), while all-*E*-β-carotene and triacylglycerols (more than 60 % triolein) were purchased from Fluka (Sigma-Aldrich, Germany). Corn oil was purchased from the local market in Graz (Austria).

Rancimat Oxidation

All-*E*-β-carotene was dissolved in acetone and added to corn oil in order to flourish a concentration of 300 µg/g ± 0.5 µg. The fortified sample was sonicated for 1 min and then kept for one hour under nitrogen in a glass bottle. A sample of 4 g of corn oil was added to the reaction vessel and oxidized using the Rancimat Metrohm model 679 (Metrohm AG, Switzerland). Other conditions of Rancimat were air flow of 20 L/h, and temperature of 110 °C. The samples were oxidized for 1 to 14 hours. After the experiment 400 µL of BHT (0.5% in methanol or acetone) was added to the oxidized samples in order to stop further possible reactions and to avoid formation of artifacts. A control sample was also oxidized under similar conditions without β-carotene. The oxidized samples

were stored at -20 °C till analysis. All preparations were carried out under nitrogen ambient environment and in the day light.

Sample Preparation

For the extraction of β-carotene, the oxidized sample of 200 mg was dissolved in 2 mL of acetone and sonicated for 20 sec. The samples were than kept at -20 °C for 24 h. The TAGs crystallizes and β-carotene moves to the acetonic fraction. The samples were then quickly filtered. This method removes about 90 % of the lipids. Saponification was avoided in order to reduce the formation of artifacts (e.g. hydrolysis of epoxides). For the analysis of TAGs and its oxidized species, a sample of 100 mg of corn oil was dissolved in 2 mL of acetone and 2 mL of HPLC solvent. The sample was then transferred to a 2 mL vial for the HPLC analysis.

HPLC-DAD-MS Analysis of β-Carotene

The HPLC system consists of an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) with vacuum degasser, quaternary pump, auto sampler, temperature-controlled column oven, and diode array detector (at 450 nm). The system was coupled to APCI-MS (Agilent Technologies, Waldbronn, Germany). The extracted β-carotene was separated using Lichrospher® 100, RP-18e (125 × 4 mm, 5 μm) column (Agilent Technologies, Germany) with a flow rate of 1 mL/min with prior equilibration over night with 0.1 mL/min. The separation of β-carotene and its oxidation products were achieved using a mobile phase consisting of methanol (solvent A), methanol/MTBE/water, 80:15:5, v/v/v (solvent B) and acetone (solvent C). The elution was started with 90 % A, 10 % B and reached 10 % A and 90 % B at 7 min and remained isocratic till 12 min. At 20 min eluent composition was 75 % B and 25 % till 30 min. The post run time was maintained for 10 min. The mass spectrometer was optimized in positive mode for the determination of β-carotene using flow injection analysis of the standard β-carotene (1 μL injection of 10 ng/μL). The optimized conditions were drying gas flow of 6 L/min, capillary voltage 2500 V, gain 8, corona current 5 μA, vaporizer temperature 330 °C, and drying gas temperature of 250 °C.

HPLC-ESI-MS Analysis of Oxidized TAGs

Liquid chromatography was carried out using an Agilent HP 1100 system coupled to ESI-MS (Agilent, Waldbronn, Germany). For the separation a Phenomenex C18 (150 × 3 mm, 5 µm) (Germany) column was used. The separation was carried out using an isocratic solvent system consists of 18 % isopropanol in methanol with 0.1 % acetic acid with a flow rate of 0.6 mL/min. Mass spectrometry was carried out using an Agilent HP 1100 system ESI-MS (Agilent, Waldbronn, Germany). Other conditions of LC and MS were the same as reported in our previous reports [16, 17].

Fatty Acid Analysis

Fatty acid methyl esters (FAME) were prepared by standard method as described by AOCS [18] using an internal standard (C11:0, 5.65 g/100 g). FAMEs were separated on a GC (HP 5890) system using Agilent-Technologies DB-Wax capillary column (30 m long, with internal diameter 0.25 mm, film thickness 0.25 µm).

Peroxide Value & Data Analysis

The peroxide value of each sample was determined using the AOCS official method (method Cd 8b-90) [18] and expressed as meq O_2/kg of fat. All samples were measured in triplicate or otherwise mentioned. Calibration data were validated using VALIDATA version 3.02 (Microsoft Excel macro). Data were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison method at $\alpha = 0.01$ or $\alpha = 0.05$ using Sigma Plot for windows version 11.0 (Systat Software, Inc, 2008).

Results and Discussion

In order to mimic the effects of thermal treatment on the carotenoids rich foods or the β-carotene rich edible oils such as red palm oil and sea buckthorn oils [19-21], we have added 300 µg/g of all-*E*-β-carotene to the corn oil. Thermal oxidation was carried out in the Rancimat at a temperature of 110 °C, which was selected as optimal temperature for studying both the oxidation of β-carotene as well as TAGs. Our previous work [17] and earlier studies [22] also showed the use of 110 °C in the Rancimat for the oxidation of oils or fats.

Validation of β-Carotene Analysis

The β-carotene degradation and its oxidation products were analyzed by HPLC-DAD and the analytical method was validated for precision, and reproducibility. The validation parameters are presented in Table 1. The coefficient of correlation of 0.9998 indicates a linear correlation of the peak area and the amount of β-carotene injected, which was confirmed by the Validata software. The limit of detection (LOD) was 4.42 ng/μL and limit of quantification (LOQ) was 16.08 ng/μL of β-carotene. Reproducibility, as intra-day and inter-day precision, was assessed in replicate (n = 5) analyses of three standard concentrations of β-carotene (100, 300, 600 ng/μL). We found no significant variation during intra and inter-day analysis with an average values of CV = 1.54 % and CV = 1.32 %, respectively.

β-Carotene degradation

All-*E*-β-carotene eluted at 15.3 min, while the retention time of 13-*Z*-β-carotene was found 16.3 min. Here we will discuss the degradation of all-*E*-β-carotene only. It was found that half of the β-carotene was degraded during the first 3 hours of thermal oxidation at 110 °C in the Rancimat. Further degradation was comparatively slow but statistically significant ($P < 0.01$) as shown in Figure 1. All-*E*-β-carotene was completely degraded or disappeared within 12 h of the thermal treatment. Previously we showed [16] that β-carotene completely degraded in high oleic model TAGs before 10 h of thermal treatment at 110 °C. That means that the stability of β-carotene in corn oil is higher than in the high oleic model TAGs, probably due to the presence of other antioxidants like tocopherols. A similar trend in the degradation of β-carotene was also found by Achir et al. [14] for palm olein and Vegetaline at four different temperatures (120-180 °C). The presence of high amounts of oleic acid was one of the factors contributing the β-carotene stability. The results of Takahashi et al. [24] show that when pure oleic acid is treated with β-carotene during oxidation, co-oxidation is the main way of degradation. However, we have shown here that in corn oil β-carotene starts degrading before the oxidation TAGs become apparent.

Effect of β-Carotene on Peroxide Index

Figure 2 shows the effects of β-carotene on the peroxide value (POV) of oxidized corn oil in presence and absence of β-carotene. It has been found that there was no significant effect of β-carotene on peroxide value till 4 h. A significant ($P < 0.05$) change in the POV was observed after 6 h. At 10th h the formation of hydroperoxides was more in control sample than β-carotene containing samples. We observed that further treatment till 14 h shows significant production of hydroperoxides in both control and β-carotene treated sample, while the β-carotene containing samples produced more peroxide than the control sample. These results showed that β-carotene induced oxidation of corn oil, which lets to the formation of more peroxides compared to the control samples oxidized under the same conditions. The longer oxidation time and increased availability of oxygen (air flow 20 L/h) also cause a higher POV than normal cooking. The results are in agreement with our previous finding of the β-carotene effects on model triacylglycerols [16]. Similarly, Heinonen et al. [24] showed that a low concentration (20 μg/g) of β-carotene acts as pro-oxidant in oil-in-water emulsions by increasing the peroxide values, hexanal and 2-pentanal formation. We observed that that the free fatty acids and mono and diacylglycerols were oxidized prior to the triacylglycerols, which was responsible for the increase in the peroxide value in the initial few hours of oxidation. Since all-*E*-β-carotene starts degrading before the corn oil TAGs, it is suggested that β-carotene oxidation products induce the oxidation of TAGs. The tremendous increase in POV at the longer times can thus be attributed to the oxidation of triacylglycerols.

β-Carotene Oxidation Products

Mass spectrometry was carried out on positive scanning mode and single ion monitoring (SIM) mode for the determination of β-carotene oxidized products. In addition, UV-vis absorption spectra and some confirmatory tests were used to correctly identify each product. Figure 3 shows typical chromatograms of β-carotene and its oxidation products in corn oil. Since the oxidation experiment was carried out at higher temperature (110 °C), only four classes of compounds were identified. The total of eight oxidation products (peak 1 to 8) was identified together with *E*- & *Z*-β-carotene. The structures of these oxidized species are shown in Figure 4. The first class of oxidized compounds was epoxides. We have identified two different mono-epoxides, which are all-

E-5,8-epoxy-5,8-dihydo-β,β-carotene (peak 6), all-*E*-5,6-epoxy-5,6-dihydo-β,β-carotene (peak 3) and its isomer 15-*Z*-5,6-epoxy-5,6-dihydo-β,β-carotene. The 5,6-epoxides were identified by the reaction with a few drops of ethanolic 0.1N HCl to get a furanoid derivatives. The formation of furanoid peak and disappearance of 5,6-epoxide peak confirm this oxidation product. Furthermore silica TLC chromatograms of the oxidized sample were developed with 5% methanol in toluene and were exposed to HCl fumes [25], the mono-epoxides were confirmed by the conversion of yellow to blue-green spots. Only one diepoxide, which was 13-*Z*-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene was identified. A hypsochromic shift of about 10 nm in the adsorption spectra and formation of blue spots under HCl fumes on TLC plate confirmed this epoxide (Table 2). The mass spectra of this compound showed a protonated molecular ion $[M+H]^+$ of m/z 569, and a fragment with the loss of one water molecule $[M+H-H_2O]^+$ of m/z 553. The elution order of these epoxide were (5) β-carotene-5,6,5′,6′-diepoxide, (6) 5,8-epoxide, (7) 5,6-epoxide and (8) 15-*Z*-5,6-epoxide. Previously Rodriguez & Rodriguez [25] showed a similar high amount of β-carotene-5,6-epoxide formed by the reaction of m-chloroperbenzoic acid in potato starch model system. Similarly 5,6- and 5,8-epoxides were also identified in the reaction of pea lipids with β-carotene in the presence of lipoxygenase enzyme. This means that during biological lipid oxidation carried out by enzymes in the presence of β-carotene produces those epoxides, which were also produced during corn oil oxidation. These are highly reactive species and potentially toxic substances.

The second class of oxidized compounds was apo-carotenals. Two types of apo-carotenals were identified, the one formed from the breakdown of *Z*-β-carotene, and the one formed from epoxides. The first type is called normal apo-carotenals. These were all-*E*-8′-apo-β-caroten-8′-al, and all-*E*-6′-apo-β-caroten-6′-al, while all-*E*-5,6-epoxy-8′-apo-β-caroten-8′-al belongs to the later class. These apo-carotenals are highly polar compounds and thus elute at the beginning of chromatographic run. All-*E*-8′-apo-β-caroten-8′-al was identified by comparison of its absorption spectrum, retention time and mass spectrum of the standard compound, while others were identified only by comparison of their absorption and mass spectra with literature data. These apo-carotenals are also formed during a widely used processing technique in food industry, called extrusion cooking. The longest chain apo-carotenals like apo-8′-, apo-10′-β-carotenals have been observed in lower to medium temperatures during extrusion cooking, while the short chain apo-

carotenals were detected at higher treatment temperatures [26-27]. Our results are also correlated with the studies of Ouyang et al. [28] on the effects of deodorization of palm oil at high temperatures (170 to 250 °C) with respect to the formation of short chain apo-carotenals. Later on Onyewu et al. [29] identified apo-carotenone and apo-carotenals formed by heating of palm oil at 210 °C. However, the effect of these oxygenated species on the oil is not presented in this reference. The third class of oxidized β-carotene is β,β-caroten-2,2′-dione (peak 4). The elution behavior of this compound showed that it is more polar than all epoxides. It could be formed directly from all-*E*-β-carotene by molecular oxygen. The fourth oxidation product was 13-*Z*-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene, which eluted just after the dione and before the mono-epoxides. We have observed only one isomer which is 13-*Z*-β,β-carotene, which eluted after all-*E*-β-carotene. Upon increasing heating time the formation of this isomer was found to increase as well.

Thus the major oxidation products were β,β-caroten-2,2′-dione (peak 4), all-*E*-5,8-epoxy-5,8-dihydo-β,β-carotene (peak 6), all-*E*-5,6-epoxy-5,6-dihydo-β,β-carotene (peak 7) and 15-*Z*-5,6-epoxy-5,6-dihydo-β,β-carotene (peak 8) were found to be formed in relatively large amounts. However, the prolongation of the thermal treatment led to a complete degradation of each intermediate product. We hypothesize that β-carotene may first be converted to its *Z*-isomers and then oxidized to the 5,6-epoxide or diones. Since we observed a large peak of 5,8-epoxide, at the beginning (first hour) of thermal treatment, which suggests a fast conversion of 5,6-epoxide to 5,8-epoxide or its degraded products like epoxy-apo-carotenals. The 5,8-epoxide was one of the stable epoxide. Apo-carotenals are also produced directly upon oxidative breakdown of β-carotene. This is in accordance with the scheme reported by Caris-Veyrat [30] for the oxidation of β-carotene in organic solution, but with different stabilities of each product.

Corn Oil Triacylglycerols Oxidation Products

Gas chromatographic results show that the corn oil has a high percentage of oleic acid (26.69 mg/100 g) and linoleic acid (52.61 mg/100 g), which could be the indicator of its stability toward thermal oxidation (Table 3). The TAGs composition of the corn oil has been previously reported [17] to contain 15 TAGs, DAGs and epoxy DAGs. Previous works of different authors are based on the characterization and identification of

standard fatty acids or single standard TAGs using GC-MS [31], NMR [32] or other simpler analytical techniques. Most of these techniques were not able to correctly identify oxidized TAGs. The uses of LC coupled to MS minimize the work load, and increase the possibility of correct structure assignment. However, only one report is available regarding the oxidized TAGs in canola oil using LC-APCI-MS [32]. We also found that our isocratic method was useful for studying the identification and characterization of TAGs and its oxidation products in model TAGs [16]. In the present study we were able to identify eight oxidized products of TAGs in corn oil at 110 °C in the Rancimat (Figure 5). These products are classed into epoxides, hydroperoxides, epoxy hydroperoxides, epidioxy hydroperoxides, and hydroxy hydroperoxides. The TAGs composition of the corn oil is shown in Table 4. We found that each class of the oxidized TAGs produced characteristic mass spectra, which could be differentiated on the basis of their relative proportions of different ions or fragments, some of which are produced by relatively similar fragmentation pathways. However, we were unable to separate and identify *E-Z* isomers of each oxidized products of TAGs, so these compounds were considered as one separate class.

Epoxides

The first class of the oxidized TAGs was epoxides. Figure 6A showed ESI-MS spectra of OOO-mono-epoxide and POO-mono-epoxide (peak g). The *m/z* 899.8, *m/z* 916.8, *m/z* 921.8 and *m/z* 881.8 correspond to the protonated molecular ion $[M+H]^+$, ammonium adduct $[M+NH_4]^+$, sodium adduct $[M+Na]^+$ and $[M+H-H_2O]^+$ of the OOO-mono-epoxide respectively. While *m/z* 873.9, *m/z* 890.8, *m/z* 895.8 and *m/z* 855.8 correspond to the protonated molecular ion $[M+H]^+$, ammonium adduct $[M+NH_4]^+$, sodium adduct $[M+Na]^+$ and $[M+H-H_2O]^+$ of the OOS-mono-epoxide. The fragment ions of *m/z* 577.5, 601.5 and 591.5 are diacylglycerols and mono-epoxy dioleoyl glycerol ions, respectively. In this spectra and other ESI-MS spectra of epoxides, we observed that epoxides are stable compounds and can be detected as adducts or in the form of an extra unsaturation at the site of epoxide by the loss of a water molecule. This loss was observed from the parent TAG molecule as well as its diacylglycerol fragment. The fragment ions of *m/z* 491.4 and 493.4 corresponds the loss of C_9H_{18} fragment leaving oxygen behind in the epoxy DAG ion. So the position of the epoxide was between C9 and C10, while the double bond was located at position of C8 and C9. Another example is POL-mono-epoxide and

OLO-mono-epoxide as shown in Figure 6B. The *m/z* 871.7 is $[M+H]^+$, *m/z* 888.8 and 893.8 show $[M+NH_4]^+$, and $[M+Na]^+$ of the POL-epoxide, respectively. Similarly a loss of a water molecule $[M+H-H_2O]^+$ and subsequent desaturation was observed at the *m/z* 853.7. In the case of OLO-epoxide, we observed that *m/z* 897.7, 914.8, 919.8, and 879.7 corresponds to $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and $[M+H-H_2O]^+$ of OLO-epoxide, respectively. The peak at the 6.6 min has also the characteristic mass spectrum of OLL-mono-epoxide. Neff and Byrdwell [34] also obtained the epoxides of OLL, LLL and OOO from the standard triolein, trilinolein sample on auto-oxidation in the dark at 50-60 °C using APCI-MS. However, they did not report ammonium adducts of epoxides; while some adducts ions were still not clear in their mass spectra. Our ESI-MS spectra show similar results, with much clearer spectra and low background.

Hydroperoxides

Figure 7A shows the ESI-MS spectra of OLO-mono-hydroperoxides. We observed a molecular ion of OLO-hydroperoxide at m/*z* 914.8, its ammonium adduct ion at *m/z* 932.8, and sodium adduct ion at *m/z* 937.8. The *m/z* 897.8 corresponds to the loss of water molecule and the formation of a stable epoxide. The intensity of this fragment ion shows that this epoxide is a stable ion. Further loss of the epoxide structure can be seen at *m/z* 881.8, which shows the formation of another unsaturation at the site of epoxide functional moiety. This can also be observed in the epoxy DAGs ions. The fragment ions at *m/z* 463.4 and 493.4 show the corresponding loss of $C_{10}H_{17}O$ and C_9H_{18} respectively from the epoxy DAG ion. This indicates that an epoxide was formed at the position of C8 and C9 and the double bond was migrated to C10 and C11, which confirmed that hydroperoxide group, was originally attached to C10 of the oleic acid moiety. Similarly, the peak at the retention time of 4.5 min was identified as LLL-mono-hydroperoxide (Figure 7B). Sjovall et al. [35] used ESI-MS single ion monitoring (SIM) for the determination of hydroperoxides of different standard TAGs. However, they did not report the exact structure of each oxidized species.

Epoxy-Hydroperoxides

Another class of oxidized TAGs which were also detected in corn oil sample oxidized at longer time and eluted earlier than epoxides was epoxy-hydroperoxides. The extracted ion chromatogram and ESI-MS spectra of OLL-epoxy hydroperoxide is shown in

Figure 8. This hydroperoxide was also detected as molecular ion at *m/z* 912.8, while its $[M+NH_4]^+$, $[M+Na]^+$, $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ ions were found to be at *m/z* 930.8, 935.8, 895.8 and 879.8 respectively. The loss of one and two oxygen atoms can also be observed at *m/z* 599.5 for LL^+, *m/z* 613.5 for LO_{ep}^+, *m/z* 615.5 for OO_{ep}^+, *m/z* 629.5 for $L_{ep}O_{ep}^+$, and *m/z* 631.5 for $O_{ep}O_{ep}^+$. The fragment ion at *m/z* 463.4 and *m/z* 493.4 explain the position of each epoxide and hydroperoxide. Sjovall et al. [35] identified the epoxy hydroperoxides from the oxidation of synthetic TAGs (SOS, OPP, SSL, and OOS) using single ion monitoring (SIM) mode of ESI-MS. They showed that epoxy hydroperoxides are produced in small quantities (3 to 7%) as compared to the mono-hydroperoxide (20 to 40%) at 37 °C for 30 hours. However, we have detected more epoxy hydroperoxides than mono-hydroperoxides. The reason may be due to the highly unsaturated nature of the corn oil and uses of high temperature oxidation.

Epoxy-bis-hydroperoxides

Figure 9 shows the extracted ion chromatogram (*m/z* 952.8) and ESI-MS spectra of LLLn-epoxy bis-hydroperoxide. Similar to other hydroperoxides, a molecular ion with *m/z* 970.8 can be found together with its lithium adduct (*m/z* 976.8). Several authors [16-17, 33-34] showed that traces of lithium in the solvent can form adducts. The *m/z* of 952.8 is believed to be the ammonium adduct of epoxide (*m/z* of 934.8) formed from the loss of two water molecule from the hydroperoxide moiety i.e. $[M-2H_2O+NH_4]^+$. Similarly, a sodium adduct $[M-2H_2O+Na]^+$ of this epoxide was also observed at *m/z* of 957.8. The complete loss of all epoxy oxygen atoms may form highly unsaturated species of *m/z* of 870.8. The ESI-MS spectra of epoxy-bis-hydroperoxides were similar to the epoxy-hydroperoxides as discussed above. Epoxy-bis-hydroperoxides are highly polar compounds and therefore was eluted before all epoxides, mono-hydroperoxides and epoxy-hydroperoxides. Neff and Byrdwell [34] identified LnLnLn-bis-hydroperoxide from the auto-oxidation of trilinolenin. However, they did not obtain a molecular ion or its protonated ion. Previously we reported [16] bis-hydroperoxides of OOO and OLL in the same conditions of oxidation as reported here. However, there was no linolenic acid in the model TAGs, which could increase the chances of oxidation of each TAGs to form this hydroperoxide.

Hydroxy-bis-Hydroperoxides

Figure 10 shows the extracted ion chromatogram of the ammonium adduct (*m/z* 976.9) and ESI-MS spectra of LLL-hydroxy bis-hydroperoxide. The *m/z* of 981.8 is believed to be the sodium adduct $[M+Na]^+$ of LLL-hydroxy bis-hydroperoxide, while *m/z* of 941.8 is formed from the loss of one water molecule from the hydroperoxide moiety i.e. $[M+H-H_2O]^+$. However, we did not see the molecular or protonated molecular ion or an expected diepoxide ion. The most probable reason could be the high reactivity of this compound towards the ammonium or sodium forming corresponding high intensity ions. Neff and Byrdwell [34] observed that hydroxides do not produce diacylglycerols ions. But in our study we obtained mono-epoxy diacylglycerols (*m/z* 613.5 for LO_{ep}^+, *m/z* 615.5 for OO_{ep}^+) as well as bis-epoxy diacylglycerols (*m/z* 629.5 for $L_{ep}O_{ep}^+$). The presence of these epoxy DAGs ions shows that loss of water and consequent formation of epoxides was the same as reported earlier. However, the high reactivity, and high fragmentor voltage used in the ESI-MS experiments prevented the detection of epoxy ions.

Conclusions

A reproducible gradient HPLC-DAD coupled with APCI-MS method is reported for the quantification and degradation of β-carotene in corn oil. The method was also very useful for the characterization of oxidation products of β-carotene in corn oil. Significant degradation of β-carotene was found in corn oil during accelerated oxidation in the Rancimat at 110 °C from 1 to 10 h. We have identified eight different oxidized products of β-carotene, which includes 8′-apo-β-carotenal, 6′-apo-β-carotenal, 5,6-epoxy-8′-apo-β-carotenal, β-carotene-2,2′-dione, all-*E*-5,8-epoxy-β-carotene, all-*E*-5,6-epoxy-β-carotene, and 15-*Z*-5,6-epoxy-β-carotene. The corn oil triacylglycerols oxidation products were identified using isocratic HPLC-ESI-MS. We found that β-carotene induced oxidation of TAGs especially at higher exposure time. For the first time we have identified two new classes of oxidized TAGs, which are epidioxy bis-hydroperoxides and hydroxy bis-hydroperoxide. Other oxidation products of TAGs were mono-epoxides, mono-hydroperoxides, and epoxy hydroperoxides. We suggest that by the supplementation of the HPLC-DAD-APCI-MS method for β-carotene reported here with ESI-MS method for TAGs oxidation provides a complete set of analytical tools to characterize carotenoids and triacylglycerols oxidation and oxidation products in edible oils or biological matrices.

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Table 1: Validation parameters for the quantification of β-carotene degradation in corn oil.

Parameter	Values
Regression	$y = 3.80 \times b + 22.09$
Correlation Coefficient	0.99984
Limit of detection (ng)	4.42
Limit of quantification (ng)	16.08
Intra-day precision (% CV)	1.54
Inter-day precision (% CV)	1.32

Figure 1: β-Carotene degradation corn oil oxidized in the Rancimat at 110 °C. Different letters indicate a significant difference ($P < 0.01$), while same letters have no significance difference.

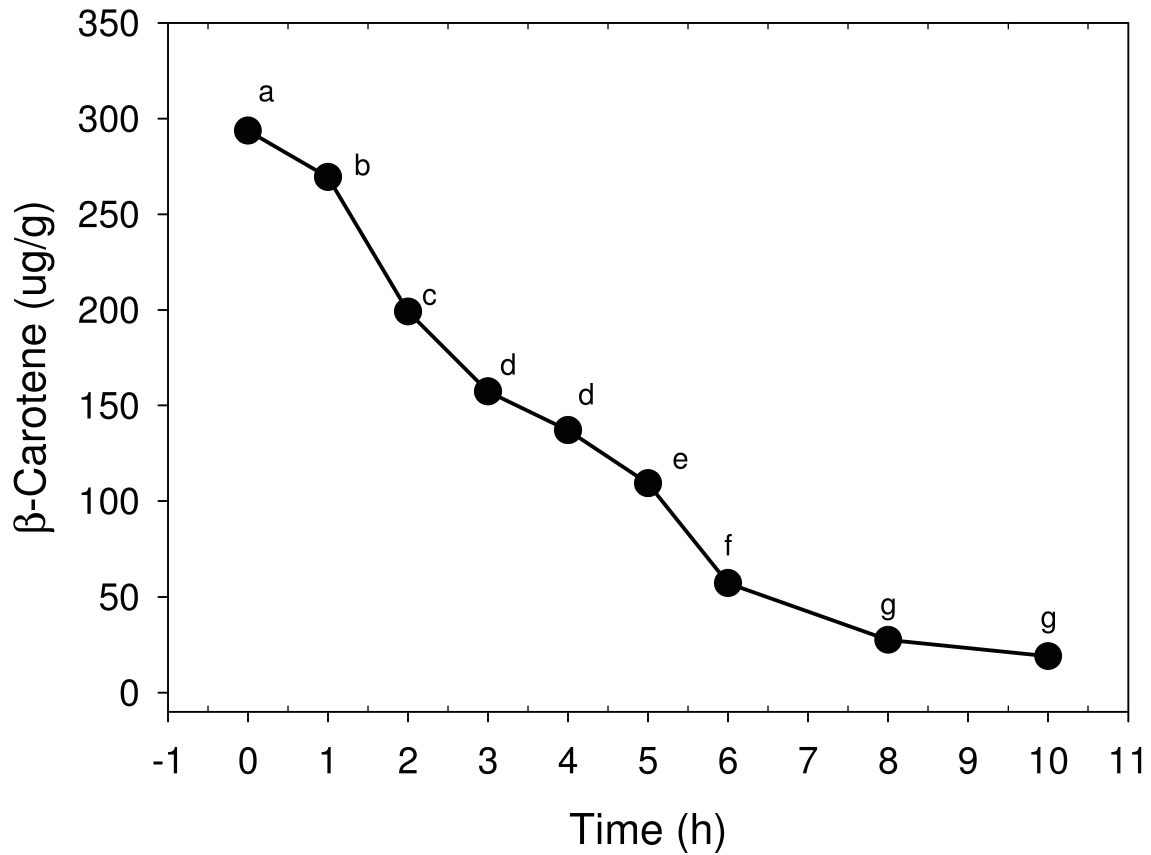


Figure 2: Effects of β-carotene (300 μg/g) on the peroxide value (meq/kg of O_2) of corn oil.

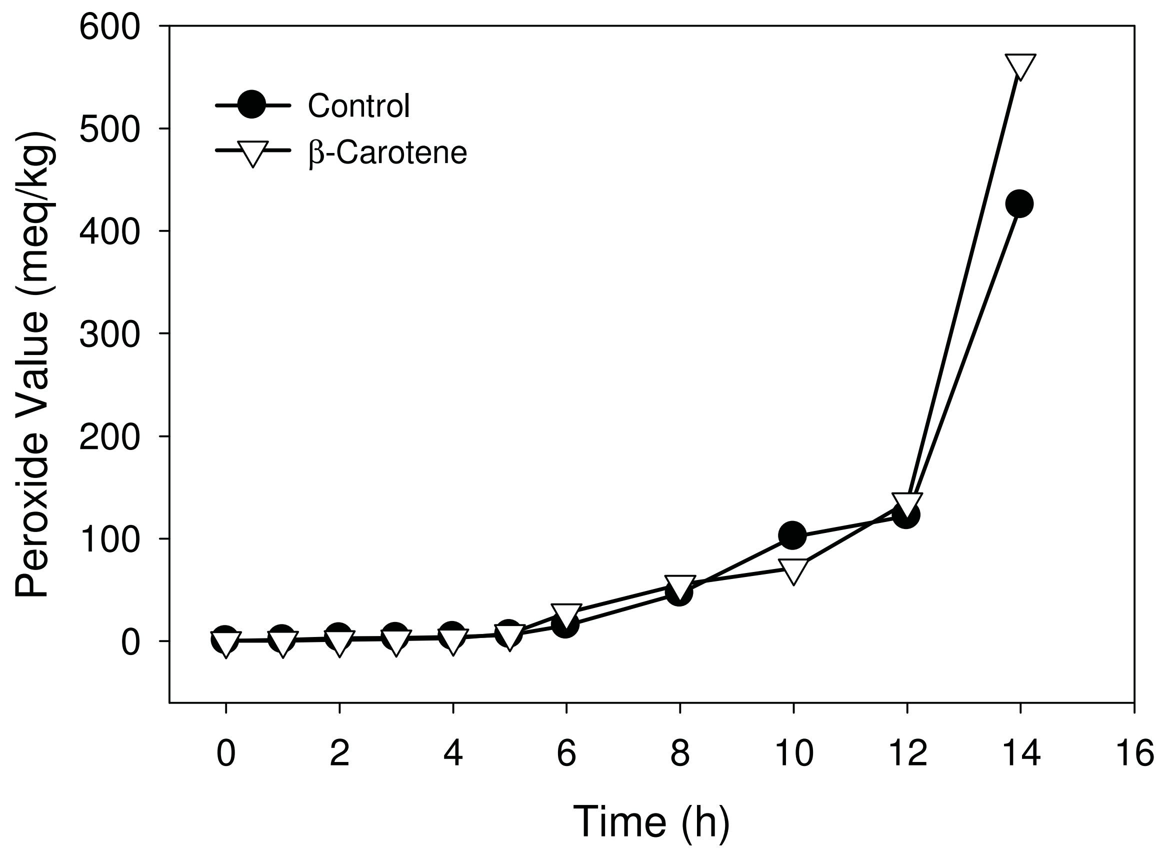


Figure 3: HPLC separation profiles of β-carotene and its oxidation products. (A) All-*E*-β-carotene standard, (B) β-carotene oxidation products from corn oil oxidized in the Rancimat at 110 °C.

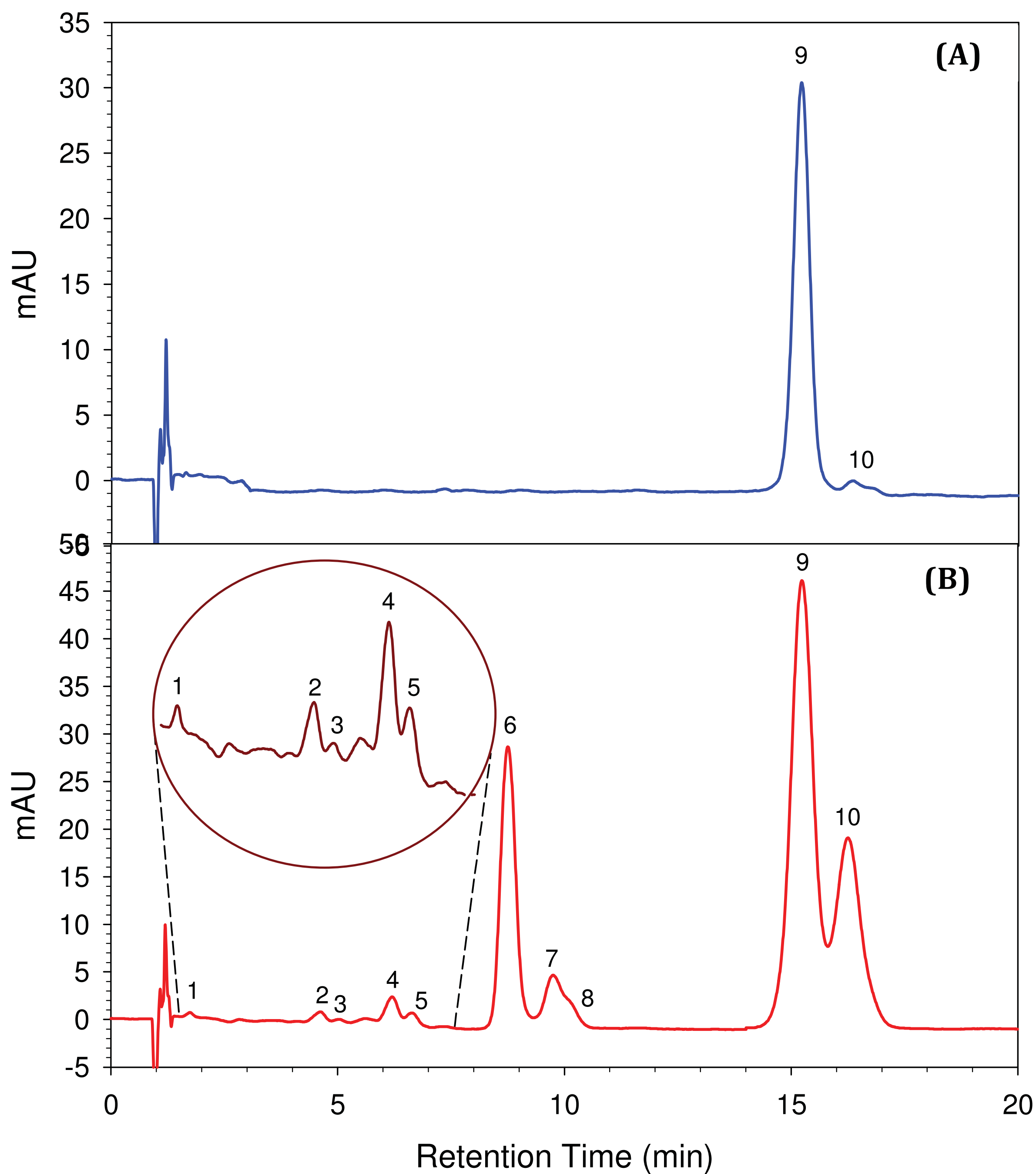


Figure 4: Structures of identified β-carotene and its oxidation products. Peak (1) 8□-apo-β-carotenal, (2) 8′-apo-β-carotenal, (3) all-*E*-5,6-epoxy-β-caroten-8′-al, (4) β-carotene-2,2′-dione, (5) 13-*Z*-5,6,5′,6′-diepoxy-β-carotene, (6) all-*E*-5,8-epoxy-β-carotene, (7) all-*E*-5,6-epoxy-β-carotene, (8) 15-*Z*-5,6-epoxy-β-carotene, (9) all-*E*-β-carotene and (10) 13-*Z*-β-carotene.

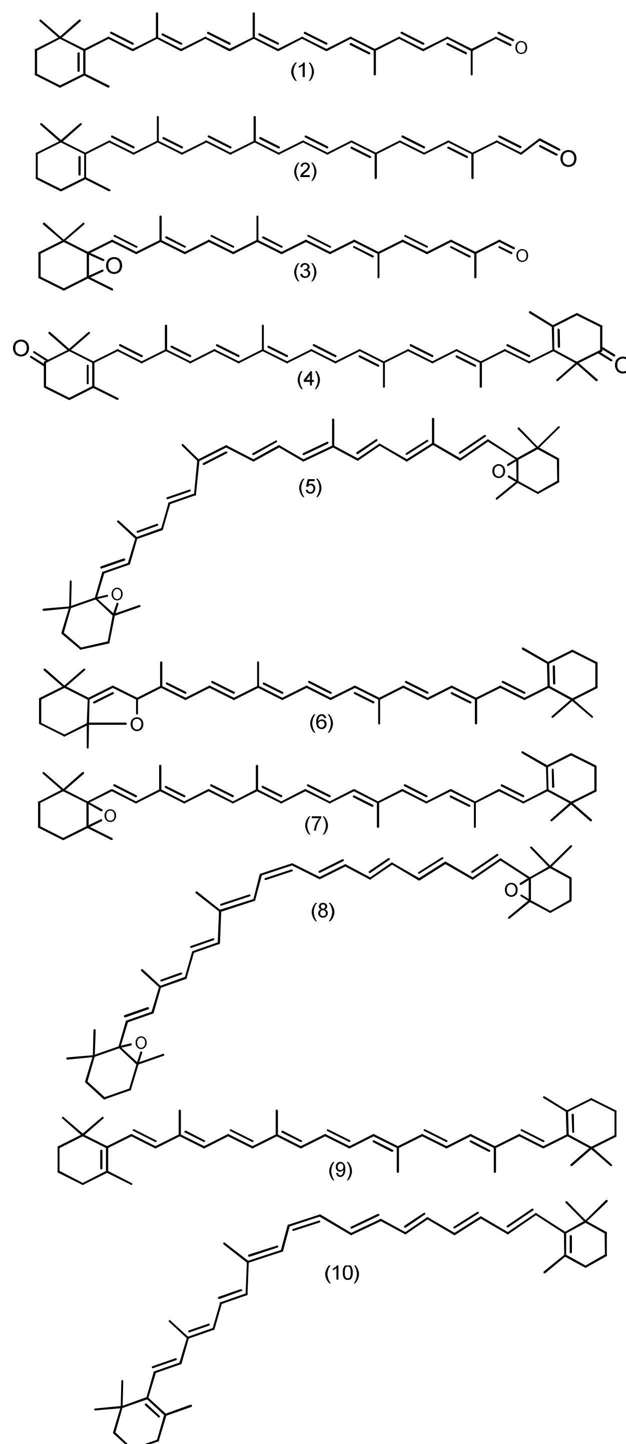


Table 2: Characteristics of β-carotene oxidation products in corn oil at 110 °C in the Rancimat.

Peak	R_t (min)	Compound	λmax	Molecular ion, formula
1	1.7	(all-*E*)-8′-apo-β-caroten-8′-al	458	417, $C_{30}H_{40}O$
2	4.6	(all-*E*)-6′-apo-β-caroten-6′-al	473	443, $C_{32}H_{42}O$
3	5.6	(all-*E*)-5,6-epoxy-8′-apo-β-caroten-8′-al	453, 474	433, $C_{30}H_{40}O_2$
4	6.1	(all-*E*)-β,β-caroten-2,2′-dione	425, 450, 478	565, $C_{40}H_{52}O_2$
5	6.6	(13-*Z*)-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene	340, 419, 442, 473	569, $C_{40}H_{56}O_2$
6	8.7	(all-*E*)-5,8-epoxy-5,8-dihydo-β,β-carotene	428, 453	553, $C_{40}H_{56}O$
7	9.7	(all-*E*)-5,6-epoxy-5,6-dihydo-β,β-carotene	451, 476	553, $C_{40}H_{56}O$
8	10.2	(15-*Z*)- 5,6-epoxy-5,6-dihydo-β,β-carotene	341, 445, 474	553, $C_{40}H_{56}O$
9	15.3	(all-*E*)- β,β-carotene	425, 451, 478	537, $C_{40}H_{56}$
10	16.2	(13-*Z*)-β,β-carotene	342, 446, 470	537, $C_{40}H_{56}$

Table 3: Fatty acid composition of corn oil.

Common Name	Fatty acid	Quantity (g/100g) Mean±SD
Myristic acid	C14:0	0.054 ± 0.01
Palmitic acid	C16:0	10.4 ± 0.69
Palmitoleic acid	*C16:1*	0.096 ± 0.02
Stearic acid	*C18:0*	1.78 ± 0.16
Oleic acid	*C18:1n-9*	26.6 ± 1.9
Linoleic acid	*C18:2*	52.6 ± 3.7
Linolenic acid	*C18:3n-3*	0.996 ± 0.10
Arachidic acid	C20:0	0.386 ± 0.01
Gadoleic acid	*C20:1*	0.347 ± 0.01
Eicosadienoic acid	*C20:2*	0.212 ± 0.02
Behenic acid	C22:0	0.170 ± 0.06

Figure 5: Total ions chromatograms of corn oils showing control, and oxidized samples for 10, 12 and 14 h in the Rancimat at 110 °C. The peaks 1-15 are explained in Table 4 and appearance of new peaks (a-h) are explained in the text.

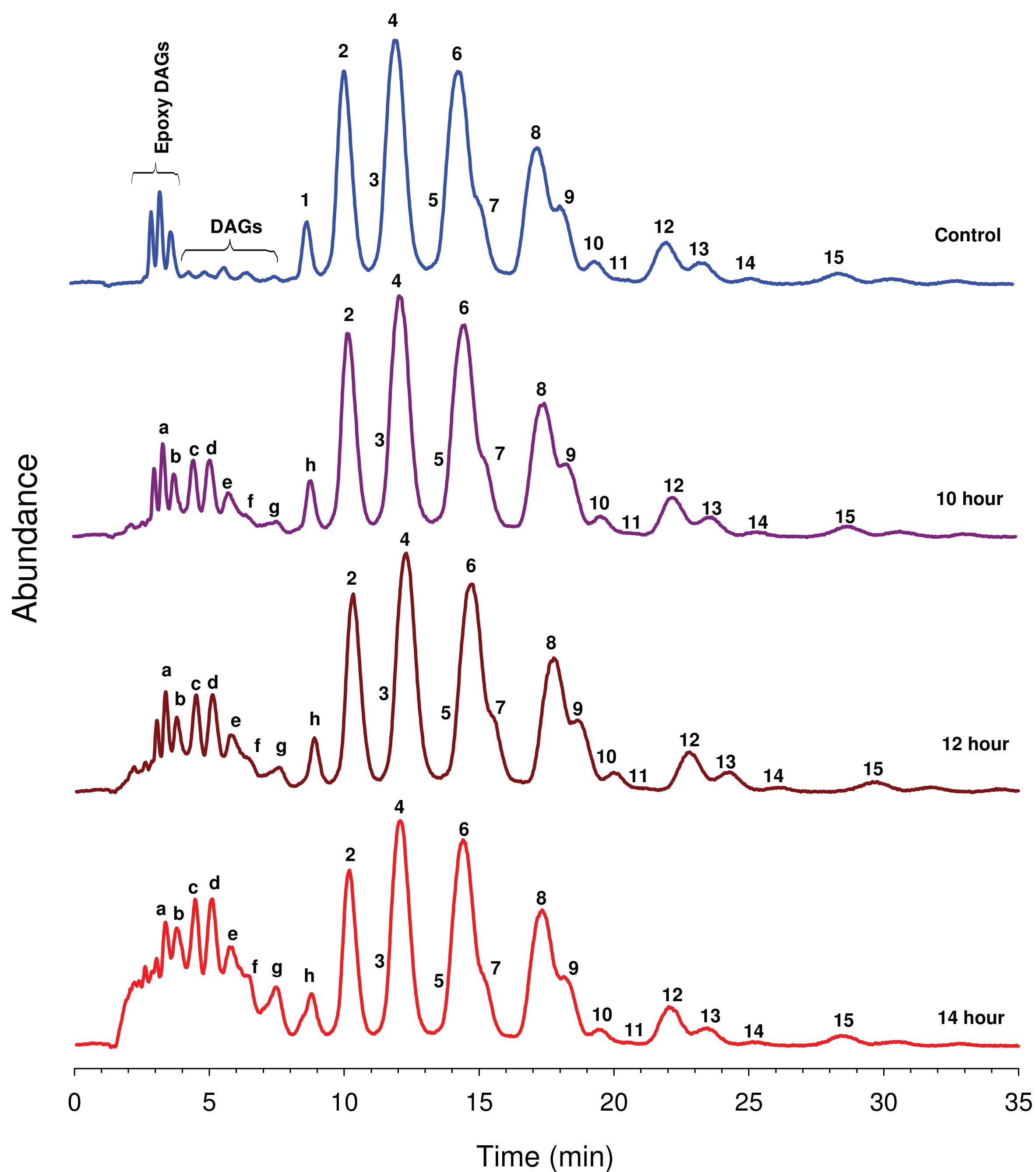
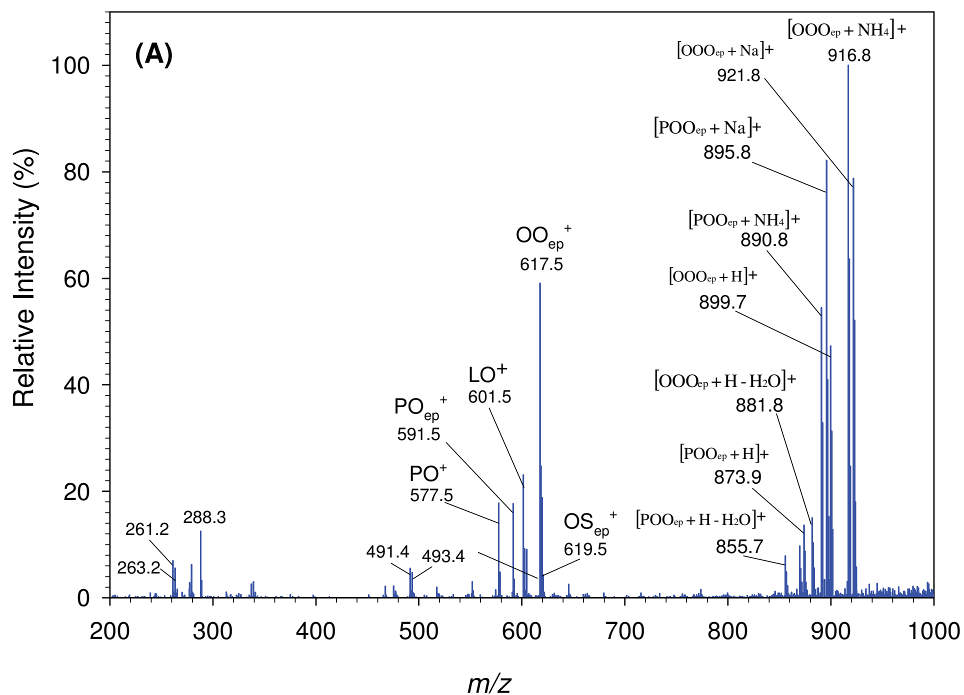


Table 4: Triacylglycerols composition of corn oil determined by ESI-MS [17].

Peak	R_t (min)	TAG	Peak Area (%)*	$M+H^+$	$M+NH_4^+$	$M+Na^+$	DG Fragments (m/z)		
1	8.9	LLLn	2.46	877	894	899	LL 599	LLn 597	
2	11.0	LLL	13.67	879	896	901	LL 599	--	--
3	12.2	PLL [a]	21.29	855	873	878	LL 599	PL 575	--
4	13.6	OLL [a]		881	898	903	LL 599	LO 601	--
5	14.5	PLP [b]	19.19	831	849	854	PP 551	PL 575	--
6	15.2	PLO [b]		857	875	880	LO 601	PL 575	PO 577
7	16.3	OLO	5.59	883	900	905	LL 599	OL 601	--
8	18.0	POO	12.89	859	877	882	PO577	OO 603	--
9	18.6	OOO	6.68	885	902	907	OO 603	--	--
10	19.3	SOL [c]	2.10	885	902	907	SO 605	SL 603	LO 601
10	19.6	SLO [c]		885	902	907	SO 605	SL 603	LO 601
11	20.3	ALL [c]		911	919	924	AL 631	LL 599	--
12	22.8	OSO	4.29	887	904	909	OO 603	OS 605	--
13	23.2	POS	2.11	861	879	884	SO 605	PS 579	PO 577
14	24.7	ALO	0.69	913	931	936	LO 601	AL 631	AO 633
15	30.0	AOO	1.01	912	933	937	AO 633	OO 603	--

* Epoxy DAGs (4.51 %) and normal DAGs (2.62 %). Similar letters (a-c) in the column represent co-eluting peaks.

Figure 6: ESI-MS spectra of oxidized TAGs (A) OOO-mono-epoxide & POO-mono-epoxide, (B) POL-mono-epoxide & OLO-mono-epoxide.



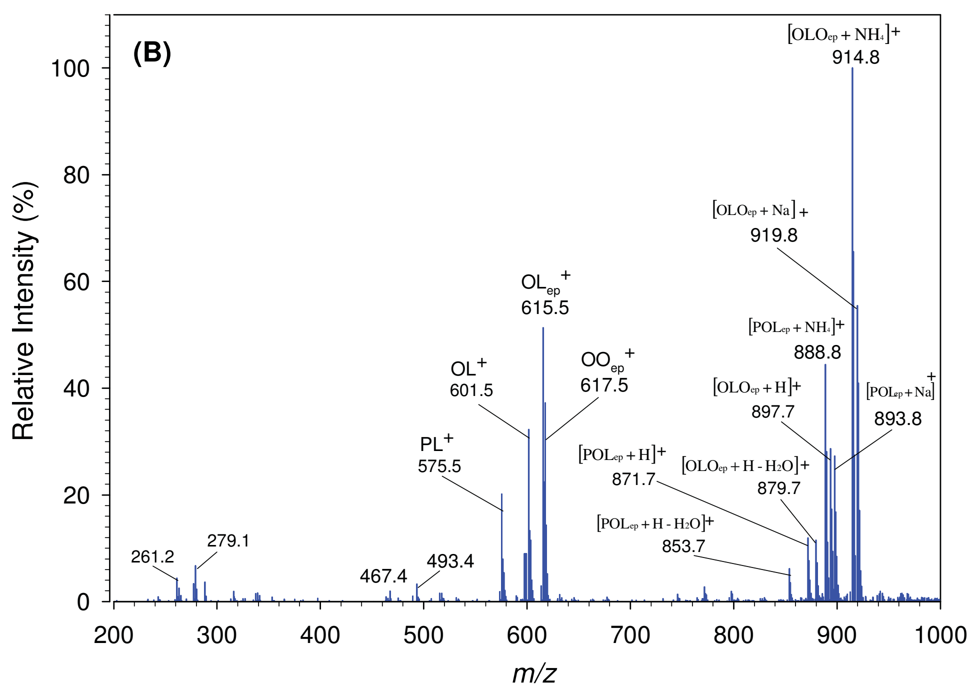


Figure 7: ESI-MS spectra of OLO-mono-hydroperoxide formed at 14 h of thermal treatment in the Rancimat at 110 °C.

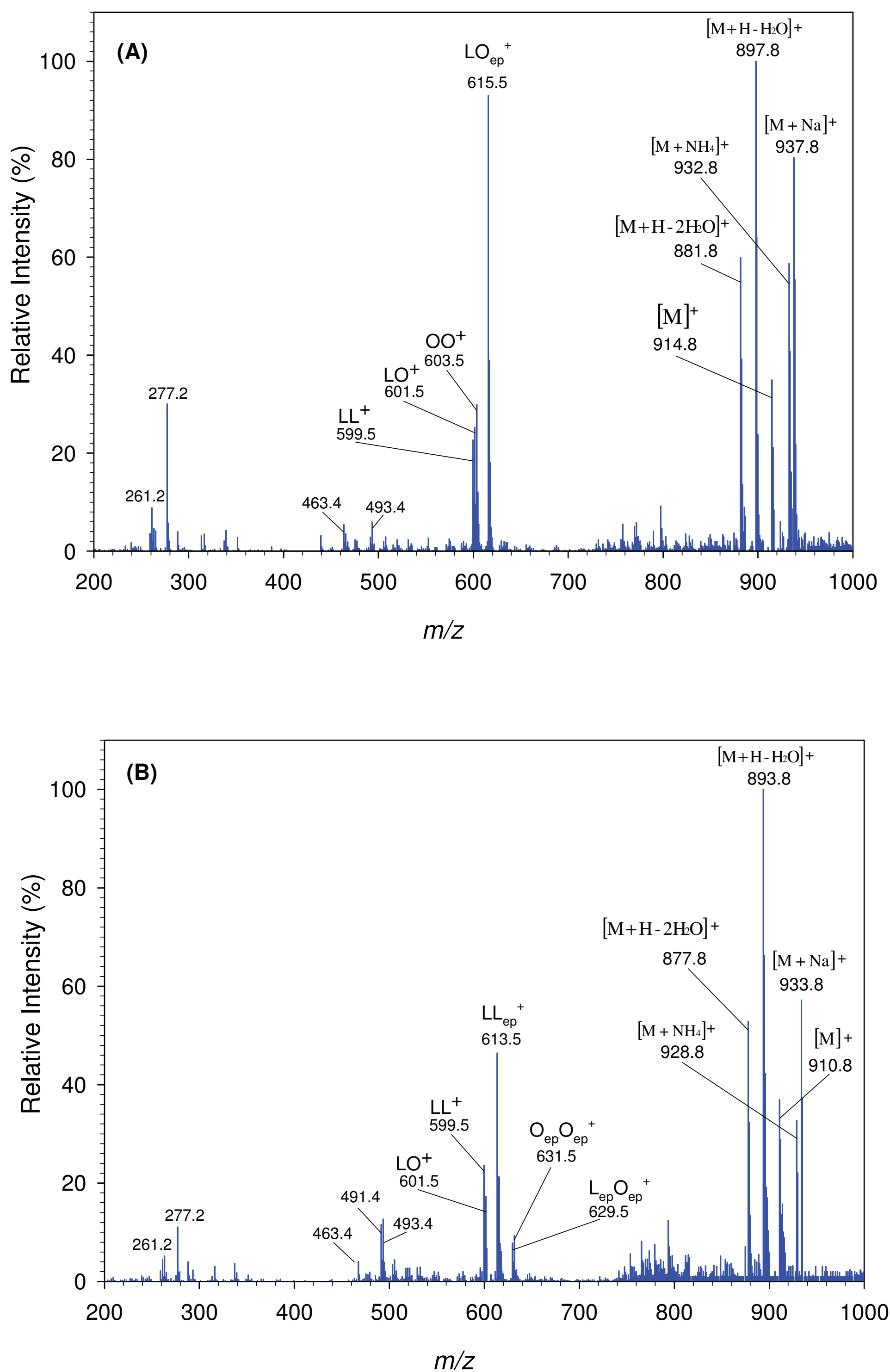


Figure 8: Extracted ion chromatograms (EIC) of *m/z* 895.8 and ESI-MS spectra of OLL-epoxy mono-hydroperoxide (*m/z* 912.8).

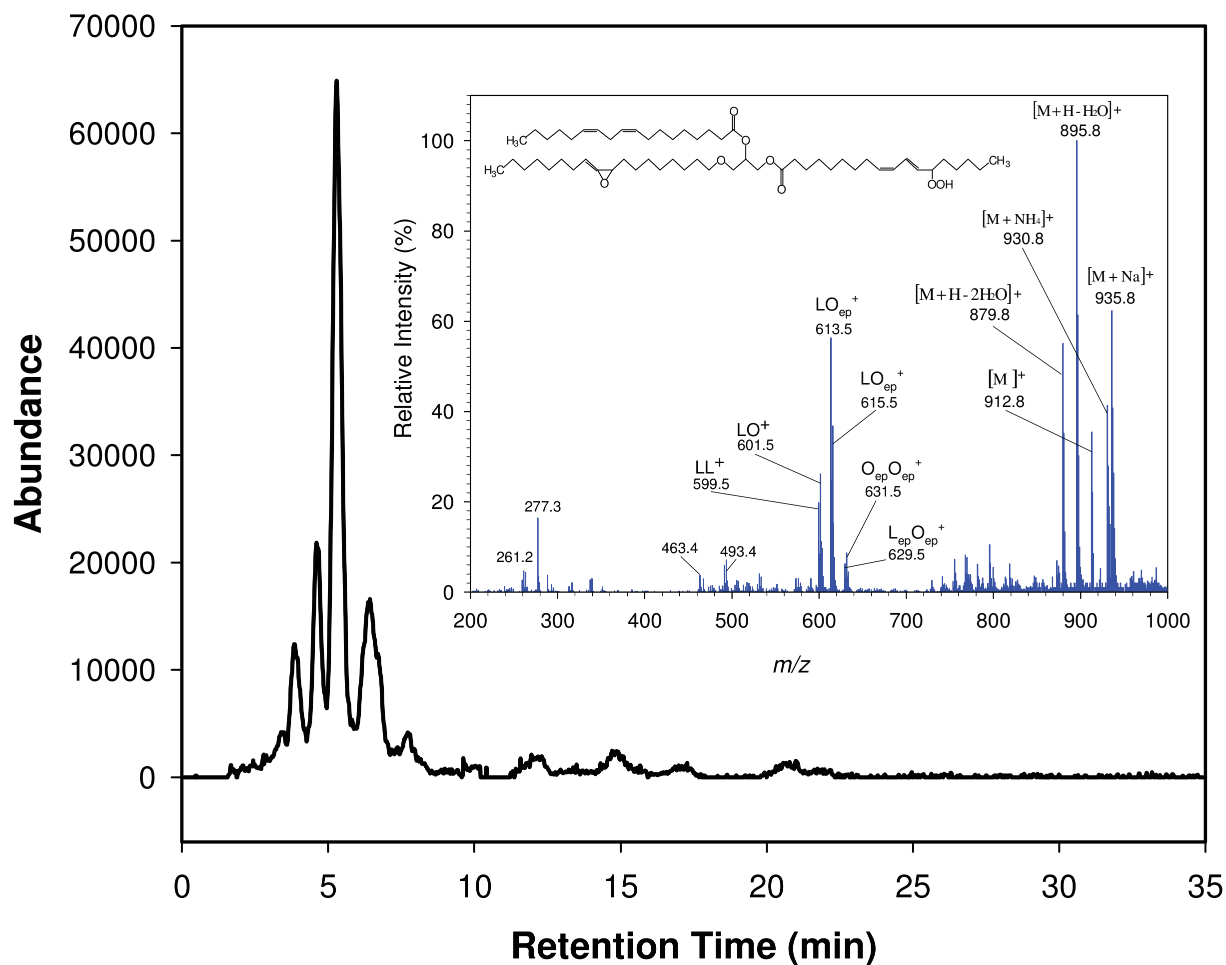


Figure 9: Extracted ion chromatograms (EIC) of *m/z* 952.8 and ESI-MS spectra of LLLn-epoxy bis-hydroperoxide (*m/z* 970.8).

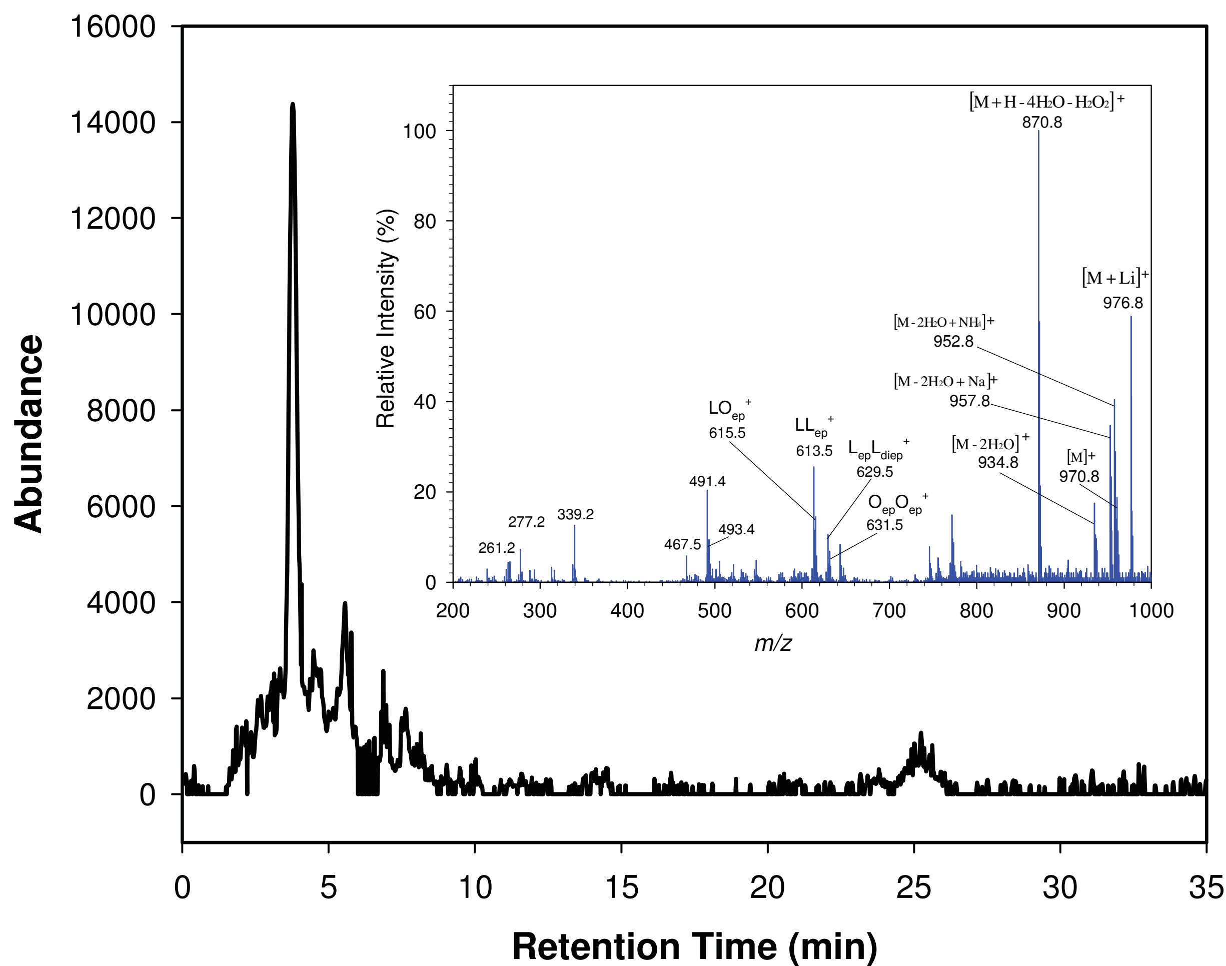
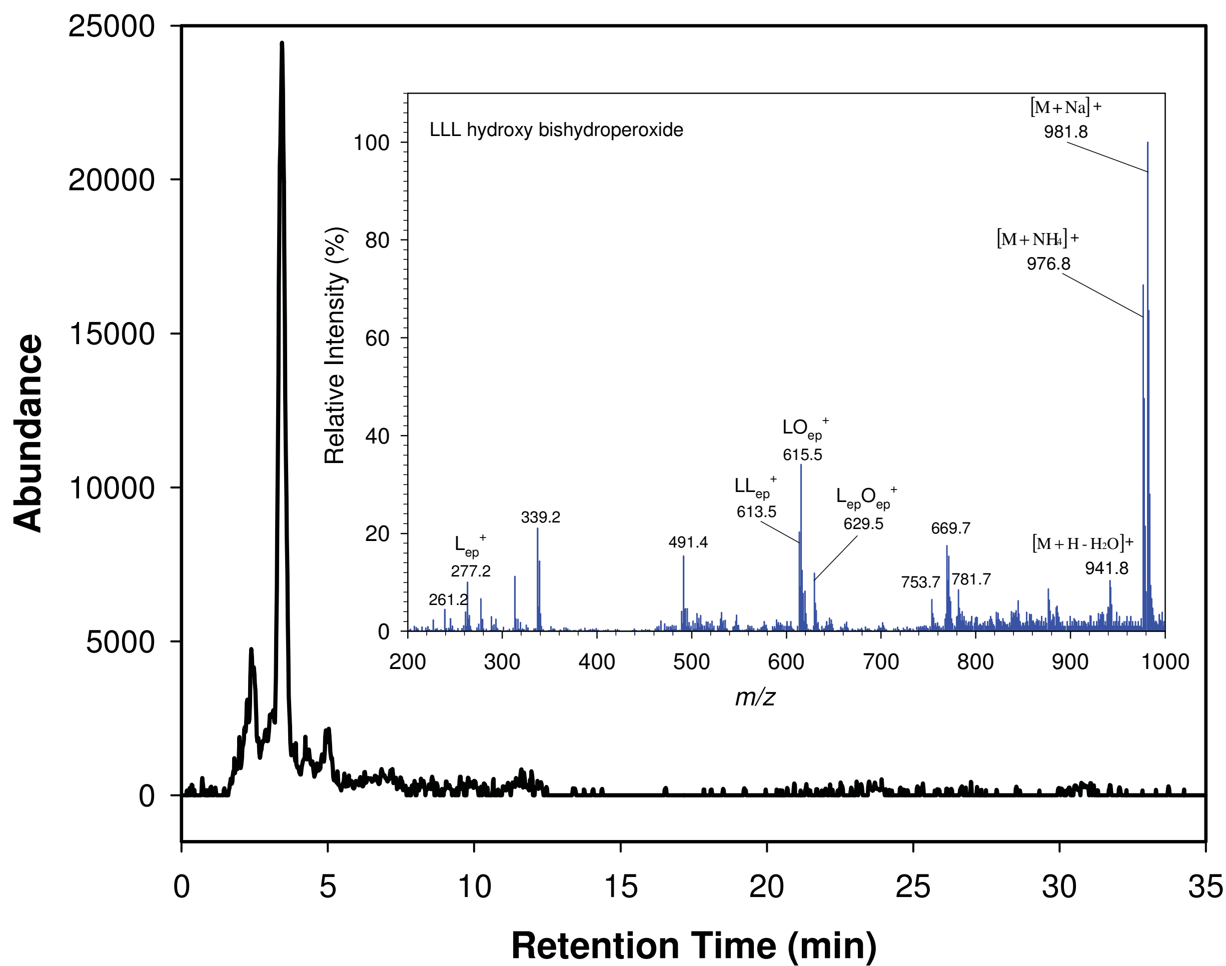


Figure 10: Extracted ion chromatograms (EIC) of *m/z* 976.8 and ESI-MS spectra of LLL-hydroxy bis-hydroperoxide (*m/z* 958.7).



Title

Carotenoids and Triacylglycerols Interactions during Thermal Oxidation of the Refined Olive Oil

Running title

Carotenoids and Triacylglycerols Thermal Oxidation in Olive Oil

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Abbreviations

TAGs, triacylglycerols; ESI-MS, electrospray ionization mass spectrometry; HPLC, high performance liquid chromatography; AOCS, American oil chemist's society; POV, peroxide value; EIC, extracted ion chromatogram. P, palmitic; S, stearic; A, arachidic; G, gadoleic; Ln, linolenic; L, linoleic; O, oleic; ep, epoxide.

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Abstract

Carotenoids (β-carotene and astaxanthin) were added to the refined olive oils and oxidized in the Rancimat at 110 °C for 1 to 14 hour. The thermal degradation of carotenoids was measured using HPLC-DAD, while triacylglycerols (TAGs) were measured using HPLC-MS. A total of 13 TAGs were identified using ESI-MS. In olive oil TAGs were found to oxidize much faster in the presence of β-carotene and astaxanthin. However, β-carotene degradation was much faster than astaxanthin. Both carotenoids were found to isomerize at the early stages of thermal oxidation in olive oil. Astaxanthin was found to protect the olive oils TAGs till 10 h of thermal treatment. Both carotenoids were found to significantly increase the peroxide values. The TAGs composition of the refined olive oils reveals a high level of oleic acid containing TAGs. A total of 11 TAGs oxidized species were identified. We have reported here for the first time a new class of oxidized species (hydroxy epidioxides), together with previously reported species. Other TAGs oxidized species were mono- and bis-hydroperoxides, epoxy hydroperoxides, hydroxy hydroperoxides, epoxy epidioxides, and mono-epoxides. The effect of carotenoids on the formation of oxidized TAGs illustrates, that the pro-oxidant action of β-carotene was much stronger than of astaxanthin. Based on these results we can suggest that astaxanthin could be beneficial to use as an alternative to β-carotene in edible oils.

Key words

β-Carotene, astaxanthin, triacylglycerols, oxidized triacylglycerols, HPLC-ESI-MS, thermal stability, carotenoids oxidation, free radical reaction.

1. Introduction

The olive tree is considered to be one of the oldest known cultivated trees in the world. It is also considered as cultural marker and a compass to explore the development of civilizations. From the very past to the present olive oil is a staple food in the Mediterranean region with special regards to its health benefits (Mataix & Barbanco, 2006). Generally, olive oil is consumed in its natural unrefined state (virgin) or as a refined or as a blend consisting of the refined and virgin oils. Virgin olive oil contains a large number of phenolic compounds, which largely contribute to the bitter taste (Servili et al., 2004). Therefore, to have more edibility it is refined using physical or chemical refining. The physical and chemical refining has been found to decrease the level of acidity and sterols (Ruiz-Méndez, López-López, Garrido-Fernández, 2008). In addition, the refined olive oils have a much lower content of phenolic compounds, because they are removed during the refining process (Garcia, Ruiz-Mendez, Romero, & Brenes, 2006). It is well known that refined oils of low quality are less stable towards thermal stress. Thus, for increasing the refined olive oil quality a number of additives have been used (Aparicio, Roda, Albi, & Gutierrez, 1999). However, some of these compounds produced undesirable effects on the quality and sensory characteristics of olive oil and are thus restricted for food uses (Bouaziz et al., 2010). So the needs for the safe alternative solutions, which would help to improve the olive oil, quality and stability during storage and oxidation is highly demanding.

Carotenoids are widely studied organic compounds present in various foods of plants and animal origin. Among carotenoids, β-carotene is one of the most important and widely studied carotenoid. It is a red-orange colored compound with highest vitamin A activity (Rodriquez-Amaya, 1999). β-Carotene serves as biological antioxidant, helpful in maintaining human health and is used as colorant in food industries. Astaxanthin is a keto-carotenoid most commonly found as a pigment in crustacean, salmonids and other sea foods. It is an important and economically valuable carotenoids due to its biological function as pro-vitamin A (Gobantes, Ghoubert, Milicua & Gomez, 1998). It provides the desirable reddish-orange color to the sea food organisms and serves as natural antioxidant (Higuera-Ciapara, Lix-Valenzuela & Goycoolea, 2006). Astaxanthin is commonly used as a feed supplement in commercial fish and crustacean farms for their proper healthy growth and to retain the beautiful colors of these organisms. It has also a

number of health-promoting properties like scavenging free radicals and quenching singlet oxygen in biological systems. It has a stronger antioxidant property than other carotenoids such as β-carotene, lutein, canthaxanthin and zeaxanthin (Naguib, 2000).

Due to the high unsaturation, carotenoids are prone to degradation during food processing, storage and thermal treatment. The major reactions are isomerization and oxidation of the parent carotenoids (Rodriguez-Amaya, 1999). Naturally occurring carotenoids are found in all-*E* form, while only small fractions of *Z*-isomers are also present. Isomerization is one of first changes in carotenoids structures during heating (Achir et al., 2010). However, carotenoids degradation in food or analogous model systems is a highly complex phenomenon. A simplified mechanism was shown by Borsarelli and Mercadante (2010) for the overall changes occurring in carotenoids during heating. Previously most of the oxidation systems rarely represent the real food matrix, especially the oxidation of carotenoids in common edible oils. We have recently observed (Zeb & Murkovic, 2010a), that β-carotene plays a significant role during the oxidation of model triacylglycerols. The structure and chemistry of oxidized TAGs in relation to its effects by the β-carotene in the model TAGs were reported. We also observed that β-carotene degraded before the oxidation of corn oil TAGs. However, the oxidation products of TAGs formed in olive oil and the effects of carotenoids on the stability of each oxidized TAG under relatively similar conditions were rarely reported, and thus is the subject of prodigious interest, and also the main purpose of the work presented here.

2. Experimental

2.1. Reagents and Materials

Methanol and acetone were purchased from Mallinckrodt Baker (The Netherlands), *tert*-butyl methyl ether (MTBE) was from Merck (Darmstadt, Germany). A Milli-Q water purification system (Millipore AS, Bedford, MA, USA) was used for the deionized water, while all-*E*-β-carotene and all-*E*-astaxanthin were purchased from Fluka (Sigma-Aldrich, Germany). Refined olive oil was kindly provided by Dr. Evangelos Katzojannos from a commercial market in Athens (Greece).

2.2. Rancimat Oxidation

All-*E*-β-carotene and all-*E*-astaxanthin were dissolved in acetone and added to olive oil in order to flourish a concentration of 300 μg/g ± 0.5 μg. The fortified sample was sonicated for 1 min and then kept for one hour under nitrogen in a glass bottle. A sample of 4 g of the fortified olive oil was oxidized using the Rancimat Metrohm model 679 (Metrohm AG, Switzerland). Rancimat conditions were air flow of 20 L/h, temperature of 110 °C, and oxidation time of 1 to 14 hours. After the experiment 400 μL of BHT (0.5 % in acetone) was added to the oxidized samples and stored at -20 °C till analysis. A control sample was also oxidized under similar conditions without carotenoids. All preparations were carried out under nitrogen ambience and in the day light.

2.3. Extraction of Carotenoids

The carotenoids were extracted from the oxidized sample of 200 mg using 2 mL of acetone and sonicated for 20 sec. The β-carotene containing samples were kept at -20 °C samples, while astaxanthin containing samples were kept at -72 °C for 24 h. The lipids crystallize and carotenoids move to the acetone phase, which is then quickly filtered. The details for this procedure are described in our recent work (Zeb & Murkovic, 2010b).

2.4. Sample Preparation

Acetone was evaporated from the carotenoids extract and the residue was re-dissolved in 1 mL of acetone for the transfer into HPLC vials for analysis. For the analysis of TAGs and its oxidized species, a sample of 100 mg of the refined olive oil was dissolved in 2 mL of acetone and 2 mL of HPLC solvent as mentioned previously (Zeb & Murkovic, 2010c). The sample was then transferred to a 2 mL vial for the HPLC analysis.

2.5. Carotenoids Analyses

The HPLC system consisted of an Agilent 1100 series system (Agilent Technologies, Waldbronn, Germany) with vacuum degasser, quaternary pump, auto sampler, temperature-controlled column oven, diode array detector (at 450 & 480 nm) and mass spectrometer (Agilent Technologies, Waldbronn, Germany). The extracted β-carotene was separated using a Lichrospher® 100, RP-18e (125×4, 5 μm) column (Agilent

Technologies, Germany) with a flow rate of 1 mL/min. The separation of β-carotene and its oxidation products were achieved using a gradient mobile phase consisting of methanol (solvent A), methanol/MTBE/water, 80:15:5, v/v/v (solvent B) and acetone (solvent C). The initial elution was 90 % A, 10 % B, which reached 10 % A and 90 % B at 7 min and remained isocratic till 12 min. At 20 min eluent composition was changed to 75 % B and 25 % till 30 min. The post run time was maintained for 10 min (Zeb & Murkovic, 2010b). The APCI-MS optimized conditions for the determination of β-carotene and astaxanthin were drying gas flow of 6 L/min, capillary voltage 2500 V, gain 8, corona current 5 μA, vaporizer temperature 330 °C, and drying gas temperature of 250 °C. Astaxanthin was analyzed using an isocratic solvent system consisting of methanol: MTBE: water (73:14:13, v/v/v) for 20 min. Other conditions were the same as above. The quantification was carried out using six point calibration curves of the standard carotenoids. Data validity was checked with VALIDATA version 3.02 (Microsoft Excel macro). Data were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison method at α = 0.01 or α = 0.05 using Sigma Plot for windows version 11.0 (Systat Software, Inc, 2008).

2.6. Triacylglycerols Analyses

Liquid chromatography was carried out using an Agilent HP 1100 system coupled to ESI-MS (Agilent, Waldbronn, Germany). A Phenomenex C18 (150×3 mm, 5 μm) (Germany) column and an isocratic solvent system consisting of 18 % isopropanol in methanol with 0.1 % acetic acid with a flow rate of 0.6 mL/min were used for separation. Mass spectrometry was carried out using an Agilent HP 1100 system ESI-MS (Agilent, Waldbronn, Germany). Other conditions of LC and MS were the same as reported previously (Zeb & Murkovic, 2010b, c).

2.7. Fatty Acid Analyses

The fatty acids were converted to its methyl esters (FAME) using a standard method as described by AOCS (1998). Briefly, aliquots of 40 μL of internal standard (C11:0, 5.65 g/100 g) were added to 20 mg (±0.5 mg) of the sample in 20 mL vials and after the evaporation of solvent, 6 mL of 0.5 M methanolic NaOH were added (80 °C for 30 min). Then the trans methylation was carried out with BF_3/Methanol (80 °C for 15 min). Phase separation was achieved with water and n-heptane. FAMEs of the samples

were separated on a GC (HP 5890) system using Agilent-Technologies DB-Wax capillary column (30 m long, with internal diameter 0.25 mm, film thickness 0.25 µm). The helium flow rate was set to 5.7 mL/min as a carrier gas. Column oven temperature was programmed from 50 to 140 °C with 15 °C/min increase, then 140 °C to 240 °C with 8 °C/min increase. Initial and final hold up time was 1 and 8 min, respectively. The injection volume was 1 µL. FAMEs were identified by comparing their relative and absolute retention times with those of authentic standards and by GC/MS detection.

2.8. Peroxide Value

The peroxide value of each sample was determined using the AOCS official method (method Cd 8b-90) (AOCS, 1998) and expressed as meq O_2/kg of fat. All samples were measured in triplicate or otherwise mentioned.

3. Results and Discussion

The olive oil containing carotenoids was oxidized at 110 °C in the Rancimat for 1 to 14 h. We have shown recently that 110 °C was the optimal temperature for studying both the degradation and oxidation products of carotenoids and TAGs (Zeb & Murkovic, 2010a). We observe that at higher temperatures, carotenoids degradation in edible oils are more faster than at 110 °C, usually in the first or two hours, thus it was not possible to obtain reproducible results regarding the degradation and oxidation products of carotenoids and triacylglycerols.

3.1. Carotenoids Analysis

Figure 1A shows the comparative chromatograms of the standard β-carotene and the oxidized sample. The retention time of all-*E*-β-carotene was 15.4 min, while its *Z*-isomers eluted after all-*E*-β-carotene. All-*E*-β-carotene was found to significantly isomerize to 13-*Z*-β-carotene during the first hour of thermal treatment. It has been found that by increasing thermal exposure time a decrease in the amount of 13-*Z*-β-carotene occurs. A small new peak appears between all-*E*- and 13-*Z*-β-carotene, which was identified from its absorption spectra to be a 9-*Z*-β-carotene. The decrease in the 13-*Z*-β-carotene and subsequent increase in the amount of 9-*Z*-β-carotene shows that 13-*Z*-β-carotene was converted to a more stable 9-*Z*-β-carotene form which is favorable under

these conditions. Henry, Catignani and Schwartz (1998) found that heating of β-carotene in safflower seed oil at several temperatures formed 13-*Z*-β-carotene in higher amounts, which was also observed in our studies. Two small peaks between 9 and 10 min of elution, were identified as 5,8-epoxy-β-carotene and its *Z*-isomer. We observed an increase in the amount of this compound during the prolonged thermal treatment (especially at 8 h). In corn oil under similar conditions, the major product occurring during the first hour of oxidation was 5,8-epoxy-β-carotene, which started disappearing with increasing exposure time of thermal treatment (Zeb & Murkovic, 2010c). In contrast to this, in olive oil, it was formed in small quantities and its amounts increases with the increase in oxidation time. It may be due to the relatively large amounts of phenolic compounds in olive oils as compared to corn oil, which may accelerate the oxidation and degradation of β-carotene. At longer treatment time in olive oils oxidized samples 6-apo-β-carotenal being the only apo-carotenal identified. This compound was also observed in corn oil oxidation at similar conditions.

During astaxanthin analysis by HPLC-DAD, all-*E*-astaxanthin eluted at 13 min as shown in Figure 1B. Only two *Z*-isomers were identified which were 9-*Z*- and 13-*Z*-astaxanthin. The elution order was all-*E*- > 9-*Z*- > 13-*Z*-astaxanthin. We have observed no peak shoulder in the standard compound, while in oxidized samples, a shoulder was observed, which may be the stereoisomer of all-*E*-astaxanthin. The absorption spectra and m/z was the same as of all-*E*-astaxanthin. However, we were unable to confirmed completed identity. Similar to β-carotene, a higher amount of 13-*Z*-isomer of astaxanthin was obtained.

3.2. Carotenoids Degradation

Although the degradation and oxidation of carotenoids in lipids especially edible oils is a complex phenomenon, this study provides a clue for the possible reactions taking places at relatively high temperature and high amounts of carotenoids. Figure 1C shows the comparative degradation of β-carotene and astaxanthin for 1 to 14 h in the Rancimat at 110 °C. The degradation of β-carotene was faster than astaxanthin in the refined olive oils. All-*E*-astaxanthin degraded slowly till 6 h and then significant degradation was observed till 12 h. Two isomers (9-*Z*-, and 13-*Z*-astaxanthin were identified in large amounts during the first hour of thermal treatment. These *Z*-isomers were found to

increase during prolonged thermal treatment. Zhao et al. (2006) reported that all-*E*-astaxanthin was converted to 13-*Z*-astaxanthin in high amounts by microwave heating. Liu and Osawa (2007) showed that *Z*-astaxanthin, especially 9-*Z*-astaxanthin, exhibited a higher antioxidant effect than the all-*E* isomer in a DPPH scavenging activity test and also in rat microsome and membrane lipid peroxidation systems. Thus the slower oxidation of astaxanthin could also be attributed to the protective effect of *Z*-astaxanthin in olive oil at 110 °C. However, at 14 h none of these carotenoids were detected in the system. This was also observed visually by the change in the color of the oxidized samples. β-Carotene degraded slowly at 4 and 5 h of thermal treatment. Further degradation was significant till the complete loss at 10 h of the thermal treatment. We observed that the degradation of β-carotene in the present studies was much slower than in the model TAGs (Zeb & Murkovic, 2010c), where half of β-carotene degraded during the first 3 hours of thermal treatment under similar conditions. This may be due to the presence of tocopherols and phenolic compounds in the olive oil. Henry, Catignani and Schwartz (1998) showed that β-carotene degradation in safflower oil follows a first order kinetics and which is more stable than lycopene but more susceptible than lutein. Similarly a recent study by Achir et al. (2010) showed a faster degradation of all-*E*-β-carotene than in all-*E*-lutein in oils during heat treatment at 120 to 180 °C. These studies however, did not provide any information about the oxidation of triacylglycerols of the treated oils. We found that β-carotene is more susceptible to degradation than astaxanthin under similar conditions in olive oils. This can be further confirmed by studying the interactions of the selected carotenoids with triacylglycerols and its oxidized species.

3.3. Effect of Carotenoids on Peroxide Value

As lipids oxidation started, among the first products formed are hydroperoxides. Highly unsaturated lipids form more hydroperoxides and thus oxidized faster than saturated or mono-unsaturated lipids (Kamal-Eldin, Mäkinen, Lampi, 2003). Figure 1D shows that till 8 h of thermal oxidation in the Rancimat at 110 °C, there were no significant changes of the peroxide value of the control, β-carotene and astaxanthin containing olive oil samples. At 10 h, the formation of hydroperoxides is more pronounced in control samples than in carotenoid treated samples. Similarly, after 12 h of oxidation, the β-carotene containing samples produced more peroxides than the astaxanthin samples and less than the control samples. At this stage all β-carotene was

oxidized completely, while some astaxanthin remained in the samples, which helped to protect the TAGs, which is shown by the reduced formation of hydroperoxides. The results are in agreement with Liu and Osawa (2007), who showed that during polyunsaturated fatty acid (PUFA) oxidation, both DHA and linoleic acid hydroperoxides formation were markedly inhibited by astaxanthin addition in the order 9-Z > 13-Z > all-E-astaxanthin. In our studies, during 14 h more peroxides were produced in β-carotene treated samples compared to the astaxanthin containing and control samples. This suggests the pro-oxidant action of the oxidized β-carotene species. These results are in agreement with our previous studies (Zeb & Murkovic, 2010a) of β-carotene oxidation in model TAGs, where the composition of model TAGs was about 90 % similar to olive oil TAGs. However, in this study the peroxide formation in the control model TAGs and β-carotene treated model TAGs are 4 and 6 times higher than the control and β-carotene containing olive oils, respectively. This shows that other ingredients such tocopherols, phenols and other antioxidant compounds may inhibit the formation of TAGs hydroperoxides, while β-carotene acts as pro-oxidant in all these experiments.

3.4. Fatty Acids and Triacylglycerols Composition

Table 1 shows the fatty acid composition of the olive oil. The refined olive oil was rich in oleic acid with 74.3 g/100 g, while linoleic acid amounts to 7.72 g/100 g and palmitic acid was 10.8 g/100 g. The content of polyunsaturated fatty acids in olive oil is very low. The high level of oleic acid was one of the determinants for the stability to the olive oils together with tocopherols and phenolic compounds. Figure 2A shows the total ion chromatogram of the refined olive oil. The peak identification and relative composition is shown in Table 2. We were able to correctly identify 13 TAGs. The relative percentage of POO, OOO, OLL and POL/PLO is higher than other TAGs. Most of the fatty acids in olive oil identified using gas chromatography were found to constitute the TAGs, however some higher fatty acids were not observed as component of TAGs, which might constitute other lipids like mono-acylglycerols (MAGs) or diacylglycerols (DAGs), esters, steroids etc. Sakouhi, Absalon, Kallel and Boukhchina (2010) identified 15 TAGs in Tunisian extra-virgin olive oils using MALDI-TOF-MS. They found that OOO was the major TAG, followed by POO and OOL. Similarly Chapagain and Wiesman (2009) identified 12 TAGs using MALDI-TOF/MS finger printing in the extra-virgin olive oils produced from the Israeli Negev desert. They found that the OOO and POO were the major TAGs in olive

oils. These results are in accordance with our results for the high amounts of OOO and POO in olive oils. Other authors also showed that refining of olive oils also does not produced significant effects on the loss of high oleic TAGs (Bouaziz et al., 2010).

3.5. Triacylglycerols Oxidation

Triacylglycerols are oxidized faster in the Rancimat and therefore cannot be correctly correlated with daily cooking. However, it will provide us a hint for the possible reactions of carotenoids in edible oils oxidation during cooking or thermal processing. Figure 2 B shows the total ion chromatogram of the β-carotene containing olive oil oxidized in the Rancimat at 110 °C for 6, 8, 10, 12, 14 h and un-oxidized control sample. Till 6 h, there was no new peak, while at 8 h of thermal oxidation we have identified five new compounds denominated as peaks, c, d, e, f, and h. At 10 h of thermal oxidation we have observed that 10 new compounds are formed. These compounds were designated as peak a-j. At 12 and 14 h the area of these peaks increases and a new peak k was also produced in relatively small amounts. At 14 h the relative percent area of the peak c, f, i, and j were found to increase significantly. In astaxanthin containing olive oil (Figure 2 C), only three peaks (c, e, and f) were identified at 8 h. The number of compounds identified increased to 6 peaks (c, d, e, f, h, and j) after 10 h. The relative amounts of these new peaks increased at 12 and 14 h of thermal treatments, with the appearance of several new peaks (a, b, g, i, and k). The relative peak areas of these peaks were smaller compared to the β-carotene containing sample, which proves that β-carotene containing samples were oxidized more readily than astaxanthin containing olive oils. This also allows us to see which oxidization product contributes to the increase of the POV in the olive oil samples. We did not observe any change in the chemistry of oxidation of olive oil TAGs by the addition of these two carotenoids. However, we observed an increase of oxidation by the addition of β-carotene and decrease or slower oxidation in the presence of astaxanthin.

3.6. Triacylglycerols Oxidation Products

Several classes of oxidation products of TAGs have been identified in the refined olive oil oxidized at 110 °C in the Rancimat. The first eluted oxidized TAGs were identified as OOO-bis-hydroperoxides (peak a) with the m/z of 948.8 as shown in Figure 3A. Unlike our previous work (Zeb & Murkovic, 2010b) we did not observe a primary fragment

coming from the loss of a complete –OOH group or outer –OH of the molecular fragments. The *m/z* 966.8 and *m/z* 971.8 represent ammonium adducts $[M+18]^+$ and sodium adducts $[M+23]^+$ being the prominent ions. The loss of an outer –OH and complete loss of –OOH was observed only in diacylglycerols fragments. These were *m/z* 601.5, 615.5, 619.5, and 641.5, which correspond to LO^+, LO_{ep}^+, SO_{ep}^+, and OO_{OOH}^+ fragments, respectively. Figure 3B shows the ESI-MS spectra of an OOO mono-hydroperoxide (peak g). The fragments at *m/z* 921.8 and 937.0 represent the sodium adducts $[M-H_2O+23]^+$ and potassium adducts $[M-H_2O+39]^+$ of the corresponding epoxide. We also detected a relatively small amount of the protonated molecular ion of mono-hydroperoxide of *m/z* 917.8, which corresponds to $[M+H]^+$. The DAGs fragment ions of *m/z* 603.5 and 617.5 are dioleoyl glycerol and mono-epoxy dioleoyl glycerol ions, respectively. The fragment at *m/z* 465.4 represents the loss of $C_{11}H_{20}$ which indicates the epoxide at position 7 and 8 on the parent ion. These results are highly correlated to the APCI-MS mass spectra of Neff and Byrdwell (1998, 1999) for hydroperoxide. They identified mono-hydroperoxide as oxidized products of standard triolein auto-oxidized at 50-60 °C. This means that triolein mono- and bis-peroxide was originally formed from triolein present in the olive oil. Peak b was identified as OLLn epoxy hydroperoxides, which was formed at a later stage of oxidation.

Figure 3C shows the ESI-MS spectra and extracted ion chromatogram of the OOO hydroxy hydroperoxide (peak c). The fragments at *m/z* 933.8, 950.8, 955.8, and 881.8 correspond to the protonated molecular ion $[M+H]^+$, ammonium adduct $[M+18]^+$, sodium adduct $[M+23]^+$, and a protonated unsaturated OOO with the loss of a hydroxyl and two hydroperoxides groups forming a $[M+H-H_2O-H_2O_2]^+$, respectively. These compounds are highly polar and therefore a higher affinity toward positive ions forming adducts. The position of these functional groups could be at C9 or C10 as shown by the fragments at *m/z* 493.4 and 505.4. Figure 3D shows the ESI-MS spectra with an extracted ion chromatogram of OOO hydroxy epidioxide (peak d). The fragments ions with *m/z* 931.8, 948.8, 953.8, 914.8 and 899.8 were identified to be the protonated molecular ion $[M+H]^+$, ammonium adduct $[M+18]^+$, sodium adduct $[M+23]^+$, $[M-H_2O]^+$, and $[M-H_2O_2]^+$, respectively. The loss of water was also observed in DAG fragment with *m/z* 615.5, while *m/z* 493.4 and 519.4 show the positions of C9 and C11 of the oxygen functional groups on the parent compound, respectively.

Peaks e and f were the epoxy epidioxides of OOO and OOS. The formation and fragmentation mechanism was previously reported (Zeb & Murkovic, 2010a). It has been observed that these compounds were formed in higher amounts as compared to other oxidized compounds. However, the relative amounts were higher in model TAGs and smaller in olive oils under similar conditions of thermal oxidation. The OOS epoxy epidioxides were produced more in β-carotene containing olive oil samples than astaxanthin.

Peaks h, i, j, and k were identified to be the mono-epoxides of OOO, POS, OOS, and SOS respectively. In epoxides, the loss of an oxygen containing functional group may produce an extra unsaturation as previously proposed by Byrdwell and Neff (1998). These authors also identified an OOO epoxide during the auto-oxidation of triolein. In our studies we found that the POS epoxide and OOS epoxide were produced more in β-carotene than astaxanthin containing samples at 14 h of thermal treatment in the Rancimat for 110 °C. In the model TAGs with a similar TAGs composition, we have identified 9 oxidized species of different classes (Zeb & Murkovic, 2010a). With the exception of OOO-hydroperoxides, OOO and OOS epoxy epidioxides, and OOO mono-epoxide, all others were reported for the first time in the refined olive oil. The changes in the chemistry and formation of new compounds in olive oil may be attributed to the presence of added carotenoids, phenolic composition, or the other species interacting during thermal oxidation.

3.7. Effects of Carotenoids on Oxidized Triacylglycerols

We have seen a tremendous increase in the peroxide values by the addition of carotenoids under similar conditions. Figure 4A shows that the formation of OOO hydroxy hydroperoxide (peak c) is highly affected by both carotenoids. The effect of β-carotene is higher at 14 h. In the case of OOO hydroxy epidioxide (peak d), there was no significant effect of astaxanthin, while β-carotene promotes the formation of these compounds. Both OOO and OOS epoxy epidioxides (peak e and f) were produced in relatively large amounts in astaxanthin containing samples as compared to control and β-carotene. However, they are formed in relatively similar amounts in control samples. The formation triolein mono-hydroperoxides (peak g) was higher in β-carotene containing samples, and lowest in astaxanthin. Triolein mono-epoxides (peak h) were produced in

higher amounts in control samples, and its formation was inhibited by both carotenoids. Figure 4B shows the correlation of peroxide values, oxidation time and the relative peak areas of OOO epoxy epidioxide. We found that this oxidized species may contribute toward the oxidation and changes in peroxide value.

4. Conclusions

Carotenoids (β-carotene and astaxanthin) were added to the refined olive oils and oxidized in the Rancimat at 110 °C for 1 to 14 hours. In olive oil TAGs were found to oxidize much faster in the presence of β-carotene than astaxanthin. Similarly β-carotene degradation was much faster than astaxanthin degradation. The isomerization of carotenoids was found to be one of the first reactions occurring during thermal oxidation in oils. Astaxanthin was found to protect the olive oils TAGs till 10 h of thermal treatment. Both carotenoids were found to significantly increase the peroxide values. The TAGs composition of the refined olive oils indicates high levels of oleic acid containing TAGs. A total of 11 TAGs oxidized species were correctly identified. The TAGs oxidized species were mono- and bis-hydroperoxides, epoxy hydroperoxides, hydroxy hydroperoxides, hydroxy epidioxides, epoxy epidioxides, and mono-epoxides. The results illustrate that the pro-oxidant action of β-carotene was much stronger than that of astaxanthin. It is therefore suggested that it could be beneficial to use as antioxidant instead of β-carotene in edible oils exposed to short time heating.

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Fig 1: (A) A typical chromatogram of β-carotene oxidation (1 & 8 h) in olive oil oxidized in the Rancimat at 110 °C. The oxidized products were 5,8-epoxides and 6-apo-β-carotenal. (B) A typical chromatogram of all-*E*-astaxanthin oxidation in olive oil oxidized in the Rancimat at 110 °C. The UV absorption spectra indicate the possible isomers.

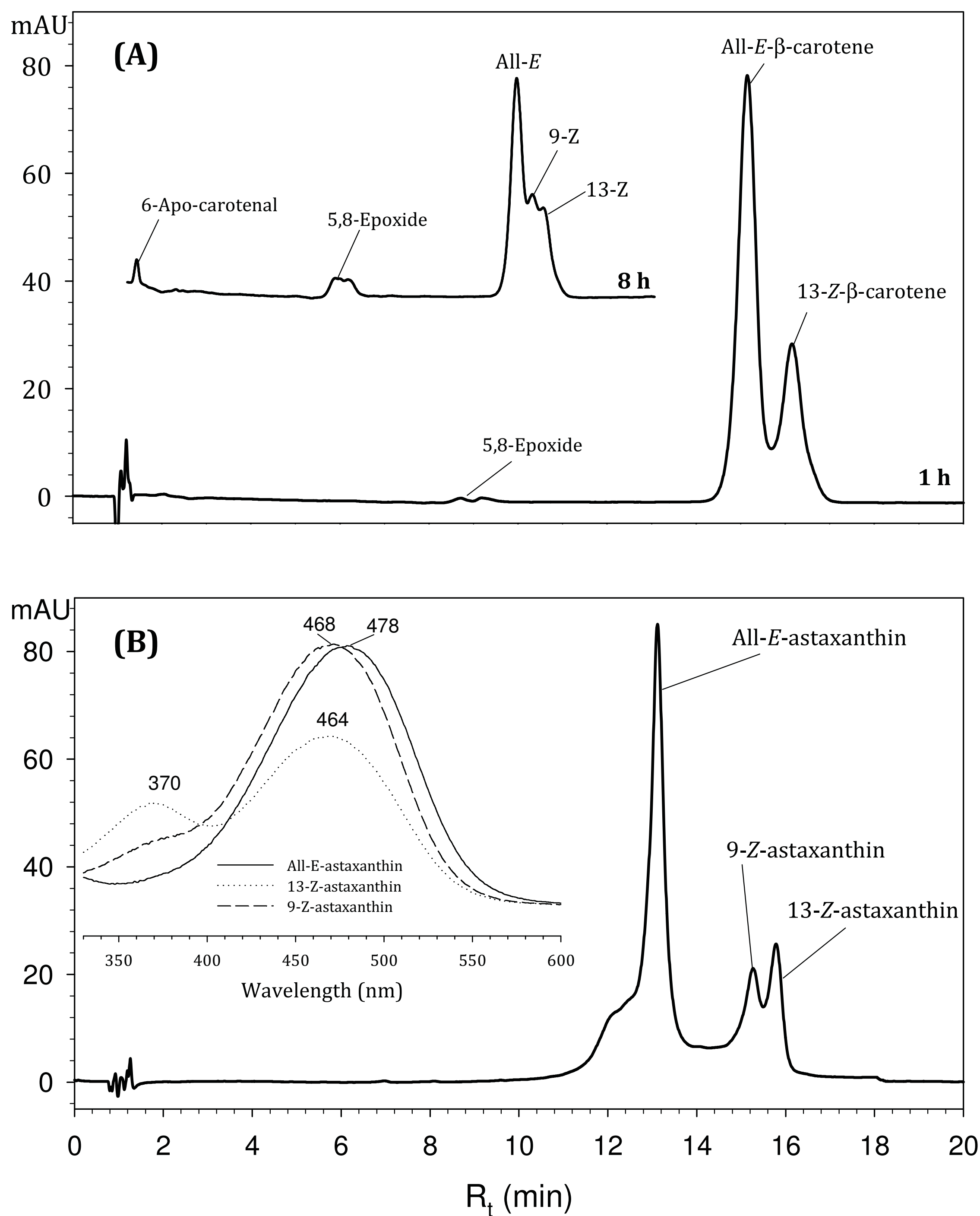


Fig 1: (C) Thermal degradation of β-carotene and astaxanthin in olive oil at 110 °C. (D) Effects of β-carotene and astaxanthin (300 μg/g) on the peroxide value (meq/kg of O_2) of olive oils.

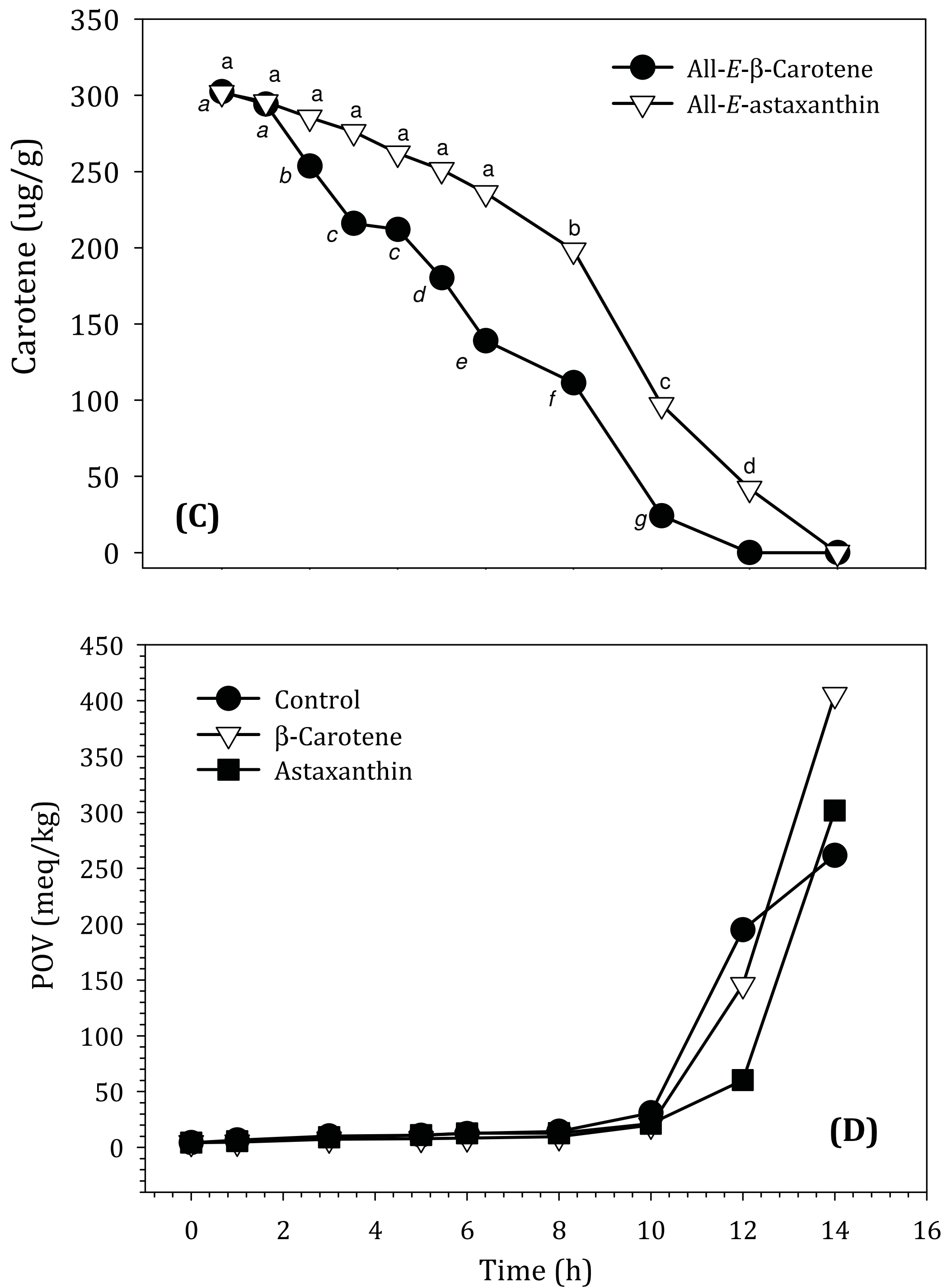


Table 1: Fatty acids composition of refined olive oil.

Common Name	Fatty acid	Quantity (g/100g)
Palmitic acid	*C16:0*	10.8
Palmitoleic acid	*C16:1*	0.75
Stearic acid	*C18:0*	2.68
Oleic acid	*C18:1n-9*	74.3
Vaccenic acid	*C18:1n-7*	1.92
Linoleic acid	*C18:2n-6*	7.72
Linolenic acid	*C18:3n-3*	0.60
Arachidic acid	*C20:0*	0.48
Gadoleic acid	*C20:1*	0.36
Eicosatrienoic acid	*C20:3*	0.03
Behenic acid	*C22:0*	0.16
Lignoceric acid	*C24:0*	0.08

Fig 2: (A) Total ions chromatograms of β-carotene containing olive oils showing control and oxidized samples for 6, 8, 10, 12, and 14 h in the Rancimat at 110 °C. The appearance of new peaks (a-h) is explained in the text.

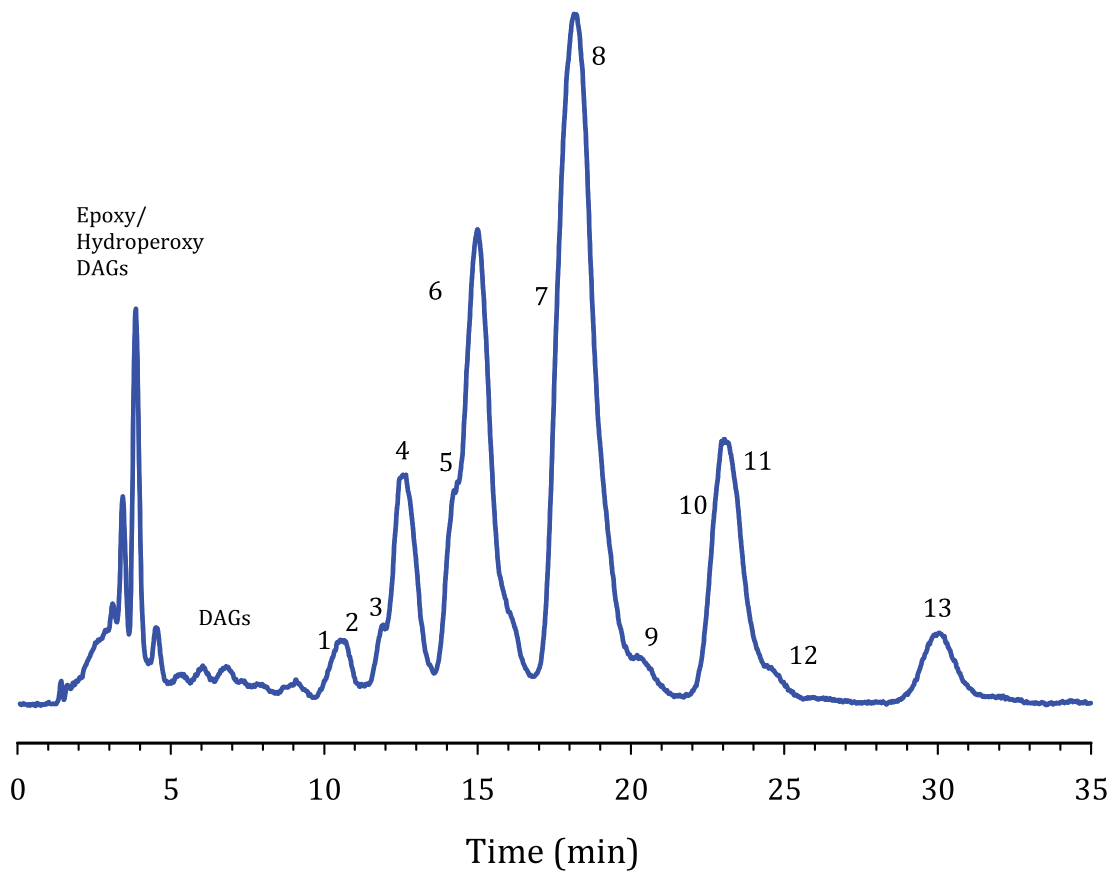


Table 2: Triacylglycerols composition of olive oil determined by ESI-MS.

Peak	R_t (min)	TAG	Peak Area (%)*	$M+H^+$	$M+NH_4^+$	$M+Na^+$	DG Fragments (m/z)		
1	10.0	PoLLn [a]	2.12	853	870	875	PoL 573	PL 575	LLn 597
2	10.8	LLL [a]		879	896	901	LL 599	--	--
3	11.5	PoOL	1.66	855	873	878	LL 599	PL 575	--
4	12.1	OLL	7.33	881	898	903	LL 599	LO 601	--
5	14.5	PLO/POL	6.17	857	875	880	PL 575	PO 577	LO 601
6	15.5	OLO	16.2	883	900	905	LL 599	OL 601	--
7	17.0	POO [b]	35.4	859	877	882	PO577	OO 603	--
8	17.8	OOO [b]		885	902	907	OO 603	--	--
9	20.5	POL_{ep}	2.60	873	890	895	PO 577	PL_{ep} 591	OL_{ep} 617
10	22.8	OSO [c]	11.5	887	904	909	OO 603	OS 605	--
11	23.2	POS [c]		861	879	884	SO 605	PS 579	PO 577
12	23.6	ALO	1.88	913	931	936	LO 601	AL 631	AO 633
13	28.7	AOO	3.83	912	933	937	AO 633	OO 603	--

* Epoxy DAGs (4.51 %) and normal DAGs (2.62 %). Similar letters (a-c) in the column represent co-eluting peaks.

Fig 2: (B) Total ions chromatograms of astaxanthin containing olive oils showing control and oxidized samples for 6, 8, 10, 12, and 14 h in the Rancimat at 110 °C. The appearance of new peaks (a-h) is explained in the text.

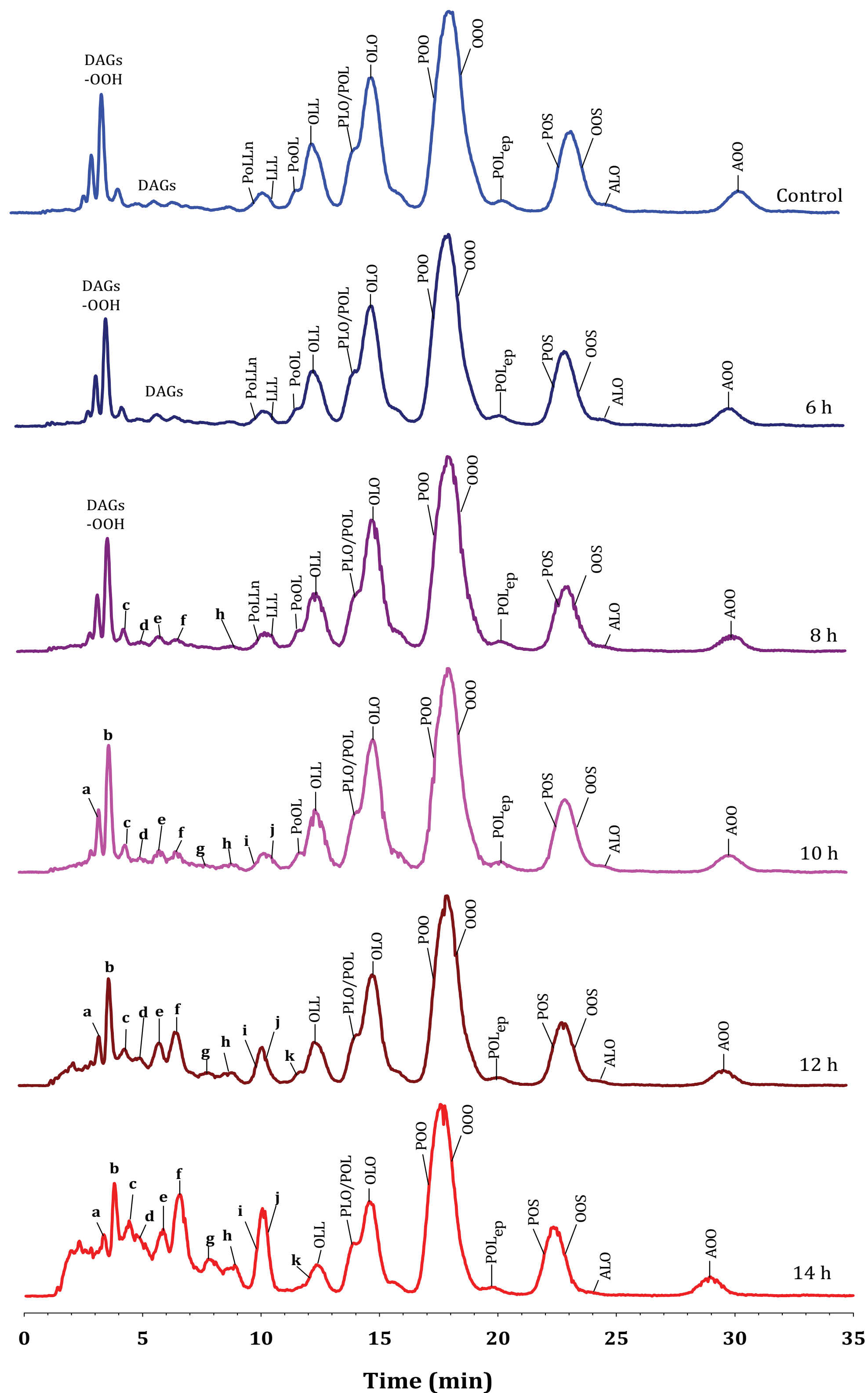


Fig 2: (C) Total ions chromatograms of astaxanthin containing olive oils showing control and oxidized samples for 6, 8, 10, 12, and 14 h in the Rancimat at 110 °C. The appearance of new peaks (a-h) is explained in the text.

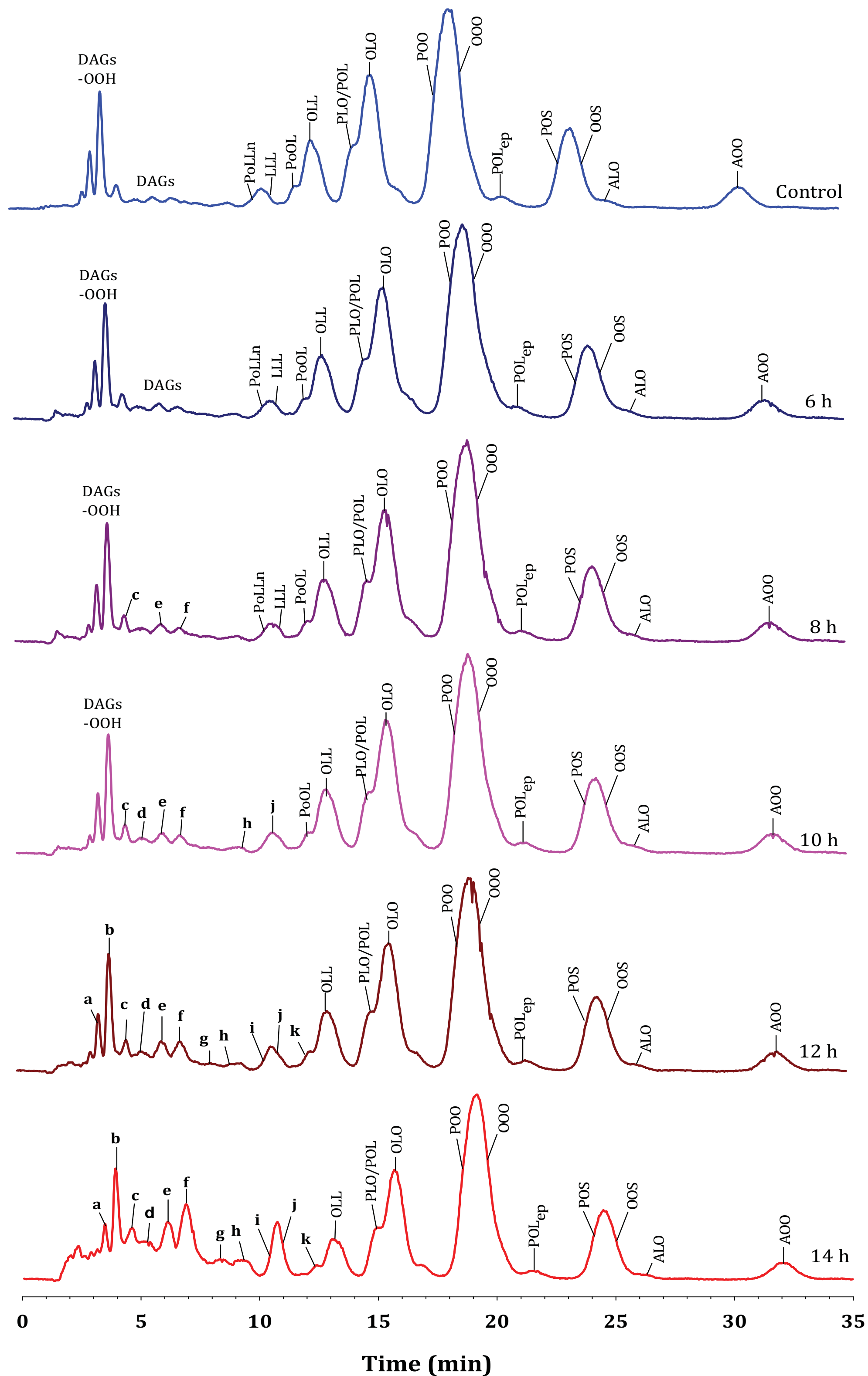


Fig 3: ESI-MS spectra of oxidized TAGs, (A) OOO bis-hydroperoxides and (B) OOO mono-hydroperoxides.

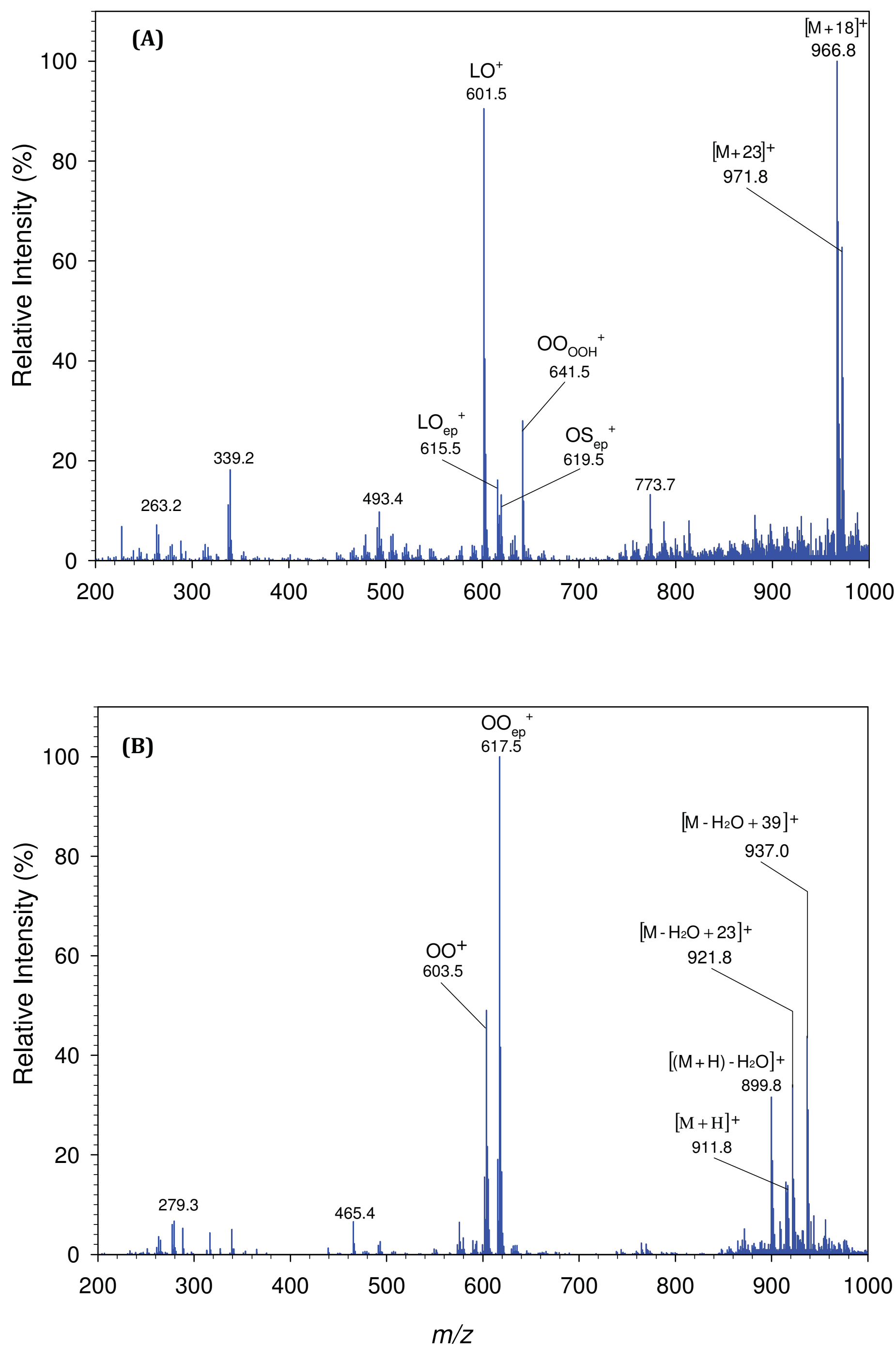


Fig 3: (C) Extracted ion chromatograms of *m/z* 950.8 and ESI-MS spectra of OOO-hydroxy hydroperoxide (*m/z* 932.8).

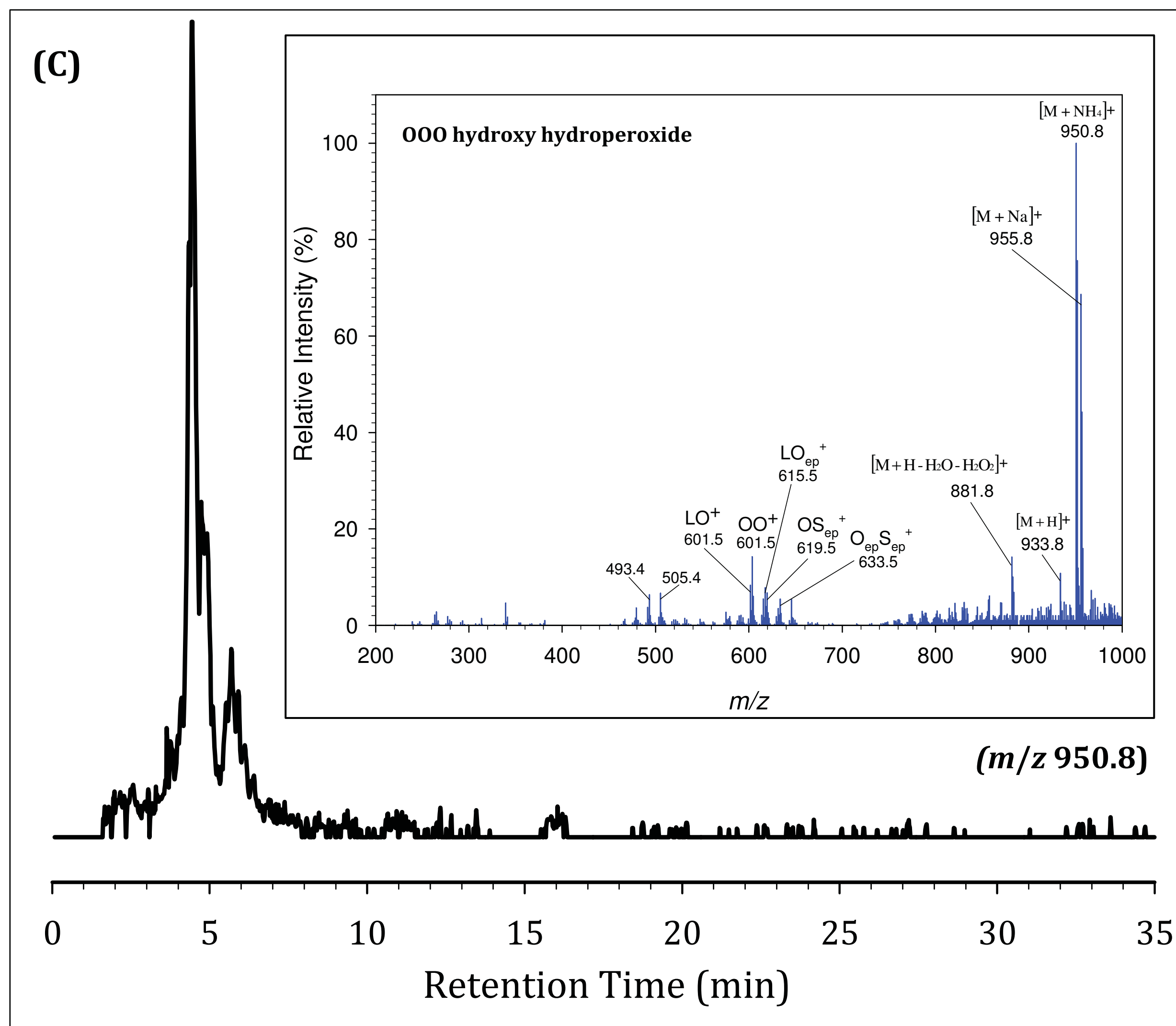


Fig 3: (D) Extracted ion chromatograms of the *m/z* 948.8 and ESI-MS spectra of OOO-hydroxy epidioxide (*m/z* 930.8).

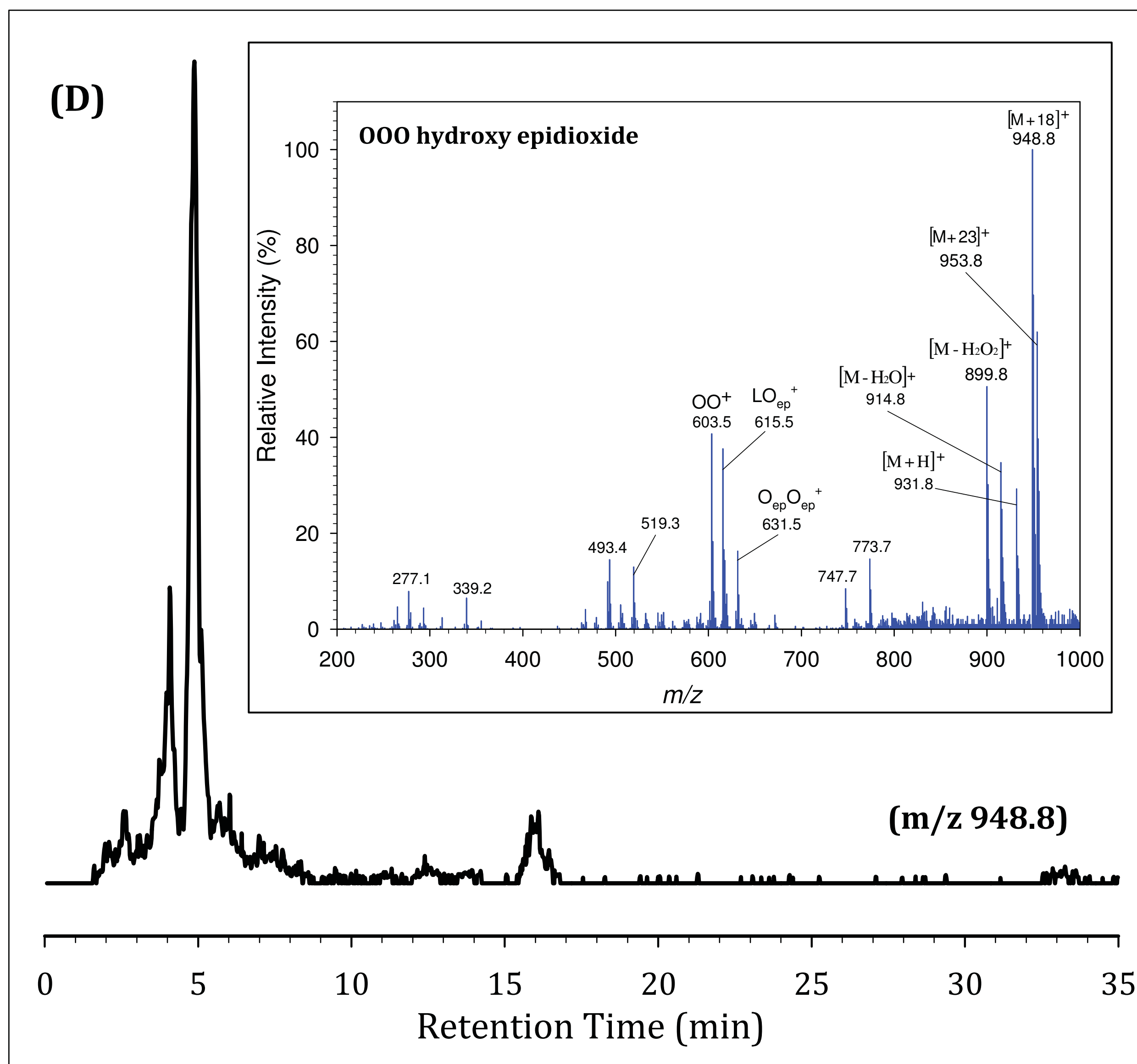


Fig 4: Carotenoids and oxidized triacylglycerols interactions. (A) Effects of carotenoids on the formation of oxidized TAGs (peaks a to k is explained in the text).

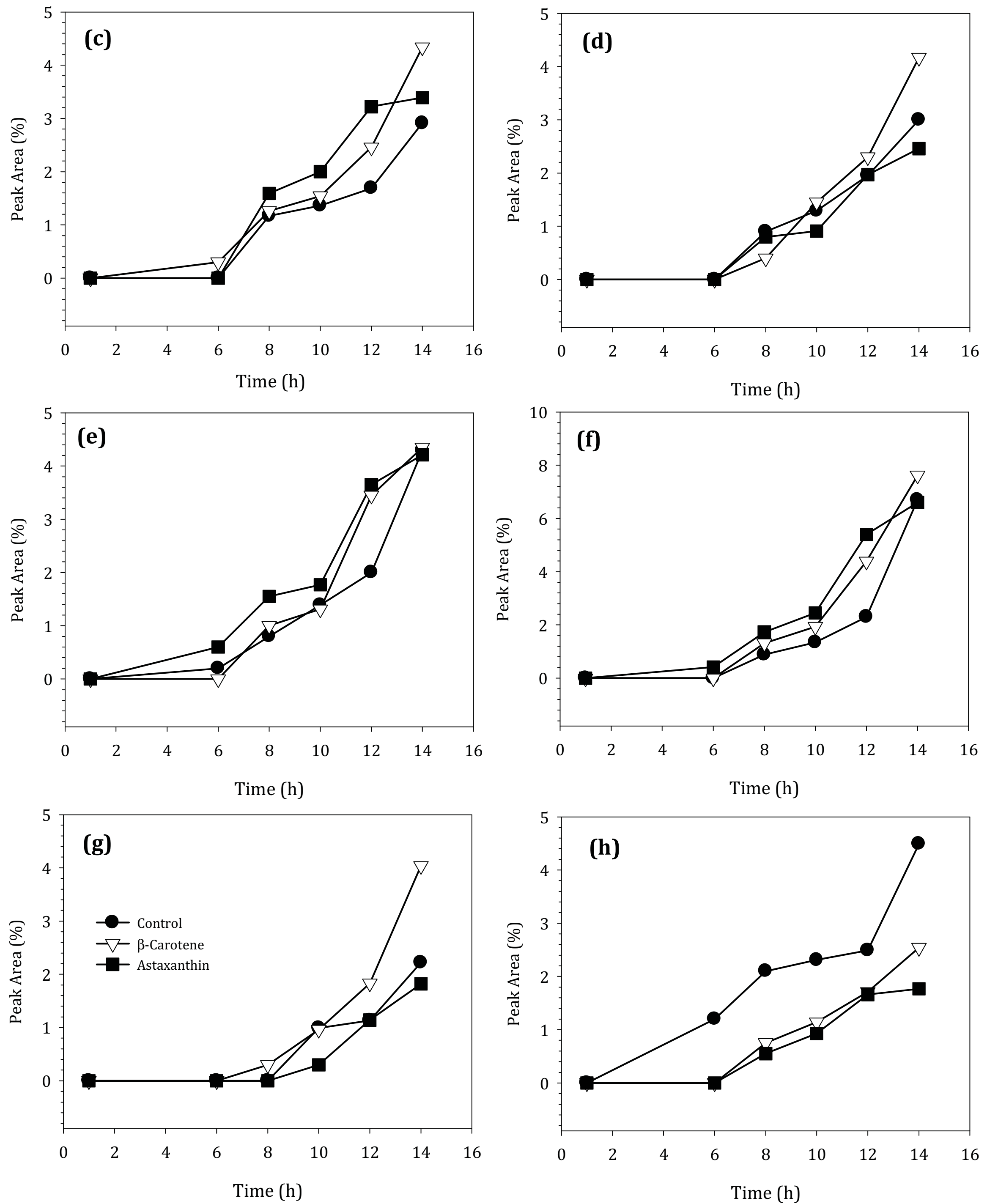
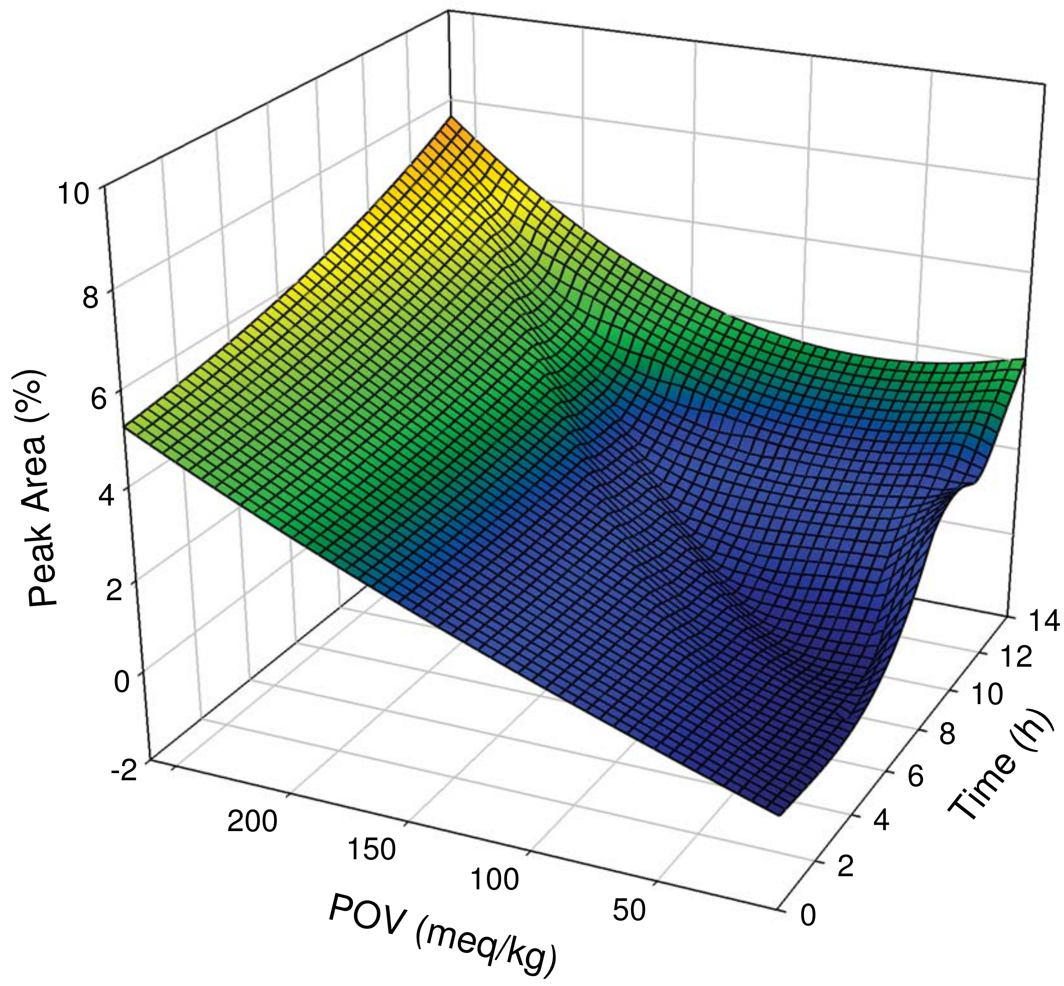


Fig 4: Carotenoids and oxidized triacylglycerols interactions. (B) Correlation of OOO epoxy-epidioxides with β-carotene and oxidation time.



3. CONCLUSIONS

The results presented in this dissertation demonstrate the role of carotenoids in triacylglycerols oxidation under the selected experimental conditions. Both carotenoids (β-carotene and astaxanthin) were added to high oleic model triacylglycerols and edible oils such as corn, rapeseed, and olive oils, and oxidized in the Rancimat. Carotenoids degradation and oxidation were studied using HPTLC and HPLC methods. Triacylglycerols and its oxidation products were studied using HPLC-ESI-MS method. The following conclusions are based on the results presented as papers and manuscripts.

1) The review of literature on the uses of TLC suggests that HPTLC have the potential to be the first choice in the analysis of carotenoids in foods. TLC coupled to mass spectrometry or scanning densitometry or image analysis could provide a fast analytical tool for the separation and characterization of carotenoids (I). We found HPTLC is a beneficial tool in the study of oxidation of β-carotene in model triacylglycerols and edible oils (II).

2) The isocratic HPLC-ESI-MS method was very useful for the fast screening and identification of triacylglycerols in edible oils. The presence of intense protonated molecular ($M+H^+$), ammonium ($M+NH_4^+$), and sodium ($M+Na^+$) adducts ions and their respective diacylglycerols ions in the ESI-MS spectra showed correct identification of TAGs. We correctly identify and separated thirteen; fourteen, fifteen and sixteen TAGs in refined olive oil, rapeseed oil, corn oil and sunflower oil, respectively (III).

3) The HPLC-ESI-MS method was also very useful in studying the oxidation products of TAGs. Epoxy epidioxides were identified as major oxidized compounds that have been identified for the first time in model TAGs (III) and olive oil (VI) under similar conditions. Among the oxidized species of TAGs, hydroxy hydroperoxides, mono-hydroperoxides, bis-hydroperoxides, epoxy-epidioxides, and epoxides were the major compounds identified (IV). In corn oil, hydroxy bis-hydroperoxides and epidioxy bis-hydroperoxides were the new classes of oxidized compounds identified (V).

4) Significant degradation of β-carotene was observed in sunflower oil (II). In high oleic model TAGs, β-carotene degraded significantly in the first three hours (IV),

however, in olive oil of relatively similar TAGs composition, β-carotene degraded slowly (VI). While in olive oil astaxanthin degradation is much slower than β-carotene.

5) An HPLC method for the degradation and oxidation of carotenoids reveals a total of eight oxidized compounds of β-carotene in corn oil. The degradation of all-*E*-β-carotene in corn oil was relatively similar to model TAGs (IV) and olive oil (VI).

6) The interactions of carotenoids and triacylglycerols reveals the pro-oxidant actions of both carotenoids studied. The pro-oxidant action of β-carotene is much stronger than astaxanthin (VI).

7) Finally, the findings presented in this dissertation help us to understand the structural characterization of TAGs using mass spectrometry and the possible role and interactions of carotenoids or its oxidation products with the normal and oxidized TAGs during thermal oxidation.

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2007	MPhil (Biochemistry) *Institute of Chemical Sciences, University of Peshawar, Pakistan*
2001	MSc (Biochemistry) *Department of Chemistry, University of Peshawar, Pakistan*
1999	BSc (Biology, Chemistry) *University of Peshawar*

Teaching

- Lecturer Biochemistry, University of Malakand, Pakistan (2003 - Present)
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Research

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- MPhil Research Student, Institute of Chemical Sciences, Univeristy of Peshawar, (2003-2007).
- Research Assistant at Nuclear Institute for Food & Agriculture, Tarnab (NIFA) Peshawar (2001)

Fellowships

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Conferences Presentations during This Dissertation

Oral Presentations

1. *Ist European Food Congress*, 4-9 November 2008, Ljubljana, Slovenia.
2. TEMPUS Joint European Project '*New Bologna Oriented Doctoral Study Programme in BA*'. 26-30 January 2009, Graz Austria.
3. *Young Investigators' Seminar on Analytical Chemistry*, 29 June-2 July 2009, Graz Austria.
4. *Österreichische Lebensmittel Chemikertage*, 19-21 May 2010, Schloss Seggau, Austria.
5. *4th Doctoral Day*, *NAWI Graz Doctoral School of Molecular Biosciences and Biotechnology*, 9th July 2010, Graz, Austria.
6. *8th Euro Fed Lipid Congress, Fats, Oils and Lipids*, 21-24 November 2010, Munich, Germany.

Poster Presentations

1. *Österreichische Lebensmittel Chemikertage*, 28-30 May 2008, Eisenstadt, Austria.
2. *9th International Symposium on Instrumental analysis*, 29 June-2 July 2008, Pecs Hungary.
3. *3rd International IUPAC Symposium on Trace Elements in Foods*, 1-3 April 2009, Rome Italy.
4. *7th Euro Fed Lipid Congress, Lipids, Fats and Oils*, 18-21 October 2009, Graz Austria.
5. *Ist Styrian Conference on Lipid Mass Spectrometry*, 21-22 October 2009, Graz Austria.
6. *Österreichische Lebensmittel Chemikertage*, 19-21 May 2010, Schloss Seggau, Austria.