DETERMINATION OF EPOXIDES AND ALCOHOLS IN EDIBLE OILS

by

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Submitted in partial fulfilment of the requirements
for the degree Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
August 2017

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ABSTRACT

Lipid oxidation refers to the oxidative degradation of lipids, leading to toxicity and rancid flavours of lipid-containing foods. This process is often measured by determining hydroperoxides and their breakdown products, such as short-chain volatiles and unsaturated carbonyls. The formation of epoxides and alcohols during lipid oxidation was described decades ago but only a few studies have reported their concentrations in oils and foods. As are all lipid oxidation products, epoxides and alcohols are present at very low concentrations so their quantification requires suitable sensitivity and selectivity of the analytical method employed. In this thesis, a ^{1}H nuclear magnetic resonance (NMR) method and a gas chromatography (GC) method are presented to quantify epoxides and alcohols in edible oils.

The ^{1}H NMR chemical shifts of epoxides were examined by epoxidation of triacylglycerol standards and oils. For quantification, the signals of glycerol protons were taken as the internal standard. The method was successfully applied to quantify epoxides formed in soybean oil oxidized at 100 ºC. The experimental procedures are simple and rapid and can be easily applied to other types of vegetable oils.

The GC method was developed to monitor epoxy and hydroxy fatty acids in vegetable oils, determining both saturated and unsaturated structures. The sample preparation employed transmethylation, solid-phase extraction (SPE), and trimethylsilyl derivatization. GC-mass spectrometry (GC-MS) enabled identification of the oxidation products, while quantification was carried out with GC-flame ionization detection (GC-FID) and GC-MS with selected ion monitoring. The new method was used to characterize and quantify individual epoxy and hydroxy fatty acids in sunflower and canola oils oxidized at 40 ºC.

The sensitivity of the GC method is superior to the ^{1}H NMR technique but is more time-consuming, mainly due to the SPE step. The ^{1}H NMR method is convenient but is limited by its sensitivity so it does not allow determination of epoxides in fresh or mildly oxidized oils. Overall, the ^{1}H NMR method is suitable for rapid analytical purposes while the GC method is appropriate for stability studies under mild oxidation conditions. Future work can apply these methods to monitor epoxides and alcohols to study competitive oxidation pathways.

LIST OF ABBREVIATIONS AND SYMBOLS USED

3-pyridylcarbinol	3-hydroxymethylpyridinyl
ACN	acetonitrile
ANCOVA	analysis of covariance
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists' Society
APCI	atmospheric pressure chemical ionization
BSTFA	N,O-bis (trimethylsilyl) trifluoroacetamide
CFIA	Canadian Food Inspection Agency
CI	chemical ionization
CI-MS	chemical ionization-mass spectrometry
COSY	correlation spectroscopy
DAG	diacylglycerol
DMOX	4,4-dimethyloxazoline
DTC	N,N-diethyldithiocarbamate
EI	electron ionization
EIC	extracted ion chromatogram
EI-MS	electron ionization-mass spectrometry
ELSD	evaporative light-scattering detection
ES	epoxystearate
ESI	electrospray ionization
eV	electron volt
FAME	fatty acid methyl ester
FID	flame ionization detection
FTICRMS	Fourier transform ion cyclotron resonance mass spectrometry
FTIR	Fourier transform infrared spectroscopy
GC	gas chromatography
GC-FID	gas chromatography-flame ionization detection
GC-MS	gas chromatography-mass spectrometry
GC-MS-SIM	gas chromatography-mass spectrometry-selected ion monitoring

GOED	Global Organization for EPA and DHA
HBr	hydrogen bromide
HFBA	heptafluorobutyrate anhydride
HIA	hydride ion affinity
HODE	hydroxyoctadecadienoic acid
HPLC	high-performance liquid chromatography
HPLC-APCI	high-performance liquid chromatography-atmospheric pressure chemical ionization
HPLC-ESI	high-performance liquid chromatography-electrospray ionization
HPLC-ELSD	high-performance liquid chromatography-evaporative light-scattering detection
HPLC-MS	high-performance liquid chromatography-mass spectrometry
HPLC-UV	high-performance liquid chromatography-ultraviolet/visible detection
IR	infrared
IUPAC	International Union of Pure and Applied Chemistry
LC-MS	liquid chromatography-mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MAG	monoacylglycerol
MALDI-FT MS	matrix assisted laser desorption ionization-Fourier transform mass spectrometry
MALDI-TOF MS	matrix-assisted laser desorption ionization-time-of-flight mass spectrometry
ME	methyl ester
MS	mass spectrometry
MSE	mean squared error
mCPBA	3-chloroperoxybenzoic acid
MS/MS	tandem mass spectrometry
MS^n	multistage mass spectrometry (n=3 or 4)
MS-SIM	mass spectrometry-selected ion monitoring
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
MTO	methyltrioxorhenium

m/z	mass to charge ratio
NARP-LC/MS	non-aqueous reversed-phase liquid chromatography/mass spectrometry
NBP	4-(*p*-nitrobenzyl) pyridine
NCI	negative chemical ionization
NMR	nuclear magnetic resonance
NP-HPLC	normal-phase high-performance liquid chromatography
OTMS	trimethylsiloxy
PA	proton affinity
pAV	*p*-anisidine value
PCI	positive chemical ionization
PFB	pentafluorobenzyl
PFBHA	pentafluorobenzyl hydroxylamine
PFB oxime	O-2,3,4,5,6-pentafluorobenzyl oxime
PUFA	polyunsaturated fatty acid
PV	peroxide value
R_f	retention factor
RP-HPLC	reversed-phase high-performance liquid chromatography
RSD	relative standard deviation
SD	standard deviation
SIM	selected ion monitoring
SPE	solid-phase extraction
TAG	triacylglycerol
TBARS	2-thiobarbituric acid-reactive substances
TIC	total ion chromatogram
TLC	thin layer chromatography
TMS	trimethylsilyl
TSP	thermospray ionization
UHPLC	ultra-high performance liquid chromatography
UV-Vis	ultraviolet-visible

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Sue Budge, for your guidance in my research and everything you have done for me. Thank you for always making time for me no matter how busy you are. Also, I'm grateful for the supports and advices from my supervisory committee, Dr. Gianfranco Mazzanti and Dr. Jenna Sullivan Ritter. I appreciate Dr. Mike Lumsden for his knowledge and help in my NMR work and for his patience and kindness all the times. I want to thank Dr. Karen M. Schaich for her knowledge and advice on my research and writing.

Special thanks go to everyone who is/has been in the Marine Lipids Lab and everyone on the third floor. This program is not just a pursuit of knowledge. It is a journey in my life. Every person I have met during this period of time has contributed to my personal growth. Many thanks to my dear friends I met in Halifax for your accompany on this journey.

Finally, I want to say thank you to my parents and my other family members for all your love and supports. And to my best friends in Wuhan, thanks for always being there for me.

CHAPTER 1 INTRODUCTION

1.1 Dissertation Overview

Lipids are one of the major components in foods, along with water, carbohydrates, and proteins (Damodaran, Parkin, & Fennema, 2008). They include a wide variety of organic compounds - fatty acids and their derivatives, steroids, carotenoids, etc. (Christie, 2003). Lipids had been considered as a group of compounds soluble in organic solvents by Christie (1982), Kates (1986), and Gurr and James (1971), as cited in O'Keefe (2002), but such a definition of lipids based on solubility remains controversial, and a precise definition of lipids does not exist (O'Keefe, 2002), because lipids encompass so many different structures. In foods, the major lipid ingredients are understood as fats and oils (Frankel, 2005). The Canadian Food Inspection Agency (CFIA) has defined fats as 'total lipid fatty acids expressed as triglycerides' (CFIA, 2014).

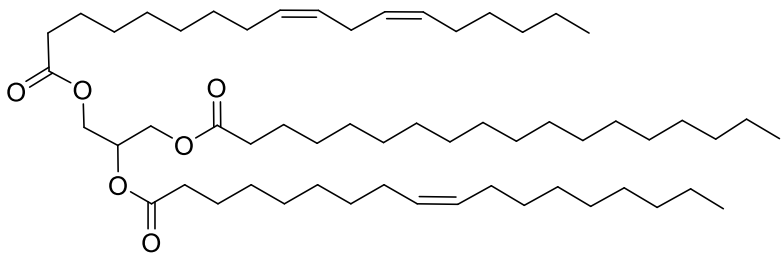


Figure 1.1 Chemical structure of a TAG molecule.

Triacylglycerols (TAGs), also called triglycerides, are glycerol molecules esterified with three fatty acid chains (Figure 1.1). Fats and oils are generally referred to as TAG extracts (>95%) from plants or animals, which may also contain small amounts

of other compounds such as diacylglycerols (DAGs), monoacylglycerols (MAGs), sterols, etc. (Gunstone, 2004). Oils are defined as TAGs that remain in a liquid state at room temperatures while fats are usually solid under the same conditions (Stauffer, 1996). The contributions of dietary fats and oils to human nutrition include providing sources of calories and essential fatty acids and carrying vitamins A, D, E, and K etc. (Stauffer, 1996; Kritchevsky, 2002). Also, they add desired flavours and textures to foods (Stauffer, 1996).

Edible oils are normally named by the original sources of the TAG extracts - vegetable oils are mainly derived from beans and seeds (Gunstone, 2011), and marine oils are fats extracted from marine animals. The various types of oils show differences in their physical, chemical, and nutritional properties due to the chemical compounds present in the oils, of which the primary constituents are TAGs (O'Brien, 2009). Different types of edible oils vary in their fatty acid compositions in TAGs. When discussing fatty acids in oils, they are described as if they are in free forms; however, in TAGs, fatty acids are esterified to the glycerol backbone.

The fatty acids in TAGs can be either saturated or unsaturated. Unsaturated fatty acids are divided into monounsaturated fatty acids, containing only one double bond, and polyunsaturated fatty acids (PUFAs), with more than one double bond. Due to the double bonds in their structures, unsaturated fatty acids are susceptible to lipid oxidation. When lipid-containing foods are exposed to oxygen and radical initiators, such as high temperatures (e.g. in frying), metals, and light, oxidation can quickly take place (Omwamba, Artz, & Mahungu, 2011). Oxidation of fatty acids in TAGs results in the formation of toxic compounds and rancid flavours of oils and lipid-containing foods so it

is considered one of the major reactions affecting the shelf-life of oils and foods (Gordon, 2004).

Many kinds of oxidation products may be generated from a single fatty acid, including hydroperoxides, carbonyls, conjugated dienes, epoxides, and alcohols. Short-chain products released from fatty acids in TAGs may be volatile and contribute to rancidity (Frankel, 1983). Products remaining bound in the TAGs, referred to as "core" products, are too large to be volatile. However, hydroperoxides and unsaturated carbonyls bound to TAGs can undergo further reactions and generate more volatile compounds even while they remain bound to TAGs (Frankel, 2005). In addition to the volatile products, common oxidation products, such as hydroperoxides and epoxides, are present in the oils with no smell or taste, but they can be absorbed by the human body (Kubow, 1990; Wilson et al., 2002; Frankel, 2005). Dietary oxidized lipids absorbed by animals can lead to a loss of appetite, harmful effects on blood circulation and inflammation, accumulation of secondary peroxidation products in the liver, and the formation of carcinogenic compounds (Kanazawa, Kanazawa, & Natake, 1985; Kubow, 1990; Frankel, 2005; Zamora & Hidalgo, 2008).

Conjugated dienes are the first primary oxidation products arising from PUFAs. Conjugated dienes are generated when the first radical forms and double bonds rearrange to stabilize the radicals (Frankel, 2005). This important indicator of lipid oxidation is monitored in most studies because it is easily followed by its optical absorbance in the 231-235 nm range (Schaich, 2016).

The measurement of lipid oxidation has been particularly focused on the formation of hydroperoxides, because hydroperoxides have been recognized as a primary

lipid oxidation product and their decomposition has always been considered an important precursor of secondary oxidation products (Frankel, 1984; Frankel, 1987). Because oils are mixtures of TAGs with different fatty acids, hydroperoxide concentrations traditionally have been measured on a per kg basis, relative to weight rather than molarity, and expressed as Peroxide Values (PVs). The most established method to measure PVs – iodometric titration – has methods standardized internationally by nearly every major analytical organization (e.g. AOCS Method Cd 8-53, IUPAC 2.501, AOAC 965.33). It requires minimal equipment, can be handled in the most limited laboratories, and is physically easy to perform. At the same time, the method has also been viewed with skepticism because it has low sensitivity (minimum detection ~ 0.5 meq/kg lipid) and is notoriously "empirical", requiring special care and exact conditions to yield reproducible results. Thus, optical methods that can detect μmol levels of hydroperoxides are increasingly being applied. Examples of such methods are the xylenol orange and ferric thiocyanate assays (Schaich, 2016).

A number of methods have also been successfully applied to measure secondary oxidation products, such as unsaturated aldehydes and volatiles (Barriuso, Astiasarán, & Ansorena, 2013).

Although it seems that lipid oxidation can be described by monitoring these oxidation products, some recent studies suggest that the reaction scheme based on hydroperoxide decomposition, which has been followed for a number of decades, may not be a comprehensive description of lipid oxidation (Schaich, 2012). Hence, accurate assessment of the extent of lipid oxidation requires measurement of many more products

than just hydroperoxides and volatiles. Two products of alternate pathways that have been notably missing from analyses are lipid epoxides and alcohols (Schaich, 2012).

Even though epoxides and alcohols have long been discussed as oxidation products (Frankel et al., 1977), they have seldom been measured in edible oils until recent years (Velasco, Berdeaux, Márquez-Ruiz, & Dobarganes, 2002; Marmesat, Velasco, & Dobarganes, 2008; Morales, Dobarganes, Márquez-Ruiz, & Velasco, 2010). The information regarding the occurrences of epoxides and alcohols in oxidized lipids has been very limited in the literature, mainly due to the limitations of the currently available methods. Therefore, the overall goal of this thesis is to develop methods to measure epoxides and hydroxy fatty acids in edible oils to enable the study of the formation of minor compounds during lipid oxidation.

The specific objectives of the study are the following:

1) Review the previous analytical methods used to quantify the minor oxygenated products in oils and foods (Chapter 2);

2) Characterize and quantify epoxides in oxidized oils using ^{1}H nuclear magnetic resonance (NMR) (Chapters 3 and 4);

3) Develop a method to qualitatively and quantitatively determine epoxy and hydroxy fatty acids in oxidized oils using gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detection (GC-FID) (Chapters 5 and 6).

CHAPTER 2 TECHNIQUES FOR THE ANALYSIS OF MINOR LIPID OXIDATION PRODUCTS DERIVED FROM TRIACYLGLYCEROLS: EPOXIDES, ALCOHOLS, AND KETONES

2.1 Publication Information

This chapter is a modified version of a manuscript, originally published as:

Xia, W., & Budge, S. M. (2017). Techniques for the Analysis of Minor Lipid Oxidation Products Derived from Triacylglycerols: Epoxides, Alcohols, and Ketones. *Comprehensive Reviews in Food Science and Food Safety*, *16*(4), 735-758.

2.2 Abstract

Lipid oxidation can lead to flavour and safety issues in fat-containing foods. In order to measure the extent of lipid oxidation, hydroperoxides and their scission products are normally targeted for analytical purposes. In recent years, the formation of rarely monitored oxidation products, including epoxides, alcohols, and ketones, has also raised concerns. These products are thought to form from alternative pathways that compete with chain scissions, and should not be neglected. In this review, a number of instrumental techniques and approaches to determine epoxides, alcohols, and ketones are discussed, with a focus on their selectivity and sensitivity in applications to food lipids and oils. Special attention is given to methods employing GC, high-performance liquid chromatography (HPLC), and NMR. For characterization purposes, GC-MS provides valuable information regarding the structures of individual oxidized fatty acids, typically as methyl esters, isolated from oxidized TAGs, while the use of liquid chromatography–

mass spectrometry (LC-MS) techniques allows analysis of intact oxidized TAGs. For quantitative purposes, traditional chromatography methods have exhibited excellent sensitivity, while spectroscopic methods, including NMR, are superior to chromatography for their rapid analytical cycles. Future studies should focus on the development of a routine quantitative method that is both selective and sensitive.

2.3 Introduction

The interaction of lipids with oxygen results in a series of complex reactions. The reactions are promoted or facilitated by heat, light, transition metals, and oxygen (McClements & Decker, 2008). Lipid molecules with double bonds, such as unsaturated fatty acids, are particularly susceptible to oxidative degradation. TAGs containing unsaturated fatty acids and present as oils and fats in foods decompose during lipid oxidation and produce off-flavours associated with oxidative rancidity. More concerning than off-aromas are other oxidation products produced at the same time, which lead to toxicity (Kubow, 1990). Thus, oxidative deterioration is one of the major concerns in food spoilage leading to the poor quality and flavours of lipid-containing foods.

Lipid oxidation is a complex process, creating real challenges when evaluating the exact extent and monitoring the progress of lipid oxidation. Previous studies that measure lipid oxidation have been conducted by evaluating reactants, intermediates, and products, and include measurement of oxygen, such as atmospheric oxygen and reactive oxygen species (Pénicaud, Peyron, Gontard, & Guillard, 2012; Roman, Courtois, Maillard, & Riquet, 2012), detection of changes in concentrations of lipid reactants (Tyagi &

Vasishtha, 1996; Kim, Yeo, Kim, Kim, & Lee, 2013), and determination of oxidation products, including primary and secondary products (Barriuso et al., 2013).

A large number of studies have measured lipid oxidation products. Although a wide variety of products are involved, hydroperoxides are among the most commonly determined, with results reported as PV. Hydroperoxides are formed from peroxyl radicals directly through hydrogen abstraction; thus, they are considered to be primary lipid oxidation products and important indicators of the extent of oxidative degradation of foods. For the study of PUFAs, where more than one double bond is involved in a chain, the formation of hydroperoxides is accompanied by conjugated structures as a result of rearrangement of double bonds (Barriuso et al., 2013). Therefore, conjugated dienes are also primary lipid oxidation products and are sometimes used to evaluate the extent of lipid oxidation; however, they are not formed with oxidation of monounsaturated fatty acids, such as oleic acid. Other methods to measure lipid oxidation, such as *p*-anisidine value (pAV), 2-thiobarbituric acid-reactive substances (TBARS), and chromatographic analysis of volatiles (Ross and Smith 2006; Barriuso et al., 2013), are widely applied to determine secondary lipid oxidation products which are thought to form from hydroperoxides through fatty acid chain cleavages. Polymers are monitored as the products of the termination stage of lipid oxidation, where recombination of radicals occurs (Neff, Frankel, & Fujimoto, 1988).

In the absence of antioxidants, oxidative degradation of lipids occurs rapidly. Therefore, antioxidants, such as tocopherols, are often added to foods to protect unsaturated lipids from oxidation. Phenolic compounds and ascorbic acid are the most important natural antioxidants in foods, but carotenoids and phospholipids have also

shown antioxidant effects (Choe & Min, 2009). The presence of antioxidants at low concentrations in food inhibits oxidation, thereby increasing the shelf-life of foods (Gülçin, 2012).

Normally the activities of antioxidant compounds are investigated by measuring the inhibition of lipid oxidation in the presence of antioxidant, compared to control samples without antioxidant added. AOCS Method Cg 7-05 for assessing the efficacy of antioxidants recommends using at least two methods for analyzing lipid oxidation (conjugated dienes, hydroperoxides, TBARS, pAV, volatiles) to determine inhibition because none of these tests provides a complete assessment of lipid oxidation. For instance, hydroperoxides are not stable and are easily decomposed or go through further reactions to form other products and radicals, which may result in missing information in the propagation stage. Tests such as pAV, TBARS, and volatile analysis monitor short-chain carbonyl compounds produced in the propagation stage of lipid oxidation. Thus, traditional tests are limited and only determine the formation of hydroperoxides and their decomposition products through scission in the chain, with little regard to other possible products derived from peroxyl radicals directly, for example, epoxides, or from hydroperoxides through pathways other than scission, such as ketones, alcohols, epoxides.

Reactions involving lipid oxidation products from hydrogen abstraction and rearrangement are rarely discussed in the literature. Formation of these alternate products may be strongly directed by antioxidants. For example, high concentrations of α-tocopherol accelerated the formation of keto- and hydroxy-compounds during the induction period of oxidation (Kamal-Eldin, Mäkinen, Lampi, & Hopia, 2002). A more

recent study discovered that the presence of either α- or δ- tocopherol caused higher levels of keto dienes than the controls (Marmesat, Morales, Ruiz-Méndez, Márquez-Ruiz, & Velasco, 2016). These effects would not be observed in common studies of antioxidants using only chemical tests such as PV or TBARS.

That concentrations of these oxidation products, epoxides, alcohols, and ketones, have rarely been reported when studying lipid oxidation in foods is due largely to a lack of suitable and applicable methods to determine those products. In this review, special focus is given to the formation of epoxides, alcohols, and ketones during lipid oxidation and to the available analytical methods for determination of these rarely monitored oxygenated lipid oxidation products.

2.4 Mechanism: Formation of Epoxides, Alcohols, and Ketones

Lipid oxidation in food is usually thought to follow a radical chain mechanism. The classic chain reaction mechanism of lipid oxidation includes three stages: initiation, propagation, and termination. In the initiation stage, initiators such as metals, light, or heat generate the *ab initio* (first) radicals at or near the double bond (Schaich, 2005).

$$LH \rightarrow L^{\bullet} \quad (1)$$

Some initiators remove electrons from oxygen to form singlet oxygen or oxygen radicals that can react directly with double bonds, unlike ground state oxygen (Min & Boff, 2002).

In the propagation stage, the radical chain becomes established and self-perpetuating. First, oxygen adds to $L^{\bullet}$ (relatively unreactive) to form reactive peroxyl radicals.

$$L_1^{\bullet} + O_2 \rightarrow L_1OO^{\bullet} \quad (2)$$

These peroxyl radicals then abstract hydrogen atoms from allylic carbons (next to or between double bonds) on adjacent acyl chains to form hydroperoxides, LOOH, and new radicals (Reaction 3) that repeat the process to keep the chain going (Reaction 4):

$$L_1OO^{\bullet} + L_2H \rightarrow L_1OOH + L_2^{\bullet} \quad (3)$$

$$L_2^{\bullet} + O_2 \rightarrow L_2OO^{\bullet} \xrightarrow{L_3H} L_2OOH + L_3^{\bullet} \quad (4)$$

The process continues until all radicals are quenched or converted to products by various secondary reactions, a stage referred to as termination.

Reactions leading to secondary products are now the subject of intense interest. Secondary products of lipid oxidation produced from scission of alkoxyl radicals have been long discussed (Frankel, 1987), where oxo- and hydroxy-fatty acids with shorter chain length are produced. However, in addition to the short-chain scission products (Berdeaux, Velasco, Márquez-Ruiz, & Dobarganes, 2002), formation of oxygenated products with the original fatty acid chain length has also been identified (Velasco et al., 2002; Morales et al., 2010). Therefore, alternative pathways to the expected scission generation of secondary products need to be investigated. Their competition with chain scissions should not be neglected.

Epoxides are functional groups in which an oxygen atom is inserted into one bond of a double bond and becomes shared between the two carbons, forming a three-membered ring structure in the chain. Lipid epoxides are formed when peroxyl radicals add directly to double bonds and the resulting peroxy dimer decomposes to an epoxide and an alkoxyl radical (Figure 2.1a and 2.2a; Frankel, 1984; Giuffrida, Destaillats, Robert, Skibsted, & Dionisi, 2004). Epoxides can also be produced from the cyclization of alkoxyl radicals (Frankel, 1984). In this case, they are thought to be secondary

products and only form from the alkoxyl radicals produced from decomposition of hydroperoxides (Figure 2.1b and 2.2b). It should be noted that the peroxyl radical addition forms an epoxy group at the position of the original double bond (Figure 2.1a); however, the cyclization of alkoxyl radicals can form positional epoxy isomers due to the electron delocalization in lipid radicals which takes place before the alkoxyl radicals are formed (Figure 2.1b). Neff and Byrdwell (1998) observed two classes of epoxides during oxidation of triolein, trilinolein and trilinolenin. In one class, the epoxy groups are formed at the position of the original double bonds. In the other, the epoxy groups are formed distant from a double bond (Neff and Byrdwell, 1998). These observations are consistent with the proposal of Schaich (2012) that epoxides can form either as a primary process via peroxyl radical addition to double bonds or as a secondary process via rearrangement of alkoxyl radicals, formed from decomposition of either peroxyl radical-double bond dimers in early oxidation or hydroperoxide decomposition as oxidation progresses. Moreover, the alkoxyl radicals released from the process can react further to generate epoxides, alcohols, ketones, and other lipid oxidation products.

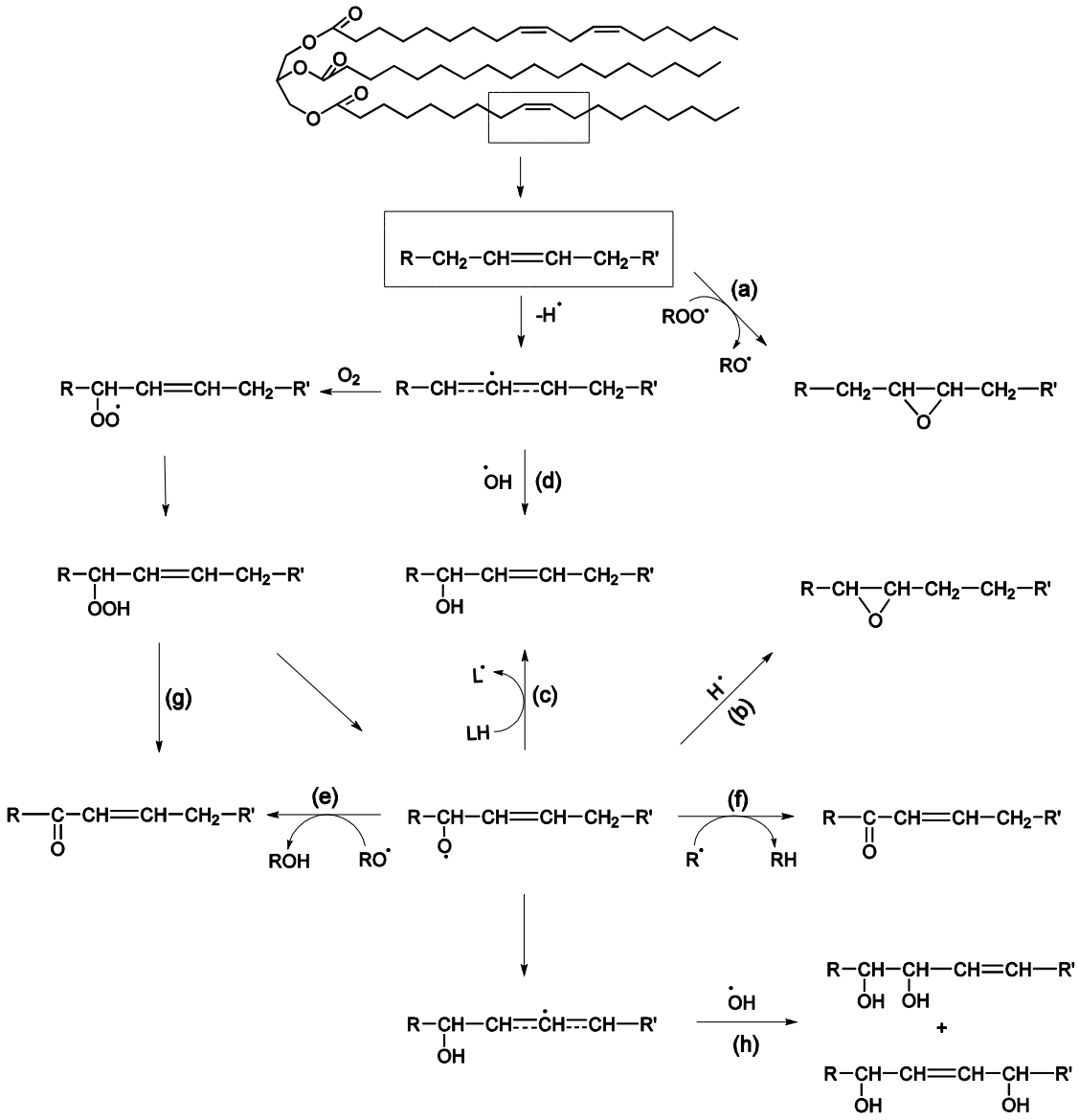


Figure 2.1 Formation of lipid oxidation products from an unsaturated site in oleic acid bound in a TAG (Terao et al., 1975; Frankel, 1984; Frankel, 1987; Schaich, 2005).

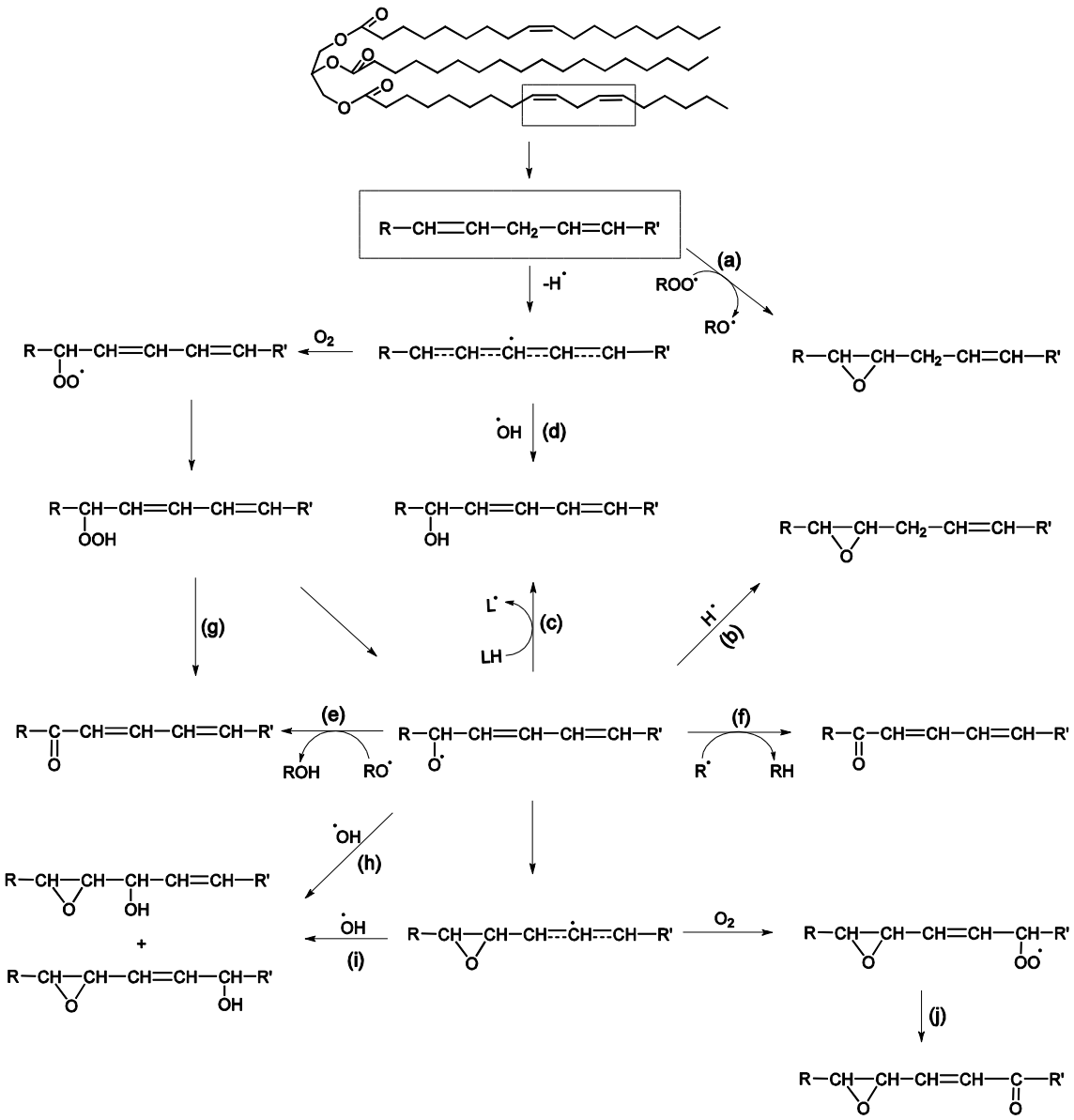


Figure 2.2 Formation of lipid oxidation products from a pentadiene system in a PUFA bound in a TAG (Terao et al., 1975; Frankel, 1984; Frankel, 1987; Schaich, 2005).

Another class of secondary products are alcohols produced from hydrogen abstraction by alkoxyl radicals (Figure 2.1c and 2.2c). Hydrogen abstraction from lipid molecules by peroxyl radicals forms hydroperoxides, the major class of primary oxidation products. As hydroperoxides decompose to alkoxyl radicals ($LO^{\bullet}$), these alkoxyl radicals can then begin to abstract hydrogens from acyl chains and hydroperoxides. The latter reaction ($LO^{\bullet} + LOOH \rightarrow LOH + LOO^{\bullet}$) slows oxidation but also expands it by enhancing chain branching (Schaich, 2005). Lipid alcohols may also be generated via addition of hydroxyl radicals, $HO^{\bullet}$, to double bonds (Schaich, 2005) or combination of hydroxyl radicals with lipid alkyl radicals, $L^{\bullet}$ (Figure 2.1d and 2.2d) (Frankel, 1984). These highly reactive hydroxyl radicals can be generated from homolytic decomposition of hydroperoxides by heat and UV light (Schaich, 2005). Surprisingly, although alcohols are always written as a major product in the classical chain reaction, lipid alcohols have been treated as minor products and seldom analyzed, perhaps due to lack of appropriate analytical methods (Schaich, 2012).

The last class of important oxidation products is ketones. Recombination of a secondary alkoxyl radical with a primary alkoxyl radical produces a ketone and an alcohol (Figure 2.1e and 2.2e) (Schaich, 2005). Ketones can also form from recombination of a secondary alkoxyl radical with an alkyl radical (Figure 2.1f and 2.2f) (Schaich, 2005) or by elimination of OH^{-} from hydroperoxides (Figure 2.1g and 2.2g) (Terao, Ogawa, & Matsushita, 1975).

Fatty acids with more than one of these oxygenated functional groups have been reported to form from both monounsaturated fatty acids and PUFA during lipid oxidation. Epoxy hydroxy monoenes result from cyclization of alkoxyl radicals and

addition of hydroxyl radicals with and without double bond migration (Figure 2.2h and 2.2i) (Frankel, 1984). Gardner, Kleiman, and Weisleder (1974) and Gardner and Kleiman (1977) characterized epoxy oxo monoenes derived from the decomposition of linoleic acid hydroperoxides in the presence of catalysts. The relevant mechanisms have been summarized by Frankel (1984) (Figure 2.2j). In addition, dihydroxy fatty acids are formed by the reaction between allylic enols and hydroxyl radicals (Figure 2.1h) (Frankel, 1987).

In summary, the locations of the oxygenated groups are highly related to the position of original double bonds. Allylic/conjugated lipid oxidation products are generated as radical formation forces double bond shifts, leading to a variety of positional and geometric isomers that cause difficulties in separation and identification of the oxidation products. In addition, previous studies on the characterization of oxidation products have mainly focused on model systems such as pure TAGs and fatty acid methyl esters (FAMEs). The extremely low levels of those oxygenated compounds have caused even more difficulties in detection in non-model systems, even in vegetable oils which primarily consist of TAGs. Thus, this review will evaluate the available methods that make use of GC, HPLC, and NMR techniques in terms of their selectivity to identify the compounds and their sensitivity to determine these products at low levels in food lipids and edible oils.

2.5 Occurrence of Epoxides, Alcohols, and Ketones

Epoxides were first monitored in oxidized methyl esters and TAGs by GC (Berdeaux, Márquez-Ruiz, & Dobarganes, 1999). Methyl oleate, methyl linoleate, triolein, and

trilinolein were heated at 180 ºC for 15 h and were tested every 5 h for epoxy fatty acid content. After heating methyl oleate for 15 h, two methyl monoepoxystearates (*trans*-9,10 epoxy and *cis*-9,10 epoxy) were present at 20.3 mg/g and 14.9 mg/g, respectively. Under the same conditions, four methyl monoepoxyoleates (*trans*-9,10, *trans*-12,13, *cis*-9,10, and *cis*-12,13 isomers) were produced from oxidation of methyl linoleate at concentrations of 5.9, 6.9, 3.1, 3.2 mg/g, respectively. Using sodium methoxide catalyzed transmethylation, the total amounts of monoepoxy fatty acids in oxidized triolein (35.7 mg/g) and trilinolein (18.3 mg/g) were similar to those present in oxidized methyl oleate (32.3 mg/g) and methyl linoleate (19.3 mg/g) (Berdeaux et al., 1999). In addition, less unsaturated substrates (methyl oleate and triolein) produced larger amounts of epoxides than more unsaturated substrates (methyl linoleate and trilinolein) (Berdeaux et al., 1999). The six monoepoxy fatty acids reported in this study, including two methyl monoepoxystearates and four methyl monoepoxyoleates, have become the six most commonly measured epoxy fatty acids in the literature.

Velasco, Marmesat, Bordeaux, Márquez-Ruiz, and Dobarganes (2004) determined monoepoxy fatty acids in oils heated using the same temperature and heating time as Berdeaux et al. (1999). The total amounts of the six common monoepoxy fatty acids were 14.24 and 9.44 mg/g in olive oil and sunflower oils, respectively (Velasco et al., 2004). The authors determined methyl epoxystearates and methyl epoxyoleates in ten used frying oils and concluded that: 1) more *trans* epoxides than *cis* epoxides were produced in monounsaturated oils; and 2) a greater concentration of epoxides were found in monounsaturated oils than polyunsaturated oils, consistent with Berdeaux et al. (1999),

which may be attributed to a lower tendency of epoxystearates to form polymers and to participate in further oxidative reactions (Velasco et al., 2004).

In addition to frying oils, epoxy fatty acids have been determined at much lower concentrations (approximately 1000 fold lower) in fresh oil, with *cis*-9,10-epoxystearate, *cis*-12,13-epoxyoleate, and *cis*-9,10-epoxyoleate being the prominent epoxy fatty acids among the six commonly determined (Mubiru, Shrestha, Papastergiadis, & De Meulenaer, 2013). *Trans* epoxy fatty acids were generally present at lower levels than *cis* epoxy fatty acids in fresh oils. For example, in sunflower oils, individual *cis* epoxy fatty acids ranged from 55 to1430 µg/g, while *trans* epoxy fatty acids ranged from 4-33 µg/g. Moreover, a strong correlation was reported between the ratio of epoxystearate to epoxyoleate and the ratio of 18:1 to 18:2 fatty acids (Mubiru et al., 2013).

Mubiru, Shrestha, Papastergiadis, and De Meulenaer (2014) measured the six common monoepoxy fatty acids in food matrices including biscuits, meat, butter, and nuts by GC. A range of 3-171 µg/g of total epoxy fatty acids were determined in the foods and no relationship was found between the sample type and the total epoxy fatty acid concentrations. A large amount of *trans*-9,10 epoxystearate (53.66 µg/g) was reported for the butter sample, while the other food matrices had lower concentrations of *trans*-9,10 epoxystearate, ranging from 0.75-17.33 µg/g (Mubiru et al., 2014). That lower epoxide concentrations were observed in complex foods than in oils should be expected considering the high reactivity of epoxides and also alkoxyl radicals (epoxide precursor) with proteins (Schaich, 2008).

Considering that the epoxy groups in the six most measured monoepoxy fatty acids occur at the position of the original double bond, i.e. C9,10 in methyl oleate and

C9,10 and C12,13 in methyl linoleate, the six epoxy fatty acids seem to be formed by peroxide addition to double bonds (Figure 1a and 2a). Positional isomers, such as 8, 9-epoxystearate and 10, 11-epoxystearate (Frankel, 1984), which may be formed by cyclization of alkoxyl radicals (Figure 1b and 2b), are not reported.

The above results reported for epoxides were determined by GC which made it possible to identify and quantify individual epoxy fatty acids. In contrast, NMR methods determine a total epoxide content. For instance, Goicoechea and Guillén (2010) determined monoepoxides and diepoxides in sunflower oils heated at 70 ºC and 100 ºC with aeration. At 100 ºC, both monoepoxides and diepoxides reached a maximum value at 161 and 107 mmol/L oil (approximately 56 and 39 mg/g) after 52 h and 35 h, respectively, of heating and showed a declining trend afterwards. At 70 ºC, the concentrations of mono- and diepoxides were detectable from 120 h and reached 41 and 123 mmol/L oil (approximately 14 and 45 mg/g) during 264 h of heating (Goicoechea & Guillén, 2010).

Available data on the occurrence of alcohols and ketones is scarcer than for epoxides. Individual C18 hydroxy fatty acids in hydrogenated ground nut oil were present between 2-494 µg/g with a total amount of 1422 µg/g, determined by GC-MS (Wilson, Smith, Wilson, Shepherd, & Riemersma, 1997). In fried sunflower oil (180 ºC for 10 h), total C18 hydroxy FAMEs ranged between 1.9 – 5.5 mg/g oil, while C18 keto FAMEs and C18 epoxy FAMEs were detected at 0.5-2.5 mg/g oil and 1.3-4.4 mg/g oil, respectively (Marmesat et al., 2008). In extra virgin olive oil heated at 190 ºC for 7.5-32 h, primary alcohols were determined by NMR at 2-5 mmol/mol TAG (approximately 0.7-1.7 mg/g TAG), while secondary alcohols were between 4-20 mmol/mol TAG

(approximately 1.4-7.0 mg/g TAG) and (E)- and (Z)-epoxides reached concentrations of 20 and 12 mmol/mol TAG (approximately 7.0 and 4.2 mg/g), respectively (Martínez-Yusta & Guillén, 2014a). Three other food types, Spanish doughnuts, pork adipose tissue, and farmed salmon fillets, were exposed to the same experimental conditions and did not show great differences in alcohol or epoxide content (Martínez-Yusta & Guillén, 2014a). However, in a later study, the same authors compared the formation of primary alcohols in fried soybean oils and the same three food types (dough, adipose tissue, and salmon fillets) and they found that primary alcohols had much higher concentrations in the salmon fillets than the other three media (Martínez-Yusta & Guillén, 2014b). The authors also reported that the primary alcohols began to be detectable after 7.5 hours of frying in soybean oil and Spanish doughnut dough, and after ten hours of frying in pork adipose tissue and salmon fillet.

Hydroxydienes and ketodienes were monitored during oxidation of sunflower oil-derived FAME at 40 ºC using HPLC-UV (Morales et al., 2010). Due to the limits of quantification, which were 0.05 mg/g for hydroxydienes and 0.02 mg/g for ketodienes, hydroxydienes and ketodienes were detectable but not quantifiable at the beginning of the experiments and the concentrations of both types of compounds increased over time. After 91 hours of heating, 3.1 mg/g of ketodienes and 1.3 mg/g of hydroxydienes were found in the FAME derived from high linoleic sunflower oil. Under the conditions of the study, more ketodienes than hydroxydienes were found in FAMEs derived from both high linoleic and high oleic sunflower oils throughout the oxidation period (Morales et al., 2010). For high linoleic sunflower oil oxidized at room temperature for the same time period, the same group found concentrations of ketodienes and hydroxydienes up to 1.6

and 0.7 mg/g, respectively (Morales, Marmesat, Ruiz-Méndez, Márquez-Ruiz, & Velasco, 2014).

Steenhorst-Slikkerveer, Louter, Janssen, and Bauer-Plank (2000) determined the concentrations of epoxy-, oxo-, and hydroxy-TAGs in oxidized rapeseed oil and linseed/safflower oil by HPLC-MS. The oils were analyzed when received, without any catalyzed oxidation. Epoxy- and oxo-TAGs co-eluted with each other, so they were determined together, at 2734 ppm (approximately 2.7 mg/g) in rapeseed oil and 28701 ppm (approximately 28.7 mg/g) in linseed/safflower oil. The concentrations of hydroxy TAGs were 8759 ppm and 3549 ppm (approximately 8.8 and 3.5 mg/g) in rapeseed oil and linseed/safflower oil, respectively (Steenhorst-Slikkerveer et al., 2000).

Levels of epoxides, alcohols, and ketones detected in heated and oxidized oils and foods are summarized in Table 2.1. Discrepancies between studies probably arise from the different techniques and oxidation conditions employed. The quantitative data show clear trends, where most oxidized oils have epoxy, hydroxy, and keto compounds at concentrations in the range of mg/g. In un-oxidized oils, these oxygenated compounds were quantifiable using chromatographic techniques but present at much lower levels at µg/g concentrations.

Table 2.1 Occurrence of epoxides, alcohols, and ketones in FAMEs, TAGs, oils, and foods with/without catalyzed oxidation.

Sample	Oxidation condition	Quantitative technique	Analyte	Range of quantity	Reference
FAME					
methyl oleate	heated at 180 °C for 5-15h	GC-FID	monoepoxy fatty acids	14.2-35.2 mg/g	Berdeaux et al. (1999a)
methyl linoleate	heated at 180 °C for 5-15h	GC-FID	monoepoxy fatty acids	9.2-19.1 mg/g	Berdeaux et al. (1999a)
FAME derived from high linoleic sunflower oil	heated at 40 °C for 0-91h	HPLC-UV	ketodienes	up to 3.094 mg/g	Morales et al. (2010)
		HPLC-UV	hydroxydienes	up to 1.338 mg/g	Morales et al. (2010)
FAME derived from high oleic sunflower oil	heated at 40 °C for 0-192h	HPLC-UV	ketodienes	up to 1.726 mg/g	Morales et al. (2010)
		HPLC-UV	hydroxydienes	up to 0.590 mg/g	Morales et al. (2010)
FAME derived from high linoleic sunflower oil	heated at 80 °C for 0-6h	HPLC-UV	ketodienes	up to 0.38 mg/g	Morales et al. (2012a)
		HPLC-UV	hydroxydienes	up to 0.46 mg/g	Morales et al. (2012a)
FAME derived from high oleic sunflower oil	heated at 80 °C for 0-17h	HPLC-UV	ketodienes	up to 0.78 mg/g	Morales et al. (2012a)
		HPLC-UV	hydroxydienes	up to 0.31 mg/g	Morales et al. (2012a)
		HPLC-ELSD	methyl *trans*-epoxystearate	up to 1.33 mg/g	Morales et al. (2012a)
		HPLC-ELSD	methyl *cis*-epoxystearate	up to 1.52 mg/g	Morales et al. (2012a)
TAG					
triolein	heated at 180 °C for 5-15h	GC-FID	monoepoxy fatty acids	13.3-35.7 mg/g after CH_3ONa catalyzed transmethylation	Berdeaux et al. (1999a)
trilinolein	heated at 180 °C for 5-15h	GC-FID	monoepoxy fatty acids	6.9-18.3 mg/g after CH_3ONa catalyzed transmethylation	Berdeaux et al. (1999a)
Oil					
hydrogenated ground nut oil	-	GC-MS-SIM	C18 hydroxy fatty acid isomers (C6-C17 substitution)	2-494 µg/g	Wilson et al. (1997)

Sample	Oxidation condition	Quantitative technique	Analyte	Range of quantity	Reference
olive oil	heated at 180 °C for 15h	GC-FID	monoepoxy fatty acids	13.52 mg/g with on-column injection, 13.72 mg/g with split injection	Velasco et al. (2002)
sunflower oil	heated at 180 °C for 15h	GC-FID	monoepoxy fatty acids	10.88 mg/g with on-column injection, 10.87 mg/g with split injection	Velasco et al. (2002)
olive oil	heated at 180 °C for 5-15h	GC-FID	monoepoxy fatty acids	4.29-14.24 mg/g	Velasco et al. (2004)
sunflower oil	heated at 180 °C for 5-15h	GC-FID	monoepoxy fatty acids	5.10-9.44 mg/g	Velasco et al. (2004)
used frying oils	-	GC-FID	monoepoxy fatty acids	3.37-14.42 mg/g	Velasco et al. (2004)
edible oils	-	GC-FID	*cis*-epoxy-oleic acid	110-2300 µg/g	Fankhauser-Noti et al. (2006)
		GC-FID	*cis*-epoxy-linoleic acid	150-1550 µg/g	Fankhauser-Noti et al. (2006)
		GC-FID	diepoxy linoleic acid	0.2-1.5 µg/g	Fankhauser-Noti et al. (2006)
frying oils	-	GC-FID	*cis*-epoxy-oleic acid	1900-7300 µg/g	Fankhauser-Noti et al. (2006)
		GC-FID	*trans*-epoxy-oleic acid	2800-16400 µg/g	Fankhauser-Noti et al. (2006)
		GC-FID	*cis*-epoxy-linoleic acid	1400-2000 µg/g	Fankhauser-Noti et al. (2006)
		GC-FID	*trans*-epoxy-linoleic acid	1800-3300 µg/g	Fankhauser-Noti et al. (2006)
		GC-FID	diepoxy linoleic acid	4.9-16 µg/g	Fankhauser-Noti et al. (2006)
sunflower oils	heated at 180 °C for 10h	GC-FID	epoxy fatty acids	1.3-4.4 mg/g	Marmesat et al. (2008)
		GC-FID	keto fatty acids	0.5-2.5 mg/g	Marmesat et al. (2008)
		GC-FID	hydroxy fatty acids	1.9-5.5 mg/g	Marmesat et al. (2008)
fresh oils	-	GC-FID	epoxy fatty acids	0.03-2 mg/g	Mubiru et al. (2013)
used frying fat/oil	-	GC-FID	epoxy fatty acids	0.05-16.57 mg/g	Brühl et al. (2016)
pumpkin seed oil	-	GC-FID	epoxy fatty acids	1.12-5.10 mg/g	Brühl et al. (2016)
sweet almond oil	-	GC-FID	epoxy fatty acids	0.72-2.65 mg/g	Brühl et al. (2016)

Sample	Oxidation condition	Quantitative technique	Analyte	Range of quantity	Reference
sunflower oil	-	GC-FID	epoxy fatty acids	0.06-1.90 mg/g	Brühl et al. (2016)
groundnut oil	-	GC-FID	epoxy fatty acids	0.10-7.25 mg/g	Brühl et al. (2016)
olive oil	-	GC-FID	epoxy fatty acids	0.10-0.32 mg/g	Brühl et al. (2016)
rapeseed oil	-	HPLC-MS	epoxy- and oxo-TAG	2734 ppm (2734 µg/g) [a]	Steenhorst-Slikkerveer et al. (2000)
		HPLC-MS	oxo-$2^1/_2$-glycerides	506 ppm (506 µg/g)[a]	Steenhorst-Slikkerveer et al. (2000)
		HPLC-MS	hydroxy-TAG	8759 ppm (8759 µg/g)[a]	Steenhorst-Slikkerveer et al. (2000)
		HPLC-MS	dihydroxy-TAG	1049 ppm (1049 µg/g)[a]	Steenhorst-Slikkerveer et al. (2000)
linseed/safflower oil	-	HPLC-MS	epoxy- and oxo-TAG	28701 ppm (28701 µg/g)[a]	Steenhorst-Slikkerveer et al. (2000)
		HPLC-MS	oxo-$2^1/_2$-glycerides	2146 ppm (2146 µg/g)[a]	Steenhorst-Slikkerveer et al. (2000)
		HPLC-MS	hydroxy-TAG	3549 ppm (3549 µg/g)[a]	Steenhorst-Slikkerveer et al. (2000)
		HPLC-MS	dihydroxy-TAG	1823 ppm (1823 µg/g)[a]	Steenhorst-Slikkerveer et al. (2000)
sunflower oil	heated at 100 °C with aeration	^{1}H NMR	monoepoxides	maximum level at 161 mmol/L (56 mg/g)[b]	Goicoechea and Guillén (2010)
			diepoxides	maximum level at 107 mmol/L (39 mg/g) [b]	Goicoechea and Guillén (2010)
sunflower oil	heated at 70 °C with aeration	^{1}H NMR	monoepoxides	maximum level at 41 mmol/L (14 mg/g) [b]	Goicoechea and Guillén (2010)
			diepoxides	maximum level at 123 mmol/L (44 mg/g) [b]	Goicoechea and Guillén (2010)
extra virgin olive oil	heated at 190°C for periods of 8h/day for 5 days	^{1}H NMR	(*E*)-9,10-epoxystearic acyl groups	22.8 mmol/L (7.9 mg/g) [b]	Guillén and Uriarte (2012a)
		^{1}H NMR	(*Z*)-9,10-epoxystearic acyl groups	14.5 mmol/L (5.0 mg/g) [b]	Guillén and Uriarte (2012a)
soybean oil	heated at 100°C for 4-20 days	^{1}H NMR	epoxides	8.4-90.7 mmol/kg (2.6-28.3 mg/g) [b]	Xia et al. (2015)

Sample	Oxidation condition	Quantitative technique	Analyte	Range of quantity	Reference
high oleic sunflower oil	heated at 40 °C for 0-86 days	HPLC-UV	ketodienes	0.13-1.34 mg/g	Morales et al. (2012b)
		HPLC-UV	hydroxydienes	0.13-0.72 mg/g	Morales et al. (2012b)
high linoleic sunflower oil	heated at 40 °C for 0-41 days	HPLC-UV	ketodienes	0.12-0.79 mg/g	Morales et al. (2012b)
		HPLC-UV	hydroxydienes	0.36-1.12 mg/g	Morales et al. (2012b)
soybean oil	heated at 40 °C for 0-53 days	HPLC-UV	ketodienes	0.07-2.97 mg/g	Morales et al. (2014)
		HPLC-UV	hydroxydienes	1.02-3.53 mg/g	Morales et al. (2014)
rapeseed oil	heated at 40 °C for 0-46 days	HPLC-UV	ketodienes	up to 2.1 mg/g	Morales et al. (2014)
		HPLC-UV	hydroxydienes	up to 3.71 mg/g	Morales et al. (2014)
Food					
infant foods	-	GC-FID	epoxidized soybean oil	9-86 µg/g	Fankhauser-Noti et al. (2005)
oily sauces	-	GC-FID	epoxidized soybean oil	47-580 µg/g	Fankhauser-Noti et al. (2005)
products in oil	-	GC-FID	epoxidized soybean oil	85-350 µg/g	Fankhauser-Noti et al. (2005)
bakery foods	-	GC-FID	*cis*-epoxy oleic acid	150-4240 µg/g	Fankhauser-Noti et al. (2006)
		GC-FID	*cis*-epoxy linoleic acid	60-3460 µg/g	Fankhauser-Noti et al. (2006)
		GC-FID	diepoxy linoleic acids	0.5-5.3 µg/g	Fankhauser-Noti et al. (2006)
products in oil	-	GC-FID/GC-MS	epoxidized soybean oil	36-374 µg/g (GC-FID) and 42-363 µg/g (GC-MS)	Biedermann-Brem et al. (2007)
biscuits	-	GC-FID	epoxy fatty acids	46.95-74.49 µg/g	Mubiru et al. (2014)
mayonnaise	-	GC-FID	epoxy fatty acids	32.6-49.16 µg/g	Mubiru et al. (2014)
nuts	-	GC-FID	epoxy fatty acids	48.88-170.78 µg/g	Mubiru et al. (2014)
butter	-	GC-FID	epoxy fatty acids	115.34 µg/g	Mubiru et al. (2014)
meat	-	GC-FID	epoxy fatty acids	3.24-5.58 µg/g	Mubiru et al. (2014)
chocolate, cocoa butter	-	GC-FID	epoxy fatty acids	0.57-3.38 mg/g	Brühl et al. (2016)

Sample	Oxidation condition	Quantitative technique	Analyte	Range of quantity	Reference
extra virgin olive oil fried with doughnut/ pork/ salmon fillets	heated at 190°C for 7.5-32 h	^{1}H NMR	(*E*)-9,10-epoxystearic acyl groups	4-20 mmol/mol TAG (1.4-7.0 mg/g) [c]	Martínez-Yusta and Guillén (2014a)
			(*Z*)-9,10-epoxystearic acyl groups	4-12 mmol/mol TAG (1.4-4.2 mg/g) [c]	Martínez-Yusta and Guillén (2014a)
			primary alcohols	2-5 mmol/mol TAG (0.7-1.7 mg/g) [c]	Martínez-Yusta and Guillén (2014a)
			secondary alcohols	4-20 mmol/mol TAG (1.4-7.0 mg/g) [c]	Martínez-Yusta and Guillén (2014a)

'-' Represents information not available

[a] The values in units of 'ppm' were converted into 'μg/g' using a 1:1 ratio.

[b] The values in units of 'mmol/kg' or 'mmol/L' were converted into 'mg/g' for comparison, using the molecular weights of methyl epoxystearate and methyl diepoxystearate and assuming that all monoepoxides were methyl epoxystearate and all diepoxides were methyl diepoxystearate. The density of oil was assumed to be 0.9 kg/L for calculation.

[c] The values in units of 'mmol/mol TAG' were converted into 'mg/g' for comparison, using the molecular weights of methyl epoxystearate for epoxides and methyl hydroxystearate for alcohols. The molecular weight of TAG was assumed to be 900 g/mol for calculation.

2.6 GC

2.6.1 Derivatization of TAGs

In the study of lipid oxidation, many GC applications focus on the analysis of small volatile compounds that form from β-scission of alkoxyl radicals, where the analytes can be subjected to GC analysis directly, normally using low temperatures. In contrast, TAGs are large, non-volatile molecules. In order to avoid the high temperatures required for analysis of glycerol-bound fatty acids, derivatives of fatty acids are synthesized for better chromatographic properties, including FAME, 3-hydroxymethylpyridinyl (3-pyridylcarbinol) esters, 4,4-dimethyloxazoline (DMOX) derivatives, and pyrrolidine derivatives (Christie, 1998).

FAMEs have been the most used derivatives for the analysis of oxygenated fatty acids. Base-catalyzed transmethylation appears to be the most appropriate method to methylate oxygenated fatty acids because some acid-catalyzed methylation methods cause artifacts or changes in structures. For example, acid-catalyzed methods resulted in the loss of a significant amount of conjugated fatty acids; methoxy artifacts also formed, which further complicated the chromatogram (Kramer et al., 1997). The common methylation reagents, BF_3 and HCl, could convert allylic hydroxy fatty acids into conjugated dienoic acids (Yurawecz et al., 1994). In addition, when applying BF_3 or HCl with methanol, a great loss of the vernolic acid (*cis*-12-epoxyoctadec-*cis*-9-enoic acid) peak was observed in GC using a thermal conductivity detector (TCD) (Kleiman, Spencer, & Earle, 1969), along with the formation of a broad peak of its derivative. Further tests confirmed the derivatives to be methoxy-hydroxy derivatives that formed during the reaction between BF_3/methanol and epoxides (Kleiman etal., 1969; Kleiman &

Spencer 1973). Although base-catalyzed methylation methods do not esterify free fatty acids, they have been the most frequently used methods for oxidized oils with excellent reproducibility and fewer artifacts produced than acid-catalyzed methods (Berdeaux, Marquez-Ruiz, & Dobarganes, 1999b), particularly when oxygen-containing fatty acids are of interest.

Less information has been reported for other derivatization methods of oxygenated fatty acids. The use of 3-pyridylcarbinol esters, DMOX derivatives, and pyrrolidine derivatives of fatty acids in the structural analysis by GC-MS has been reviewed by Christie, Brechany, Johnson, and Holman (1986), Christie (1998), and Harvey (1998). Although direct preparation of 3-pyridylcarbinol esters from TAGs was initially considered impossible (Christie, 1998), a one-step transesterification method was later applied to prepare 3-pyridylcarbinol esters from TAGs (Destaillats & Angers, 2002; Dubois, Barthomeuf, & Bergé, 2006). However, transesterification of oxidized TAGs using this method needs verification.

2.6.2 Characterization of oxygenated fatty acids by GC-MS

GC-MS has been a powerful tool to characterize oxygenated fatty acids, derivatized as methyl esters or as one of the above derivatives. This section will focus on the applications of GC-MS in structural analysis with regard to the structures likely encountered during lipid oxidation, such as those with double bonds conjugated to an oxygenated group. Characteristic major ions or fragments from epoxy, hydroxy, and keto fatty acids are reported in Table 2.2.

Table 2.2 Selected GC-MS characterization of oxygenated fatty acids in literature sources.

Ionization	Major ions (fragments)	Systematic name[a]	Reference
Epoxy-			
EI	199 ($CH_3O_2C(CH_2)_7CHOCH^+$), 155 ($CH_3(CH_2)_7CHOCH^+$)	9,10-epoxyoctadecanoic acid ME	Kleiman and Spencer (1973)
EI	199 ($CH_3O_2C(CH_2)_7CHOCH^+$), 153 ($CH_3(CH_2)_4CH=CHCH_2CHOCH^+$)	9,10-epoxyoctadec-12-enoic acid ME	Kleiman and Spencer (1973)
EI (20eV)	308 (M-18), 187 ($CH_3O_2C(CH_2)_7CH=OH^+$), 155 (187-32)	9,10:12,13-diepoxyoctadecanoic acid ME	Piazza et al. (2003)
EI	164, 207	12,13-epoxyoctadec-9-enoic acid ME	Mubiru et al. (2013)
EI	207, 111	12,13-epoxyoctadeca-9,15-dienoic acid ME	Mubiru et al. (2013)
EI	236, 247	15,16-epoxyoctadeca-9,12-dienoic acid ME	Mubiru et al. (2013)
EI	185, 155, 108	9,10-epoxyoctadeca-12,15-dienoic acid ME	Mubiru et al. (2013)
PCI (isobutane)	313 (M+H), 295 (M+H-18), 281 (M+H-32), 263 (M+H-18-32)	12,13-epoxyoctadecanoic acid ME and 9,10-epoxyoctadecanoic acid ME	Plattner et al. (1983)
PCI (methane)	327(M+H), 309, 291, 277	9,10:12,13-diepoxyoctadecanoic acid ME	Biedermann-Brem et al. (2007)
PCI (ammonia)	344($M+NH_4$), 309(M+H-18)	9,10:12,13-diepoxyoctadecanoic acid ME	Biedermann-Brem et al. (2007)
NCI (ammonia)	325(M-H)	9,10:12,13-diepoxyoctadecanoic acid ME	Biedermann-Brem et al. (2007)
Hydroxy-			
EI	229 ($CH_3O_2C(CH_2)_{10}$-CH(OH)), 200 ($CH_3O_2C(CH_2)_9CH_3$), 197 (229-MeOH)	12-hydroxyoctadecanoic acid ME	Wilson et al. (1997)
EI	243 ($CH_3O_2C(CH_2)_{10}$-$CH(OCH_3)$), 129 ($CH_3(CH_2)_5$-$CH(OCH_3)$)	12-methoxyoctadecanoic acid ME	Wilson et al. (1997)

Ionization	Major ions (fragments)	Systematic name	Reference
EI	371 (M-15), 301 ($CH_3O_2C(CH_2)_{10}CHOSi(CH_3)_3^+$) , 187 ($CH_3(CH_2)_5CHOSi(CH_3)_3^+$)	12-hydroxyoctadecanoic acid ME TMS ether	Kleiman and Spencer (1971)
EI	369 (M-15), 299 ($CH_3O_2C(CH_2)_7CH=CH-CH_2-CHOSi(CH_3)_3^+$) , 270 (migration ion, $CH_3OC^+(OTMS)(CH_2)_7-CH=CH-CH_2$), 187 ($CH_3(CH_2)_5CHOSi(CH_3)_3^+$)	12-hydroxyoctadec-9-enoic acid ME TMS ether	Kleiman and Spencer (1973); Mubiru et al. (2013)
EI	369 (M-15), 294 (M-trimethylsilanol), 259 ($CH_3O_2C(CH_2)_7-CHOSi(CH_3)_3^+$), 230 (migration ion, $CH_3OC^+(OTMS)(CH_2)_7$), 227 ($CH_3(CH_2)_4CH=CH-(CH_2)_2-CHOSi(CH_3)_3^+$)	9-hydroxyoctadec-12-enoic acid ME TMS ether	Kleiman and Spencer (1973)
EI	369 (M-15), 313 ($CH_3O_2C(CH_2)_7CH=CH-(CH_2)_2-CHOSi(CH_3)_3^+$) , 294 (M-90), 173 ($CH_3(CH_2)_4CHOSi(CH_3)_3^+$)	13-hydroxyoctadec-9-enoic acid ME TMS ether	Kleiman and Spencer (1973)
EI	369 (M-15), 327 ($CH_3O_2C(CH_2)_9-CH=CH-CH_2-CHOSi(CH_3)_3^+$) , 298 (migration ion, $CH_3OC^+(OTMS)(CH_2)_9-CH=CH-CH_2$), 185 ($CH_3-CH_2-CH=CH-(CH_2)_2CHOSi(CH_3)_3^+$)	14-hydroxyeicosa-11,17-dienoic acid ME TMS ether	Kleiman and Spencer (1973)
EI	285 ($CH_3O_2C(CH_2)_7-CHOSi(CH_3)_3-CH=CH^+$), 227 ($CH_3(CH_2)_6-CH=CH-CHOSi(CH_3)_3^+$)	9-hydroxyoctadec-10-enoic acid ME TMS ether	Kleiman and Spencer (1973)
EI	311 ($CH_3O_2C(CH_2)_7CHOSi(CH_3)_3-CH=CH-CH=CH^+$), 225 ($CH_3(CH_2)_4CH=CH-CH=CH-CHOSi(CH_3)_3^+$)	9-hydroxyoctadeca-10,12-dienoic acid ME TMS ether	Kleiman and Spencer (1973)
EI	313 ($CH_3O_2C(CH_2)_9-CH=CH-CHOSi(CH_3)_3^+$), 199 ($CH_3(CH_2)_4-CHOSi(CH_3)_3CH=CH^+$)	13-hydroxyoctadec-11-enoic acid ME TMS ether	Kleiman and Spencer (1973)
EI	311 ($CH_3O_2C(CH_2)_7-CH=CH-CH=CH-CHOSi(CH_3)_3^+$), 225 ($CH_3(CH_2)_4-CHOSi(CH_3)_3CH=CH-CH=CH^+$)	13-hydroxyoctadeca-9,11-dienoic acid ME TMS ether	Kleiman and Spencer (1973)

Ionization	Major ions (fragments)	Systematic name	Reference
EI (25eV)	487 (M-15), 471 (M-31), 431 ($CH_3O_2C(CH_2)_3$-$CHOSi(CH_3)_3$-$(CH_2)_9$-$CHOSi(CH_3)_3^+$), 401 ($CH_3(CH_2)_4$-$CHOSi(CH_3)_3$-$(CH_2)_9$-$CHOSi(CH_3)_3^+$),203 ($CH_3O_2C(CH_2)_3$ -$CHOSi(CH_3)_3^+$), 173($CH_3(CH_2)_4$-$CHOSi(CH_3)_3^+$)	5,15-dihydroxyeicosanoic acid ME TMS ether	Borgeat et al. (1982)
EI (25eV)	494 (M), 479 (M-15), 463 (M-31), 404 (M-trimethylsilanol), 255 ($CH_3O_2C(CH_2)_3$-$CHOSi(CH_3)_3$-$CH=CH$-$CH=CH^+$), 225 ($CH_3(CH_2)_4$-$CH(OSi(CH_3)_3)$-$CH=CH$-$CH=CH^+$), 203 ($CH_3O_2C(CH_2)_3$-$CHOSi(CH_3)_3^+$),173($CH_3(CH_2)_4$-$CHOSi(CH_3)_3^+$)	5,15-dihydroxyeicosatetraenoic acid ME TMS ether	Borgeat et al. (1982)
EI	229, 259	9-hydroxyoctadecanoic acid ME TMS ether	Mubiru et al. (2013)
EI	215, 273	10-hydroxyoctadecanoic acid ME TMS ether	Mubiru et al. (2013)
EI	213, 273	10-hydroxyoctadec-12-enoic acid ME TMS ether	Mubiru et al. (2013)
EI	227, 259	9-hydroxyoctadec-12-enoic acid ME TMS ether	Mubiru et al. (2013)
EI	173, 313	13-hydroxyoctadec-9-enoic acid ME TMS ether	Mubiru et al. (2013)
EI	259 ($CH_3O_2C(CH_2)_7$-$CHOSi(CH_3)_3^+$), 215 ($CH_3(CH_2)_7CHOSi(CH_3)_3^+$)	9,10-dihydroxyoctadecanoic acid ME TMS ether	Street et al. (1996)
EI	299 ($CH_3O_2C(CH_2)_7CH=CH$-CH_2-$CHOSi(CH_3)_3^+$), 275 (($CH_3(CH_2)_4CHOSi(CH_3)_3$-$CHOSi(CH_3)_3^+$), 173 ($CH_3(CH_2)_4CHOSi(CH_3)_3^+$)	12,13-dihydroxyoctadec-9-enoic acid ME TMS ether	Street et al. (1996)
EI	361 ($CH_3O_2C(CH_2)_7CHOSi(CH_3)_3$-$CHOSi(CH_3)_3^+$), 271 (361-trimethylsilanol), 259 ($CH_3O_2C(CH_2)_7$-$CHOSi(CH_3)_3^+$), 213 (($CH_3(CH_2)_4CH=CH$-$CH_2CHOSi(CH_3)_3^+$)	9,10-dihydroxyoctadec-12-enoic acid ME TMS ether	Street et al. (1996)
EI	275 ($CH_3(CH_2)_4$ $CH(OSi(CH_3)_3)$-$CHOSi(CH_3)_3^+$), 173 ($CH_3(CH_2)_4CHOSi(CH_3)_3^+$)	12,13-dihydroxyoctadeca-6,9-dienoic acid ME TMS ether	Street et al. (1996)

Ionization	Major ions (fragments)	Systematic name	Reference
EI	339 ($CH_3O_2C(CH_2)_7$-CH=CH-CH_2-CH=CH-$CH_2CHOSi(CH_3)_3^+$), 233($CH_3CH_2CH(OSi(CH_3)_3^+$)-$CHOSi(CH_3)_3^+$), 131 ($CH_3CH_2CHOSi(CH_3)_3^+$)	15,16-dihydroxyoctadeca-9,12-dienoic acid ME TMS ether	Street et al. (1996)
EI	259, 171	9, 13-dihydroxyoctadeca-11,15-dienoic acid ME bisTMS ether	Miakar and Spiteller (1994)
EI	299, 273, 131	12, 16-dihydroxyoctadeca-9,13-dienoic acid ME bisTMS ether	Miakar and Spiteller (1994)
PCI (ammonia)	404 ($M+NH_4$), 387 (M+H), 314 ($M+NH_4$-90), 297(M+H-90)	12-hydroxyoctadecanoic acid ME TMS ether	Plattner et al. (1983)
PCI (isobutane)	387(M+H), 385 (M-H), 297(M+H-90), 301, 187	12-hydroxyoctadecanoic acid ME TMS ether	Plattner et al. (1983)
Keto-			
EI	422(M),390 (M-32), 312 ($CH_3O_2C(CH_2)_{15}$-C^+(O)-CH_3) , 297 ($CH_3O_2C(CH_2)_{15}$-C^+(O))	17-oxohexacosa-20-enoic acid ME	Kleiman and Spencer (1973)
EI	310(M),279 (M-31), 239 ($CH_3O_2C(CH_2)_7$-CH=CH-$(CH_2)_2$-C $^+$(O)), 99 ($CH_3(CH_2)_4$-C^+(O))	13-oxooctadec-9-enoic acid ME	Weihrauch et al. (1974)
EI	310(M),279 (M-31), 185 ($CH_3O_2C(CH_2)_7$-C^+(O)), 153 ($CH_3(CH_2)_4$-CH=CH-$(CH_2)_2$-C^+(O))	9-oxooctadec-12-enoic acid ME	Weihrauch et al. (1974)
EI	310(M), 239 ($CH_3O_2C(CH_2)_5$-C^+(O) $(CH_2)_3$CH=CH-CH_2^+), 181 ($CH_3(CH_2)_5$-CH=CH$(CH_2)_3C^+$(O)), 157 ($CH_3O_2C(CH_2)_5$-C^+(O), 125(CH_3-$(CH_2)_5$CH=CH-CH_2^+)	7-oxooctadec-11-enoic acid ME	Daulatabad and Jamkhandi (1997)
EI	262, 249, 234, 206, 192	3-pyridylcarbinol 7-oxostearate	Brechany and Christie (1992)
EI	304, 291, 276, 248, 234	3-pyridylcarbinol 10-oxostearate	Brechany and Christie (1992)
EI	302, 274, 260, 234	3-pyridylcarbinol 12-oxooctadec-9-enoate	Brechany and Christie (1994)
EI	304, 276, 248, 234	3-pyridylcarbinol 10-oxooctadec-14-enoate	Brechany and Christie (1994)

Ionization	Major ions (fragments)	Systematic name	Reference
PCI(isobutane)	313(M+1),281(M+1-32)	13-ketooctadecanoic acid ME	Plattner et al. (1983)
Multi-oxygenated			
EI	241($CH_3(CH_2)_4CH$=CH-CH(OTMS)-$CHOCH^+$), 199($CH_3O_2C(CH_2)_7CHOCH^+$) or 199($CH_3(CH_2)_4CH$=CH-CH(OTMS)$^+$)	9,10-epoxy-11-hydroxy-12-octadecenoic acid ME TMS ether	Gardner et al. (1974)
EI	327($CH_3O_2C(CH_2)_7CH$=CH-CH(OTMS)-$CHOCH^+$), 298($CH_3O_2C(CH_2)_7CH$=CH-CH(OTMS)-CH^+), 285($CH_3O_2C(CH_2)_7CH$=CH-CH(OTMS)$^+$), 241($CH_3(CH_2)_4$-CHOCH-CH(OTMS)-CH=CH^+)	12,13-epoxy-11-hydroxy-9-octadecenoic acid ME TMS ether	Gardner et al. (1974)
PCI(isobutane)	325 (M+H), 309 (M+H-16), 307 (M+H-18), 293 (MH-32)	9,10-*trans*-epoxy-13-keto-*trans*-11-octadecenoic acid ME and 12,13-*trans*-epoxy-9-keto-*trans*-11-octadecenoic acid ME	Plattner et al. (1983)

[a] methyl ester is abbreviated to ME.

In electron ionization (EI), fragmentation is usually distinct for different structures. However, whether a mass spectrum is informative or not depends on the type of derivative and the location of functional groups. Direct analysis of methyl esters of oxygenated fatty acids has been conducted by GC-MS, where molecular ions are barely detectable for epoxy-, hydroxy-, and keto-FAMEs (Kleiman & Spencer, 1973; Weihrauch, Brewington, & Schwartz, 1974). EI fragmentation can provide information on the location of a hydroxy or a keto group in the chain, as cleavages occur on each side of the functional group (Figure 2.3a and 2.3b) (Weihrauch et al., 1974; Wilson et al., 1997). For keto-FAMEs, both cleavages α- and β- to the carbonyl group take place (Figure 2.3b) (Weihrauch et al., 1974). When a double bond is present, allylic cleavage results in more intense ions than α- and β-cleavages to the carbonyl group, such as that observed with methyl 7-ketooctadec-11-enoate (Daulatabad & Jamkhandi, 1997). The least characteristic spectra are found for epoxy methyl esters, where only a single diagnostic ion is formed. For methyl 9,10-epoxyoctadecanoate, for example, the diagnostic ion is *m/z* 155 (Kleiman & Spencer, 1973); the same diagnostic ion was found for methyl 9,10:12,13-diepoxyoctadecanoate (Blee & Schuber, 1990) and methyl 9,10-epoxyoctadec-12-enoate (Piazza, Nuñez, & Foglia, 2003). Two mechanisms of formation of *m/z* 155 have been proposed in previous work (Kleiman & Spencer, 1973; Piazza et al., 2003): 1) by cleavage on each side of the epoxy group and loss of *m/z* 157 [$(CH_2)_7COOCH_3$] (Figure 2.3c); and 2) by cleavage between the C-O bond in the epoxy group and generation of *m/z* 155 from loss of CH_3OH from *m/z* 187 $[HO{=}CH(CH_2)_7COOCH_3]^+$ (Figures 2.3d, 2.3e, and 2.3f) (Piazza et al., 2003). Mubiru et al. (2013) presented spectra for monoepoxy FAMEs with zero, one, and two sites of

unsaturation, where characteristic ions were identified for each epoxy FAME. Although the spectra of unsaturated epoxy FAMEs were distinguishable from each other, the ions with $m/z < 100$ were intense and the relative intensities of the diagnostic ions were low, which caused difficulties in determining the location of epoxy groups and double bonds in unknown structures.

Compared to EI, chemical ionization (CI) is a lower energy process and results in less fragmentation in the spectra so structure identification can be difficult, but molecular weights of oxygenated FAMEs can be obtained (Plattner, Gardner, & Kleiman, 1983; Fankhauser-Noti, Fiselier, Biedermann-Brem, & Grob, 2006). A significant advantage of CI is that retention times of the target peaks are the same as EI when employing the same GC conditions.

The functional groups in FAMEs can also be derivatized to increase sensitivity and selectivity for a specific class of compounds and, in some cases, to produce better peak shape in chromatograms (Brondz, 2002). Adsorption of hydroxy fatty acids on GC columns results in poor peak shape in the chromatogram (Orata, 2012). This can be solved by derivatization of the hydroxy groups using various methods, including alkylsilyl (Poole, 2013), acyl (Orata, 2012), and methoxy derivatives (Wilson et al., 1997).

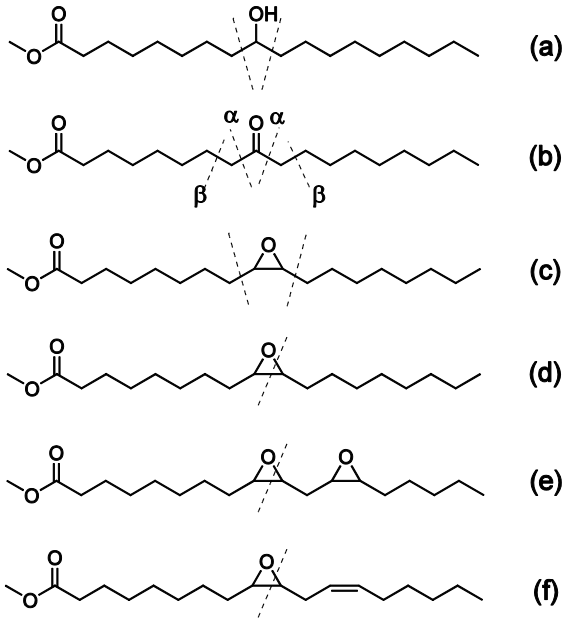


Figure 2.3 Major fragmentations of oxygenated FAMEs in EI.

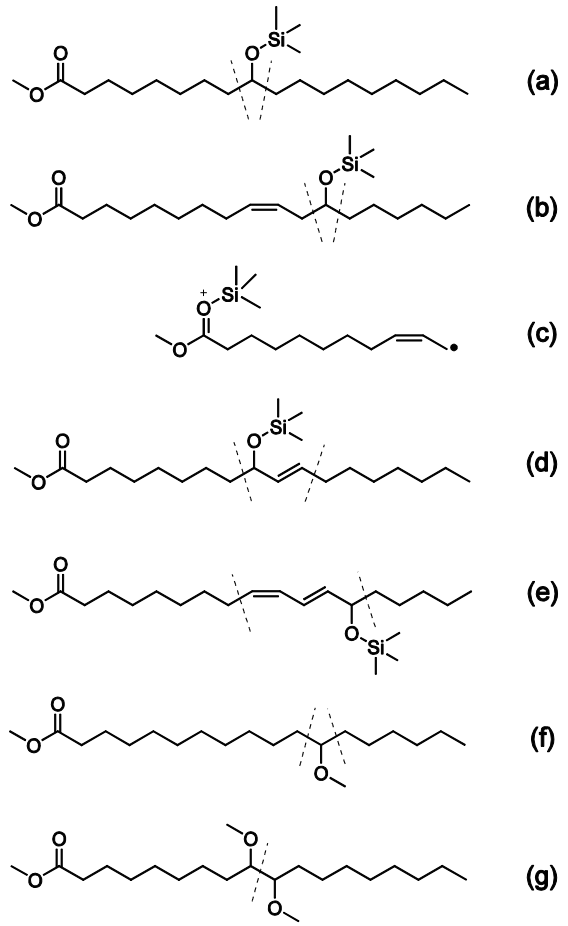


Figure 2.4 Major fragmentations of derivatives of hydroxy FAMEs in EI.

Alkylsilyl derivatives include the trimethylsilyl (TMS) derivative, one of the most widely used for hydroxy groups (Poole, 2013). In general, a major peak of *m/z* 73 and a few intense ions of large mass indicate the presence of a TMS group in a FAME. In the spectra of saturated TMS derivatized hydroxy FAMEs, intense ions were found representing the cleavage on each side of the carbon attached to the trimethylsiloxy (OTMS) groups (Figure 2.4a). However, this rule does not apply to unsaturated hydroxy FAMEs, and the fragmentation highly depends on the relative location of the hydroxy groups and the double bonds. When at least one methylene group separated the hydroxy groups from the double bond, cleavages occurred on both sides of the OTMS group (Figure 2.4b), while a 'migration ion' with an even-numbered mass was generated from the movement of the TMS group to the ester group (CH_3-O-C($OTMS^+$)), such as that observed with methyl ricinoleate (Figure 2.4c) (Kleiman & Spencer, 1973). The 'migration ion' has also been found for pyrrolidides of TMS-derivatized hydroxy fatty acids (Tulloch, 1985). When the OTMS group was allylic to a double bond or a conjugated diene, the location of the corresponding allylic or conjugated dienol system was indicated by two of the major ions in the spectra, as for methyl 9-hydroxy-10-octadecenoate (Figure 2.4d) and 9-hydroxy-10,12-octadecadienoate (Figure 2.4e), where cleavages occurred on both sides of the allylic or conjugated dienol system (Kleiman & Spencer, 1973). The assignments of the ions presented by Kleiman and Spencer (1973) have been useful for the identification of hydroxy fatty acids derived from oxidation of edible oils, although several chromatographic peaks remained unknown (Mubiru et al., 2013). Moreover, the rule that allylic OTMS cleaved as a whole in EI spectra has been extensively used to characterize methyl esters and pentafluorobenzyl (PFB) esters of

monohydroxy fatty acids containing more than two double bonds (Wheelan, Zirrolli, & Murphy, 1995). However, that rule did not apply to TMS-dihydroxy fatty acid PFB esters with three conjugated double bonds (Wheelan et al., 1995), which means that it remains challenging to interpret spectra derived from PUFAs with more than three double bonds, as in fish oils, without reference standards of hydroxy fatty acids.

Common methods for acylating hydroxy groups use carboxylic acid anhydride and fluorinated reagents. Fluorinated reagents, such as heptafluorobutyrate anhydride (HFBA), have been used in combination with negative chemical ionization (NCI) to improve quantification limits of hydroxy fatty acids, as discussed in the next section. Structural interpretation of HFBA derivatives has been studied for 2- and 3-hydroxy saturated FAMEs (Pons et al., 2000). However, precise determination of the position of the double bond in unsaturated hydroxy FAMEs was not possible (Pons et al., 2000) and little information regarding hydroxy fatty acids as oxidation products was provided.

Methoxy derivatives are more stable than TMS derivatives and have been used to identify hydrogenated C18 and C20 fatty acids with hydroxy groups in several locations (Wilson et al., 1997). Hydrogenation simplified the interpretation of spectra with fragments formed by cleavages of the fatty acid chain on both sides of the methoxy group, giving two intense ions in the spectrum (Figure 2.4f) (Wilson et al., 1997).

Few derivatization methods have been developed for epoxides since reactions result in ring opening. Methanolysis of epoxy FAME was discussed by Kleiman and Spencer (1973), where the use of BF_3-methanol resulted in ring opening of the epoxy group. The reaction produced a hydroxy-methoxy derivative. In an earlier work, this reaction was applied to epoxy fatty acids, followed by conversion of the resulting

hydroxy group using tetramethylammonium hydroxide and production of dimethoxy products (Wilson & Lyall 2002). Two major fragments were formed from the cleavage between the two vicinal methoxy groups (Figure 2.4g), characteristic in MS spectra (Wilson & Lyall 2002).

Ketones do not contain active hydrogens as do alcohols. Derivatization can be made using pentafluorobenzyl hydroxylamine (PFBHA) to convert keto-fatty acids into their O-2,3,4,5,6-pentafluorobenzyl oximes (PFB oxime) (Figure 2.5a). Generally, in EI, *m/z* 181 characterized PFB oximes as 181 is the mass of the $[C_6F_5CH_2]$ moiety (Hachey, Patterson, Reeds, & Elsas, 1991). When present, *m/z* $[M-181]^+$ resulted from the loss of the $C_6F_5CH_2$, and *m/z* $[M-197]^+$ originated from the loss of the $C_6F_5CH_2O$ moiety. In contrast to EI, *m/z* 167 $[C_6F_5]^-$ and *m/z* 197 $[C_6F_5CH_2O]^-$ were characteristic ions for the PFB oxime in NCI (Hachey et al., 1991; Schulze, Lauchli, Sonwa, Schmidt, & Boland, 2006), with methane as the reagent gas. The combination of TMS and PFBHA derivatizing reagents was also employed for the identification of ketols (Schulze et al., 2006). In α- and γ-ketols, hydroxy groups were derivatized using TMS, and α-cleavage of the OTMS group resulted in ions in the spectra indicating the location of their OTMS groups (Figure 2.5b). The derivatization method did not contribute to isomerization or artifacts when applied to unsaturated keto-FAMEs with conjugated double bonds or α- and γ-ketols (Schulze et al., 2006). An exception was 9,12-dioxododec-10-enoic acid which went through enolization in the presence of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) to form methyl 12-O-2,3,4,5,6-pentafluorobenzyl oximino-9-trimethylsilyloxy-8,10-dodecadienoate (Gallasch & Spiteller 2000). Overall, structural

information can be obtained using the combination of EI and NCI spectra of PFB oximes, although their applications in lipid oxidation have not been reported.

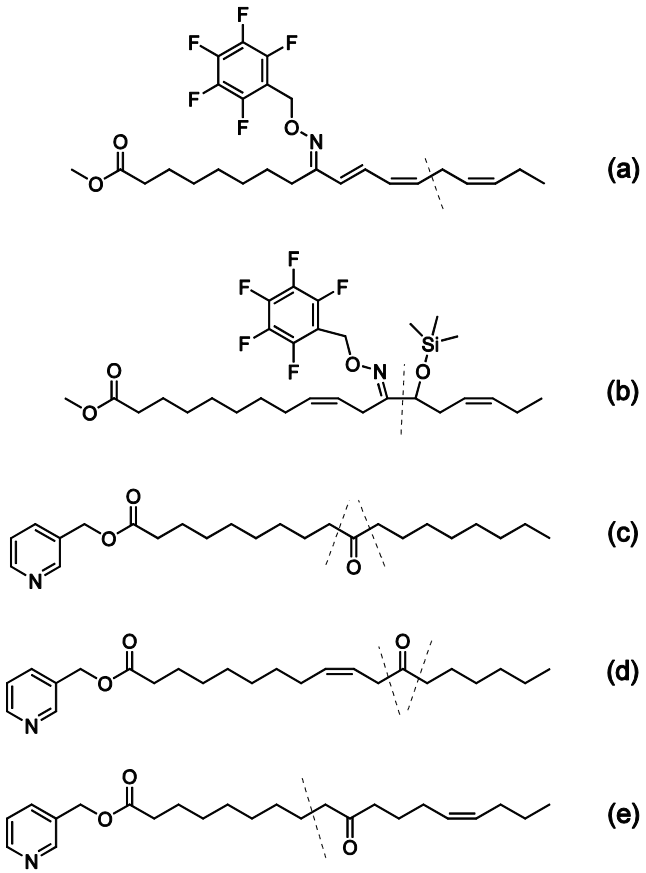


Figure 2.5 Major fragmentations of derivatives of keto fatty acids in EI.

In addition to FAMEs, 3-pyridylcarbinol esters have been employed to identify keto fatty acids. A gap of 28 within an obvious series of ions separated by 14 indicated the keto position in several saturated 3-pyridylcarbinyl-oxo-stearates (Figure 2.5c) except for 3-pyridylcarbinyl 5-oxo-stearate (Brechany & Christie, 1992). In spectra of unsaturated keto fatty acid 3-pyridylcarbinol esters, the gap of 28 *m/z* in the spectrum continued to indicate the location of the keto group, while the double bond position was determined by a gap of 26, although conjugated structures were not involved in the study

(Brechany & Christie, 1994). It was noted that when the double bond was separated from the keto functionality by only one methylene group, the cleavage α to the keto group was more dominant (Figure 2.5d); otherwise, the cleavage β to the keto group resulted in intense ions in the spectra (Figure 2.5e).

In summary, GC-MS is particularly useful in determining the location of oxygenated groups in the fatty acid chain, although derivatization is necessary. The use of derivatization requires more time to prepare samples for GC analysis but allows better interpretations of MS spectra, particularly when used with derivatives such as TMS ethers and PFB oximes.

2.6.3 Quantification of oxygenated fatty acids by GC

While GC-MS may help to identify the structures of the oxygenated fatty acids, quantitation of these compounds requires determination of retention times and response factors in GC-FID using standards of commercial fatty acids. The major challenge to measuring oxidized fatty acids in oils is that they are present at low concentrations. To make those products detectable, it is necessary to separate them from large amounts of the non-altered fatty acids, then concentrate them to detectable levels. One useful approach to such separation employs solid-phase extraction (SPE) with a silica column as the stationary phase. Dobarganes, Velasco, & Dieffenbacher (2000) applied this technique directly to oxidized fats and oils to separate the relatively polar compounds (DAGs, MAGs, and free fatty acids) from TAGs. SPE separates non-oxidized and oxidized FAMEs in steps, starting with a low polarity solvent mixture such as hexane:ethyl acetate 98:2 (v/v) (Jenske & Vetter, 2008), hexane: diethyl ether 95:5 (v/v)

(Velasco et al., 2002), and hexane: diethyl ether 98:2 (v/v) (Marmesat et al., 2008), to remove unaltered FAMEs, leaving oxidized FAMEs on the column. A relatively more polar solvent such as ethyl acetate or diethyl ether is then applied to elute the oxidized FAMEs from the silica column. This method has been considered a concentration step to quantify trace levels of oxidized fatty acids (Velasco et al., 2002; Jenske & Vetter, 2008; Marmesat et al., 2008). The SPE steps can also be modified for specific purposes. For example, Wilson et al. (1997) used three elutions on a silica column to separate monohydroxy fatty acids from dihydroxy fatty acids and other more polar compounds. Such SPE separations can be particularly useful in improving resolution with GC. For instance, Mubiru et al. (2013) also used three elutions on a silica column to elute epoxy fatty acids with n-hexane: diethyl ether (90:10, v/v), and they successfully removed co-eluting hydroxy fatty acid interferences from monoepoxy fatty acids. The pre-treatment methods and detection conditions are summarized in Table 2.3.

Table 2.3 GC quantitative methods and corresponding pre-treatment and detection conditions for analysis of oxygenated fatty acids in foods.

Analytes	Samples	Pre-treatment	Extraction condition	Detection	Column	LOD/LOQ	References
monohydroxy (plus hydroperoxy) fatty acids	nut oil	hydrogenation, transmethylation, SPE, and methylation of OH group	SPE: First elution: 95:5 hexane: ethyl acetate; Second elution: 80:20 hexane: ethyl acetate	MS-SIM	CP-Sil 19 fused silica (25 m × 0.25 mm i.d.)	LOD=0.2 ng (injection volume=1μL)	Wilson et al. (1997)
mono epoxy fatty acids	thermoxidized olive and sunflower oils	transmethylation and SPE	SPE: First elution: 95:5 hexane: diethyl ether; Second elution: diethyl ether	FID	DB-Wax fused silica (30 m × 0.25 mm i.d.)	not reported	Velasco et al. (2002)
mono epoxy fatty acids	thermoxidized olive and sunflower oils/ used frying oils	transmethylation and SPE	SPE: First elution: 95:5 hexane: diethyl ether; Second elution: diethyl ether	FID	HP-Innowax fused silica (30 m × 0.25 mm i.d.)	not reported	Velasco et al. (2004);
methyl diepoxy linoleate	oil/ sauce/ infant food	transmethylation, normal phase high performance liquid chromatography (NPLC) extraction	NPLC column: packed with a cyano phase (25 cm × 0.2 mm i.d.) Mobile phase: 20% methyl tert. butyl ether (MTBE)/ pentane	FID	A column coated with a 0.2 mm film of PS-255, a methyl polysiloxan (30 m × 0.25 mm i.d.)	LOD= 2mg/kg extract and LOQ= 6 mg/kg	Fankhauser-Noti et al. (2005); Fankhauser-Noti et al. (2006)

Analytes	Samples	Pre-treatment	Extraction condition	Detection	Column	LOD/LOQ	References
monoepoxy fatty acids	oils/snacks	transmethylation	N/A	FID	SP-2560 (100 m × 0.25 mm i.d.)	not reported	Fankhauser-Noti et al. (2006); Biedermann-Brem et al. (2007)
methyl mono-/ di-epoxy linoleate	oily foods	transmethylation	N/A	EI/CI-MS	Rtx 2330 (20 m × 0.25 mm i.d.)	LOD<1mg/kg (CI) LOQ=3mg/kg (EI)	Biedermann-Brem et al. (2007)
epoxy, keto and hydroxy fatty acids	thermoxidized sunflower oils	transmethylation, SPE, and hydrogenation	SPE: First elution: 98:2 hexane: diethyl ether; Second elution: diethyl ether	FID	DB-Wax fused silica (60 m × 0.25 mm i.d.)	LOQ=1.6-2.1 µg/mL	Marmesat et al. (2008)
keto and hydroxy fatty acids	milk fat	transmethylation and SPE	SPE: First elution: 98:2 hexane: diethyl ether; Second elution: diethyl ether	FID	DB-Wax fused silica (30 m × 0.32 mm i.d.)	not reported	Márquez-Ruiz et al. (2011)
mono epoxy fatty acids	arachid/colza/corn/frying/ olive/soya/ sunflower/ salad/mixed/ soya oils	transmethylation and SPE	SPE: First elution: 98:2 hexane: diethyl ether; Second elution: 90:10 hexane: diethyl ether; Third elution: 70:30 hexane: diethyl ether	FID	CP-Sil 88 (60 m × 0.25 mm i.d.)	LOD=1.45 µg/g and LOQ=2.9 µg/g	Mubiru et al. (2013)

Analytes	Samples	Pre-treatment	Extraction condition	Detection	Column	LOD/LOQ	References
mono epoxy fatty acids	oil/ crisps/ pork/ milk powder	modified Bligh and Dyer method, transmethylation and SPE	SPE: First elution: 98:2 hexane: diethyl ether; Second elution: 90:10 hexane: diethyl ether	FID	CP-Sil 88 (50 m × 0.25 mm i.d.)	LOD=1.7-10.2 µg/g and LOQ=3.3-20.5 µg/g	Mubiru et al. (2014)

With appropriate pre-treatment, including transmethylation and SPE, GC-FID has been reported to be a powerful tool to simultaneously determine epoxy, hydroxy, and keto fatty acids (Marmesat et al., 2008), and also monoepoxy fatty acids alone (Berdeaux et al., 1999; Velasco et al., 2002). In those studies, hydrogenation led to simpler chromatograms and better peak shapes for epoxy fatty acids. This step was particularly important for the resolution of keto and hydroxy fatty acids by GC as their peaks severely co-eluted in their unsaturated forms. An obvious disadvantage is that hydrogenation resulted in loss of information about number and position of double bonds in those hydroxy and keto fatty acids.

Unsaturated oxygenated fatty acids have been discussed, but the limited information concerning the identities of the GC peaks has prevented their quantification. Measurements of unsaturated monoepoxy fatty acids in fresh oils have been conducted by Mubiru et al. (2013). The limit of detection (LOD) and limit of quantification (LOQ) were 1.45 and 2.9 μg/g of oil, respectively. This method has also been applied to monitor epoxy fatty acid formation in fats, oils, and chocolates (Brühl, Weisshaar, & Matthäus, 2016).

Methoxy derivatives of C18 hydroxy FAME isomers were employed as markers of lipid peroxidation (Wilson et al., 1997), where hydrogenation was conducted to reduce hydroperoxy groups to hydroxy groups and to eliminate double bonds; thus, this approach did not differentiate between hydroxy and hydroperoxy fatty acids. Two ion fragments resulted from cleavage at each side of the OCH_3 group, thus giving the location of the original hydroxy/hydroperoxy group (Figure 2.4f) and enabling quantification in selective ion monitoring (SIM) mode. This approach has been applied to food and oil

samples, with a detection limit of 0.2 ng when monitoring a single isomer (Wilson et al., 1997). The method was further developed for simultaneous determination of hydroxy and epoxy fatty acids in human plasma, where hydroxy and epoxy fatty acids were converted into methoxy and dimethoxy derivatives, respectively (Wilson & Lyall 2002); however, no applications have been reported for food.

Fluorinated reagents, such as fluorinated anhydride and PFB chloride, have been used to analyze hydroxy FAMEs in biological samples where hydroxy groups were derivatized into fluorinated groups. The utilization of fluorinated derivatives with NCI increased sensitivity and selectivity for hydroxy fatty acids (Stan & Scheutwinkel-Reich 1980; Jenske & Vetter 2008). A recent study reported the detection limits to be 300 fg - 2 pg for 2-hydroxy FAME and 50 - 500 fg for 3-hydroxy FAME in bovine milk fat (Jenske & Vetter 2008). However, since fluorinated derivatives of hydroxy FAMEs have been seldom used for the study of lipid oxidation, their applicability to unsaturated fatty acids needs verification. Similar to hydroxy FAMEs, use of fluorinated reagents facilitated the quantification of keto FAMEs. With improved sensitivity in NCI mode, PFB derivatives have been mainly applied for quantification, with a detection limit of 0.1 pg (Hachey et al., 1991). No applications of these fluorinated derivatives have been reported for the study of lipid oxidation in food and oil.

In summary, GC has shown excellent sensitivity for the determination of oxidized fatty acids as minor products of lipid oxidation but there remain unresolved problems in co-elution and identification of unknown compounds. Co-elution results in even less characteristic spectra and further difficulties in identification of the peaks. Instead of traditional one-dimensional GC, two-dimensional GC, employing 2 columns of distinct

polarity, has also been used to analyze food fatty acids, which significantly improved the separations of both the major and the minor components (Hyötyläinen et al., 2004). This technique has potential in obtaining improved separations of complex oxygenated oxidation products in GC.

2.7 HPLC

2.7.1 HPLC Characterization of oxygenated TAG

HPLC has long been used to separate epoxy fatty acids (Gérard, Moreau, Fett, & Osman, 1992), hydroxy fatty acids (Bussell & Miller, 1979; Hennion et al., 1983; Bandi & Reynolds, 1985; Bandi & Ansari, 1986), and keto fatty acids (MacMillan & Murphycor 1995; Byrdwell & Neff 1999), with and without utilization of derivatives. Unlike GC, regular HPLC allows analysis of intact TAGs without derivatization, in addition to fatty acids in their acid or methyl ester forms.

Normal-phase HPLC (NP-HPLC) and reversed-phase HPLC (RP-HPLC) are classified based on the relative polarity of stationary and mobile phases, which are normally selected in developing HPLC methods for different purposes. NP-HPLC, employing a polar stationary phase and non-polar mobile phase, is more often used for analysis of polar lipids such as fatty acids in their free or methyl ester forms or polar oxidation products, particularly hydroperoxides. In contrast, RP-HPLC uses a non-polar stationary phase and a polar mobile phase to separate non-polar compounds such as TAGs. However, there are exceptions: 1) NP-HPLC may result in high retention of oxidation products on the column and even possible oxidation (Zeb, 2015); and 2) compared to RP-HPLC, which was excellent at separating individual TAGs by molecular

size, NP-HPLC proved to be a better choice to separate TAGs according to their polarities, especially when a specific functional group or a class was desired (Steenhorst-Slikkerveer et al., 2000).

Although HPLC allows analysis of intact TAGs, it is a challenging task because a large number of individual TAG species are encountered as a result of the many possible combinations of esterified fatty acids (Buchgraber, Ulberth, Emons, & Anklam, 2004). In addition, TAGs are normally homologs or regio- and geometric isomers overlapping with each other in chromatograms (Buchgraber et al., 2004). A third problem is that separations focusing on TAGs necessarily lose small scission products at the solvent front. Detection of all products requires use of complex solvent gradients for elution.

Because standards cannot be found for every conceivable product or TAG, HPLC coupled with MS has been useful in identifying their structures in vegetable oil, lard, and plasma samples (Marai, Myher, & Kuksis, 1983). Among the MS techniques that have been applied with HPLC, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been the most discussed techniques to analyze intact TAGs, while thermospray ionization (TSP) has been mainly applied for fatty acids and FAMEs. In contrast to GC-MS, these MS techniques are soft ionization techniques; therefore, diagnostic ions result from molecular ions, and DAG fragments are more often used for identification of compounds than are the fragments generated from cleavage in the hydrocarbon chain.

While APCI and ESI have been both applied to identify lipid oxidation products, APCI was introduced earlier than ESI for this application and is thought to produce more fragmentation than ESI (Byrdwell, 2001). The analysis of lipid samples using HPLC-

APCI has been reviewed by Byrdwell (2001), where studies on applications of HPLC-APCI to oxygenated fatty acids and oxygenated TAGs were both discussed. Here, we summarize that material to allow for a comparison with HPLC-ESI. In terms of analyzing intact oxygenated TAGs, HPLC-APCI has been applied to a variety of naturally occurring TAGs containing epoxy groups (Neff & Byrdwell 1995a) and hydroxy groups (Byrdwell & Neff, 1998), as well as mono- and diepoxides (Neff & Byrdwell, 1998) and a ketone (Byrdwell & Neff, 1999) derived from the oxidation of TAG standards.

Using the APCI technique, TAGs were generally identified by the proton adduct of molecular ion $[M+H]^+$ and $[M-RCOO]^+$ ions resulting from 1, 2-DAG, 2, 3-DAG and l, 3-DAG fragments (Neff & Byrdwell, 1995b). The relative intensities of the protonated molecular ion and the DAG ions were closely related to the degree of unsaturation in the TAGs, where higher unsaturation led to higher abundance of $[M+H]^+$ ions (Byrdwell & Emken, 1995); protonated molecular ions were not observable in the mass spectra of saturated TAGs (Byrdwell & Emken, 1995).

With oxidized TAGs, the protonated molecular ions were present, while the molecular adducts derived from the mobile phase further confirmed the molecular weight of the oxidation products, for example, $[M+23]^+$ and $[M+39]^+$ as acetonitrile adducts (Neff & Byrdwell, 1998; Byrdwell & Neff, 1998). Two different mechanisms have been reported for non-methylene-interrupted and methylene-interrupted situations for a double bond and an epoxy group (Neff & Byrdwell, 1995a, 1998). When an epoxy group was adjacent to a double bond (non-methylene-interrupted), $[M-O+H]^+$ was observed due to the loss of an oxygen. In contrast, when the epoxy group was not directly adjacent to a

double bond, as in vernolic acid (12, 13-epoxyoctadec-*cis*-9-enoic acid), $[M-H_2O+H]^+$ was observed due to the loss of H_2O (Neff & Byrdwell, 1995a, 1998).

A ketone was also characterized in APCI (Byrdwell & Neff, 1999), but the spectra of keto-OOS (dioleoyl-9-ketostearoyl-glycerol) and epoxy-OOS (dioleoyl-9,10-epoxy stearyl-glycerol) both showed major ions of *m/z* 603 and *m/z* 619, resulting from DAG fragments. Only the low intensities of high-mass ions and the retention times could enable the identification of the keto-OOS.

For hydroxy TAGs, using an acetonitrile/methylene chloride solvent system, four acetonitrile adducts $[M+23]^+$, $[M+39]^+$, $[M+54]^+$, and $[M+59]^+$ were formed. The base peak for mono-, di-, and trihydroxy TAGs was $[M-nH_2O+H]^+$, where n is the number of hydroxy groups, thus allowing determination of the number of hydroxy groups in TAGs (Byrdwell & Neff, 1998).

ESI was introduced more recently than APCI in lipid oxidation studies. ESI analysis of non-oxygenated TAGs produced molecular adduct ions derived from the mobile phase instead of protonated molecules (Holčapek, Jandera, Zderadička, & Hrubá, 2003), with low or no abundance of DAG fragments, in contrast to APCI. Although relative abundances of protonated DAG fragments were low in ESI spectra, identification of individual acyl chains of a TAG was possible (Cozzolino & De Giulio, 2011). However, when DAG fragments were absent, a tandem mass spectrometry (MS/MS) technique was applied to characterize the ammonia adduct of the molecular ion (Duffin, Henion, & Shieh, 1991). By increasing the collision energy, fatty acids were identified by the DAG fragments, which resulted from the loss of a fatty acid from the ammoniated adduct (Duffin et al., 1991).

Giuffrida, Destaillats, Skibsted, and Dionisi (2004) studied the MS/MS spectra of ammonium adducts of saturated epoxy TAG standards. The MS/MS fragmentation pathway was confirmed by analysis of hydroperoxides and epoxides derived from $^{18}O_2$ oxidation of pure TAG standards in sealed containers. For interpretation of MS/MS spectra of epoxy TAGs, the DAG product ion was prone to dehydration, which resulted in an ion formed by loss of $H_2{}^{18}O$ from the DAG ion. The use of the stable isotope characterized the epoxide fragmentation pathway. Comparison of the spectra confirmed the mono-oxygenated TAG, which was derived from thermo-oxidation, to be an epoxide, not a ketone, although a keto-TAG and an epoxy-TAG would have the same molecular mass (Giuffrida et al., 2004).

When determining oxygenated TAGs as minor or trace amounts in complex biological samples, MS/MS alone was not enough to make correct assignments and the use of MS^3 enabled more complete and unambiguous molecular identifications (McAnoy, Wu, & Murphy, 2005). ESI-MS has been applied to characterize TAGs containing naturally-occurring hydroxy fatty acids such as ricinoleic acid (12-hydroxyoctadec-9-enoic acid) and lesquerolic acid (14-hydroxyeicos-11-enoic acid) in plant oils with subsequent fragmentation using MS/MS, MS^3, and MS^4 (Lin &Arcinas, 2008; Lin, Arcinas, & Harden, 2008; Lin 2009; Lin & Chen, 2010; Lin & Chen, 2014). MS^n has been mainly used for the study of naturally-occurring hydroxy fatty acids rather than oxidation products.

Recently, Zeb and Murkovic (2010) added ammonium acetate (0.05%) and sodium acetate (0.001%) to HPLC solvents to increase the ionization efficiency in ESI, which facilitated identification of the molecular adducts of TAGs. Under these

conditions, MS/MS was not required for identification. The prominent ions observed with APCI were still applicable. For example, an epoxy TAG gave $[M+H-H_2O]^+$ in addition to its sodium or potassium adduct, while *m/z* 603.5 and 617.5 could differentiate between dioleoyl glycerol and mono-epoxy dioleoyl glycerol ions. Similarly, TAGs containing an epoxy group and a double bond gave DAG fragments of *m/z* 601.5 and 615.5 as well as $[M+H-H_2O]^+$. For the identification of triolein diepoxide, the epoxy DAG fragments also played an important role, giving ions of *m/z* 617.4 and m/z 631.5 (Zeb & Murkovic, 2010). This approach has been applied to the study of oxidation products, including epoxides, epoxy-epidioxides, epoxy-hydroperoxides, hydroxy-epidioxides, and hydroxy-hydroperoxides derived from olive oil (Zeb & Murkovic, 2011), corn oil (Zeb & Murkovic, 2013), and camellia oil (Zeb, 2012).

Structural identification from ESI-MS was supplemented with information about product structures derived from elution orders of synthetic oxygenated TAGs, such as epoxy and hydroxy TAGs, with RP-HPLC-ELSD, using a linear gradient of 20–80% isopropanol in methanol as eluting solvent (Sjövall, Kuksis, Marai, & Myher, 1997). Specifically, the effect of double bonds and functional groups on retention times was estimated using a plot of the theoretical carbon numbers of both non-oxygenated and oxygenated TAG standards versus their retention times. The theoretical carbon numbers of oxygenated TAGs were obtained by applying correction factors, assuming that the correction factors of saturated TAGs were zero. With reference to a series of the saturated TAG standards, the incremental elution factors of the saturated and unsaturated oxidized TAGs were calculated for the distribution of functional groups and double bonds in TAGs. Therefore, when analyzing oxidized TAGs, by referring to the retention times of

saturated TAG standards, the elution factors of unknown oxygenated TAG could be estimated, which allowed identification of oxidized TAGs (Sjovall, Kuksis, & Kallio, 2001). Based on this research, keto-TAG were also synthesized and identified using elution factors (Suomela, Ahotupa, Sjövall, Kurvinen, & Kallio, 2004a). This method has been mainly applied to the study of TAG oxidation in biological samples rather than foods (Suomela, Ahotupa, Sjövall, Kurvinen, & Kallio, 2004b; Suomela, Ahotupa, & Kallio, 2005).

ESI and APCI have also been used for a dual parallel MS approach to determine the oxidation products produced in canola oil (Byrdwell & Neff, 2002). While APCI was more sensitive to saturated TAGs than unsaturated TAGs, ESI showed the opposite being more sensitive to unsaturated TAGs than saturated TAGs, and was generally more sensitive overall to TAGs (Byrdwell & Neff, 2002). With 20 mM ammonium formate solution in H_2O/ACN (1:4) at 20 μL/min, ESI produced abundant $[M+NH_4]^+$ ions for hydroxy- and keto-TAGs, which barely had a proton adduct $[M+H]^+$ using APCI. Using the dual parallel MS approach, APCI and ESI gave complementary information about the structure of TAGs.

Comprehensive two-dimensional liquid chromatography has been employed to detect oxygenated TAGs but has yet to be applied to the study of lipid oxidation products (van der Klift, Vivó-Truyols, Claassen, van Holthoon, & van Beek, 2008). Eight epoxy TAGs were identified in corn oil by APCI. These oxygenated TAGs were shown to be minor components by evaporative light-scattering detection (ELSD), although quantitative results were not shown (van der Klift et al., 2008).

2.7.2 HPLC characterization of oxygenated fatty acids

Compared to the direct analysis of TAGs, fewer HPLC studies have been reported for oxygenated fatty acids in their free or methyl ester forms. In the literature, reports of RP-HPLC-APCI applied to oxygenated fatty acids have been limited to hydroxy fatty acids in the literature (Ikeda & Kusaka, 1992; Kusaka & Ikeda, 1993). Ikeda and Kusaka (1992) compared several amide derivatives for the analysis of fatty acids from C_{10} to C_{30}, including several hydroxy fatty acids. With N-propyl amide derivatization, the yield of the reaction could be quantitative, although the location of the hydroxy group in the fatty acid chain had an effect on its CI spectra such that 2-hydroxy fatty acids had a base ion of $[M+H]^+$, while the spectra of 12-hydroxy fatty acids, including 12-hydroxy-C18:0 and ricinoleic acid, showed a base peak of $[M-H_2O+H]^+$. Kusaka and Ikeda (1993) investigated 3-methyl-7-methoxy-1,4-benzoxazin-2-one derivatives of hydroxy fatty acids with HPLC-APCI, where the base peak was $[M+H-H_2O]^+$, while $[M+H]^+$ was small. However, the same derivatives of 2-hydroxy-C18:0 and 12-hydroxy-C18:0 gave similar spectra (Kusaka & Ikeda, 1993); therefore, the technique gave no indication of the location of the hydroxy group. In a different approach, RPLC–ESI/APCI-MS/MS analysis in negative ion mode was useful in identifying epoxy and hydroxy fatty acids in biological samples, as cleavage of bonds occurred in the oxirane ring of epoxy PUFAs and adjacent to the hydroxy groups in hydroxy PUFAs (Bylund, Ericsson, & Oliw, 1998), similar to fragmentations observed using GC-MS.

RP-HPLC coupled with TSP has been reviewed for its applications to characterize oxidation products of PUFAs, including acetyl-derivatized and underivatized hydroxy fatty acids, epoxy fatty acids, and epoxyhydroxy fatty acids (Yamane, 2002). The TSP-

interface temperature was optimized for high sensitivity in positive ion mode. Epoxy fatty acids and epoxyhydroxy fatty acids gave major ions of $[M+H]^+$, $[M+NH4]^+$, $[M+Na]^+$ and $[M+H-H_2O]^+$ (Yamane, Abe, & Yamane, 1994). With the electron beam on, cleavage occurred at the epoxy ring and formed ions indicating the location of the epoxy group (Yamane et al., 1994). Underivatized hydroxy PUFAs gave a single base ion of $[M+H-H_2O]^+$, different from epoxy fatty acids. However, it was not possible to locate the hydroxy group in underivatized hydroxy-PUFAs because cleavage of the bond near the hydroxy group was not observed in the spectra. For underivatized polyhydroxy-PUFAs, stepwise elimination of water molecules from the hydroxy groups generated many fragment ions and decreased sensitivity. Polyhydroxy-PUFAs with large ranges of polarity were necessarily separated using a gradient elution solvent; however, the composition of the mobile phase was altered on the TSP-interface and this change affected the sensitivity. Therefore, it was necessary to use simultaneous detection of deuterium-labeled internal standards and analytes to ensure reasonable reproducibility (Yamane, 2002). With acetylation, the range of polarities of polyhydroxy-PUFAs was reduced to a limited level (Yamane & Abe, 1992). Acetylation of hydroxy- and hydroperoxy-PUFAs gave a $[M + H - n\times(60)]^+$ (n= number of the hydroxy/hydroperoxy groups) ion as the base ion, which was generated by eliminating acetic acid, for the identification of hydroxy and hydroperoxy acids in the spectra; for hydroperoxy-PUFAs, a $[M + H - n(60) - n(H2O)]^+$ ion was also present (Yamane & Abe, 1992; Yamane, Abe, Yamane, & Ishikawa, 1992). Sensitivity was much improved with acetylation as a result of less fragmentation and higher intensity of the base ion (Yamane & Abe, 1992). TSP in the discharge mode facilitated the observation of molecular ions of analytes at low levels

(Chapman, Pfannkoch & Kupper, 1994). Oxygenated FAMEs were identified based on both the molecular weights and fragmentation diagnostics for oxygenated functional groups, which resulted from the loss of a methanol and two water molecules from $[M+H]^+$ (Chapman et al., 1994).

Overall, HPLC analysis of oxygenated fatty acids and TAGs does not allow determination of the location of functional groups in fatty acid chains. In this aspect, GC-MS is more useful. However, in terms of applications to food samples, where most oxygenated fatty acids are expected to be present in TAGs, HPLC is a powerful approach to analyze intact TAG by taking advantage of simpler procedures. In addition, HPLC allows the determination of oxygenated acyl chains in TAGs which is not possible with GC since derivatization is necessary.

2.7.3 Quantification by HPLC

Quantification of oxygenated products with HPLC techniques has been mainly reported for their FAME forms (Table 2.4). As mentioned in the GC section, base-catalyzed methylation methods have been considered more suitable for oxygenated fatty acids, particularly with conjugated structures. Two transmethylation methods employing KOH/methanol and sodium methoxide were compared using HPLC-UV by Morales, Marmesat, Dobarganes, Márquez-Ruiz, and Velasco (2012b). Losses of hydroperoxy-dienes were ~90% wt. in KOH/methanol but less than 10% wt. in sodium methoxide/methanol. Therefore, transmethylation with sodium methoxide was shown to

Table 2.4 HPLC quantitative methods and corresponding pre-treatment and detection conditions for analysis of oxygenated fatty acids in foods.

Analytes	Samples	Pre-treatment	Stationary phase	Mobile phase	Detection	LOD/LOQ	Reference
Normal-phase							
epoxy- and hydroxy fatty acids	free fatty acid standards	esterification with diazomethane	Si 60 (10 cm×3 mm)	binary gradient: Eluent A(hexane/ isopropanol/ acetic acid (99.3:0.5:0.2)) and Eluent B(hexane/ isopropanol/ acetic acid (79.8:20:0.2))	ELSD	LOD=1 µg (nonlinear response below 10 µg)	Gérard et al. (1992)
hydroxy- and ketodienes	methyl linoleate hydroperoxides	N/A	Si (25 cm×2.1 mm, 5 µm particle size)	85:15 hexane: diethyl ether	UV	absolute contents not reported	Hopia et al. (1996)
epoxy-, hydroxy-, and oxo-TAG	rapeseed oil and linseed/ safflower oil	N/A	Si (25cm×2.1mm , 5 µm particle size)	gradient starting with pure n-hexane to a mixture of n-hexane/ isopropyl alcohol	ESI-MS	LOD=5 ng for total ion chromatogram and LOD=0.1-0.5 ng for extracted ion chromatogram	Steenhorst-Slikkerveer et al. (2000)

Analytes	Samples	Pre-treatment	Stationary phase	Mobile phase	Detection	LOD/LOQ	Reference
hydroxy- and ketodienes	sunflower oils	transmethylation	Si 60, 5 µm particle size	82:18 heptane: diethyl ether	UV	LOD=0.1 µg/mL and LOQ=0.3 µg/mL for ketodienes; LOD=0.2 µg/mL and LOQ=0.6 µg/mL for hydroxydienes	Morales et al. (2010); Morales et al. (2012b) Morales et al. (2012c)
epoxy fatty acids, and hydroxy- and ketodienes	methyl oleate and methyl linoleate/ sunflower oil/ soybean oil/ rapeseed oil	N/A	Si 60 (25 cm×4 mm, 5 µm particle size)	82:18 heptane: diethyl ether	ELSD nebulizer temperature: 35 ◦C tube temperature: 40 ◦C gas flow: 25 psi nebulization gas: nitrogen	LOD=0.9-5.2 µg/mL and LOQ=2.5-11.6 µg/mL	Morales et al. (2012a); Morales et al. (2014)
Reversed-phase							
TAG containing ricinoleic acid	castor oil	N/A	C_{18} (25 cm×4.6 mm, 5 µm particle size)	linear gradient: starting with 100% methanol to 100% 2-propanol in 40 min	ELSD tube temperature: 80 ◦C nebulization gas: nitrogen	absolute contents not reported	Lin et al. (2003)

Analytes	Samples	Pre-treatment	Stationary phase	Mobile phase	Detection	LOD/LOQ	Reference
epoxy- and hydroxy-fatty acids	chemically and thermally oxidized rapeseed oil	N/A	C_{18} (10 cm×2.1 mm, 1.8 µm particle size)	gradient: solvent A: acetonitrile/ water/ formic acid(50:50:0.1) and Solvent B: acetone/ formic acid(100:0.1).	ESI-MS (1mM lithium formate as ionization enhancer)	LOD=40.3 pg and LOQ=134.3 pg for 12-hydroxystearic acid (injection volume: 3 µL)	Tarvainen et al. (2012)

be a more appropriate method and was employed with HPLC-UV and HPLC-ELSD to analyze epoxy, hydroxy, and keto fatty acids (Morales et al., 2012b; Morales et al., 2014).

Conjugated diene structures have enabled the detection of a number of oxidized compounds by HPLC-UV, such as ketodienes and hydroxydienes. Ketodienes exhibit maximum absorbance at 268 nm, while hydroxydienes and hydroperoxydienes are monitored at 233 nm (Hopia, Huang, & Frankel, 1996). A significant improvement in this method was made by Morales et al. (2010) who chemically synthesized conjugated dienes with C13-substituted hydroperoxy, hydroxy, and keto groups to determine response factors for each class of compounds; therefore, absolute contents of the oxidation products were obtained instead of relative contents reported by Hopia et al. (1996). The HPLC-UV method was then extensively applied to sunflower oils with different levels of oleic and linoleic acids, with LOQ of 0.3 µg/mL for hydroperoxy- and ketodienes and 0.6 µg/mL for hydroxydienes (Morales et al., 2012b).

Esterified glycerols and methyl esters do not absorb at wavelengths higher than 220 nm, except for those with conjugated structures (Holčapek, Jandera, Fischer, & Prokeš, 1999), causing difficulties in the detection of non-conjugated oxidized fatty acids. As an alternative, ELSD has been coupled with HPLC to detect compounds that do not absorb UV light (Morales, Marmesat, Dobarganes, Márquez-Ruiz, & Velasco, 2012a). By connecting HPLC with UV and ELSD in series, it was possible to quantify hydroperoxides, monoepoxy fatty acids, ketodienes, and hydroxydienes in oxidized sunflower oils (Morales et al., 2014). Response curves were established for methyl 13-hydroperoxy-, 13-hydroxy-, and 13-ketooctadecadienoate, as well as methyl *cis*- and *trans*-9,10-epoxystearates, with LOQ of 5.7, 11.6, 5.4, 5.1, and 2.5 µg/mL, respectively.

A substantial advantage in the use of ELSD over UV is that it enabled the detection of methyl *cis*- and *trans*-9,10-epoxystearates, although the peaks were not completely resolved from the non-oxidized FAME converted from sunflower oils (Morales et al., 2012a). A major limitation of the approach is that, the relatively low sensitivity of ELSD compared to UV only allowed detection of hydroperoxides and ketodienes in the sunflower oils with high oxidation levels, while hydroxydienes were not detectable after oxidation for 17 hours at 80 ºC. The LOQ for ketodienes was 0.4 mg/g of FAME (Morales et al., 2012a). Thus, the relatively low sensitivity of ELSD limited its applications in quantifying oxidized fatty acids in minimally oxidized oils.

Applications of RP-HPLC for the quantification of intact TAGs have been developed mainly for the analysis of molecular species of naturally occurring oxygenated acylglycerols, such as castor bean oil (Lin, Turner, Liao, & McKeon, 2003). The large number of structures of oxidized TAG products resulted in highly complex RP-HPLC chromatograms, which caused difficulties in interpretation. To avoid this problem, Steenhorst-Slikkerveer et al. (2000) synthesized reference standards for each class of oxidation products and employed NP-HPLC to resolve the oxidized TAG based on their classes. A similar response was obtained for each of the different oxygenated classes by ESI. With identification by ESI, the LOD was 5 ng in the total ion chromatograms (TIC) and 0.1 to 0.5 ng for the extracted ion chromatograms (EIC) (Steenhorst-Slikkerveer et al., 2000). This method was applied to determine epoxy- and oxo- (co-eluting), and hydroxy TAGs derived from storage of rapeseed oil and a mixture of linseed oil and safflower oil.

Simultaneous resolution of oxygenated free fatty acids and TAGs was achieved using ultra-high performance liquid chromatography-ESI-MS (UHPLC-ESI-MS). Lithium was used as an ionization enhancer to increase the sensitivity (Tarvainen, Phuphusit, Suomela, Kuksis, & Kallio, 2012). Relative contents of individual TAGs were estimated by the intensities of the molecular ion represented by $[M+Li]^+$ (Lin & Chen, 2014). Epoxy and hydroxy fatty acids in artificially digested rapeseed oils were successfully separated from other compounds using UHPLC, where the LOQ for 12-hydroxystearic acid was 134 pg with an injection volume of 3 μL (Tarvainen et al., 2012). This method has been applied to analyze oxidized TAG in Atlantic salmon fillets but absolute contents were not reported (Tarvainen, Nuora, Quirin, Kallio, & Yang, 2015).

Compared to GC, more types of detectors have been used for quantitative HPLC analysis in the study of oxygenated lipid oxidation products and each type of detector has its own advantages and disadvantages. UV detection is the most sensitive in analyzing fatty acids but is limited to conjugated structures, while ELSD is more useful for the detection of epoxy fatty acids but is limited by its sensitivity and response factors that vary with structures. ESI-MS has been the most popular choice for the analysis of intact TAGs. However, direct analysis of intact oxidized TAGs remains challenging with HPLC, as such approaches are plagued by difficulties in chromatographic resolution and resulting uncertainties in the identification of the large number of possible structures.

2.8 NMR Spectroscopy

NMR has been widely used in the study of edible oils, including analysis of fatty acid profiles (Knothe & Kenar, 2004), acyl positional distribution of TAGs (Mannina, Luchinat, Emanuele, & Segre, 1999), determination of ω-3 fatty acids in fish oil (Sacchi, Savarese, Falcigno, Giudicianni, & Paolillo, 2008), analysis of adulteration (Mannina et al., 2009), and quality control and authentication (Dais & Spyros, 2007; Dais & Hatzakis, 2013), where ^{1}H, ^{13}C, and ^{31}P NMR were all applied. In contrast, previous NMR studies on lipid oxidation products mainly focused on ^{1}H NMR because of its relatively higher sensitivity among the commonly studied nuclei (Table 2.5). Martínez-Yusta, Goicoechea, and Guillén (2014) have recently reviewed ^{1}H NMR methods for the determination of thermal degradation products of food lipids, with special focus on the effects of oxidation conditions. In their review, chemical shifts of alcohol-, epoxide-, and ketone-related structures were summarized mainly based on standard compounds.

In ^{1}H NMR, signals of the epoxy protons (-C**H**OC**H**-) are normally observable and can be used for identification. In $CDCl_3$, monoepoxy protons gave signals at 2.9 ppm, while in diepoxides, epoxy protons gave signals at 2.9 and 3.1 ppm, differentiating them from other chemical groups in oils (Goicoechea & Guillén, 2010).

Alcohol protons (-O**H**) have a large range of chemical shifts, from 1 - 5 ppm; the exact chemical shift depends on a number of conditions, such as temperature, solvent, and concentration. In conjugated hydroxydiene structures, alcohol protons appeared as a broad peak around 7 ppm (Gardner & Weisleder, 1972). Therefore, in contrast to epoxides, identification of hydroxy groups in ^{1}H NMR spectra generally relies on the signals of the CH group where the hydroxy group is bonded to the main chain, to identify

hydroxy groups in a ^{1}H NMR spectrum, rather than targeting the alcohol –O**H** protons directly. In $CDCl_3$, the CH groups (-C**H**OH) in unsaturated hydroxy FAME gave signals between 4.0-4.4 ppm (Porter, Mills, & Carter, 1994); when using CCl_4 as solvent, the CH groups (-C**H**OH) exhibited signals between 3.38 – 3.93 ppm (Gunstone, Harwood, & Dijkstra, 2007).

Unlike an aldehyde, ketones do not contain protons that can be observed in a ^{1}H NMR spectrum. As an alternative, the protons α to the carbonyl groups, giving signals between 2.27–2.38 ppm, can be monitored, but these coincide with the acyl C2 protons in esterified glycerols (Kuo, Lanser, Kaneshiro, & Hou, 1999). It is only when a double bond is conjugated to a carbonyl group that two usable signals at 6.8 and 6.1 ppm (R_1-C**H**=C**H**-C(O)-R_2) become apparent (Porter & Wujek, 1987). Thus, unless the carbonyl group is in a conjugated ketone, the ketone structure is difficult to observe in a ^{1}H NMR spectrum.

NMR analyses offer several advantages over other methods (Martínez-Yusta et al., 2014). Unlike chromatographic techniques such as GC or HPLC, derivatization and pre-treatment of a lipid sample is not necessary for NMR analysis. Instead, samples are simply dissolved in a deuterated solvent. Another very attractive feature of NMR is that it allows simultaneous determination of several kinds of oxidation products in a single run. For example, Goicoechea and Guillén (2010) monitored mono- and diepoxides along with hydroperoxides and aldehydes in oxidized sunflower oils using ^{1}H NMR. The distinct signals of epoxides allowed their quantification, although alcohols and ketones were more often evaluated in shorter aldehyde forms, such as 4-hydroxy-(E)-2-nonenal, 4-hydroxy-(E)-2-hexenal, and 4-oxoalkanals (Guillén & Uriarte, 2009; Guillén & Uriarte,

2012b, 2012c), which resulted from scissions in the chain. Conjugated hydroxy dienes, showing two multiplet signals near 6.48 and 5.98 ppm, were observed in sunflower oil by Guillén and Goicoechea (2007) but were not quantified. Quantitative work was also carried out for primary and secondary alcohols in olive oil and soybean oil under deep-frying conditions (Martínez-Yusta & Guillén, 2014a, 2014b). Ketones have been seldom monitored on their own.

NMR has also been compared to several standard methods typically used to study oxidative degradation of oil and lipid samples, including PV, conjugated diene and triene contents, pAV, TBARS, and acid value (Shahidi, Wanasundara, & Brunet, 1994; Wanasundara, Shahidi, & Jablonski, 1995; Skiera, Steliopoulos, Kuballa, Holzgrabe, & Diehl, 2012a, 2012b). Good correlation was reported between NMR and the standard methods, suggesting that NMR was an effective way to determine both primary and secondary oxidation products. At the same time, it must be noted that NMR has one major drawback in its low sensitivity compared to the chromatographic methods (Table 2.5). In a very recent study, the LOQ of epoxides using ^{1}H NMR was 6.3 mmol per kg of oil (Xia, Budge, & Lumsden, 2015). Although there have rarely been other limits of detection or of quantification reported for epoxides, alcohols, or ketones, recent studies suggested that mmol/mol TAG was the most used unit when reporting concentrations of the oxidation products (Martínez-Yusta & Guillén, 2014a, 2014b). Therefore, considering the trace levels of oxygenated fatty acids, especially alcohols and ketones, it is very difficult to apply the convenient NMR technique to determine those compounds. Future studies of oxidized lipids can improve the selectivity and sensitivity through pre-treatment before NMR analysis in order to meet the requirements for analysis of those

trace compounds; however, pre-treatments are time-consuming and run the risk of modifying oxidation products, thereby offsetting any advantage gained by the speed and ease of NMR methods.

Table 2.5 NMR quantitative studies of epoxides and alcohols as non-volatiles in foods.

Analytes	Samples	Oxidation condition	Aeration	Chemical shift	LOD/LOQ	Reference
epoxides	sunflower oil	up to 55 h at 100 °C and 264 h at 70 °C in open Petri dishes	circulating air	2.9 ppm (monoepoxide and diepoxide) and 3.1 ppm (diepoxide only)	not reported	Goicoechea and Guillén (2010)
epoxides	frying extra virgin olive oil	up to 40 hours at 190 °C (8 h/day for five days) in an industrial fryer	natural air	2.88 and 2.63 ppm for (Z)- and (E)-9,10-epoxystearyl groups respectively	not reported	Guillén and Uriarte (2012a)
alcohols	frying soybean oil with dough, pork adipose tissue, and farmed salmon fillets	up to 32 hours at 190 °C (8 h/day for four days) in an industrial fryer	natural air	3.61 ppm (primary alcohols)	not reported	Martínez-Yusta and Guillén (2014b)
epoxides and alcohols	frying extra virgin olive oil with doughnut, pork adipose tissue, and farmed salmon fillets	up to 32 hours at 190 °C (8 h/day for four days) in an industrial fryer	natural air	2.63 ppm ((E)-9,10-epoxystearyl), 2.88 ppm ((Z)-9,10-epoxystearyl), 3.54–3.59 ppm (secondary alcohols), 3.62 ppm (primary alcohols)	not reported	Martínez-Yusta and Guillén (2014a)
epoxides	soybean oil	up to 12 days in amber bottles at 100 °C	natural air	between 2.90 and 3.24 ppm (total epoxides)	6.3 mmol/kg oil	Xia et al. (2015)

2.9 Other Techniques

In addition to the methods discussed above, other techniques have been employed to analyze oxygenated products, including a number of modern MS ionization techniques, including matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Picariello, Paduano, Sacchi, & Addeo, 2009; Badu & Awudza, 2017), high-resolution matrix assisted laser desorption ionization-Fourier transform mass spectrometry (MALDI-FT MS) (van den Brink, Boon, O'Connor, Duursma, & Heeren, 2001), and Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS) (Proschogo et al., 2012). Taking advantage of their ability to analyze compounds with large masses, oxygenated TAGs could be detected, but assignment of functional groups was not made (van den Brink et al., 2001; Picariello et al., 2009; Proschogo et al., 2012). Quantitative determinations are still to be verified.

Oxidized lipids have been studied by mid-infrared (IR) spectroscopy to detect the presence of oxygen-containing functional groups. In IR, ester carbonyl groups (C=O) exhibit absorbance at 1746 cm^{-1}, overlapping with the stretching vibration at 1728 cm^{-1} of the secondary oxidation products containing carbonyl groups, such as aldehydes and ketones (Guillén & Cabo, 2000; Vlachos et al., 2006). Ketones and aldehydes showed maximum absorbance at 1715 cm^{-1} and 1725 cm^{-1}, respectively, and also broadened the ester peak at 1746 cm^{-1} (Vlachos et al., 2006). Even so, these studies monitored C=O bonds at 1728 cm^{-1} mainly as a composite of carbonyl secondary oxidation products. Characteristic bands for alcohols appeared at 3535 cm^{-1} (Ledreau, Dupuy, Artaud, Ollivier, & Kister, 2009). Alcohols were detected as a widening band in the Fourier transform infrared spectroscopy (FTIR) spectra of sunflower oils stored at room

temperature and oxidized at 70 ºC with aeration (Guillén & Goicoechea, 2007). However, FTIR is even less sensitive than ^{1}H NMR for detecting low levels of oxidation (Guillén & Goicoechea, 2007). Little information is available in the literature regarding quantification of epoxide, alcohols, and ketones specifically using this technique. Most studies have focused on the detection of PV and commonly-studied secondary products (Yildiz, Wehling, & Cuppett, 2001; Guillén & Cabo, 2002).

Overall, the modern MS ionization and IR techniques mentioned above are convenient in that they require little sample preparation as derivatization is not necessary. However, IR is considered mostly semi-quantitative due to the presence of overlapping bands. IR techniques require further validation for quantitative applications.

2.10 Conclusions and Future Prospects

Recent progress in the characterization and quantification of oxidized fatty acids has facilitated a better understanding of lipid oxidation, but the currently available methods still suffer from unresolved problems in sensitivity and selectivity. MS has been the most widely used detector to identify oxidation products with GC and HPLC. GC-MS, mostly used in the EI mode, has helped to characterize the structures, providing more information than the soft ionization methods (such as ESI, APCI) utilized with HPLC, especially for the determination of the locations of functional groups. However, the large number of structures encountered, poor resolution of the chromatographic peaks, and unclear fragmentation in spectra have caused difficulties in the identification of the compounds with GC, especially when double bonds, isomers, and multiple oxygenated groups are present. In this review, we summarized commonly used rules to interpret MS

spectra of derivatized and underivatized oxygenated fatty acids which have been applied to characterize oxidation products in real samples. However, there remain unassigned GC peaks in previous work due to the lack of information on oxidation products with even more complicated structures which are not commercially available as standards. Chemical synthesis of fatty acids with multiple functional groups may help to better interpret the mass spectra of unassigned peaks. Otherwise, hydrogenation results in better peak shapes and resolution in chromatograms, reducing the number of possible structures. With hydrogenation, GC-FID has shown excellent sensitivity for the quantification of oxygenated fatty acid esters but with a loss of information about double bond positions.

HPLC-MS techniques allow analysis of intact TAGs and offer information regarding the distribution of oxygenated fatty acids in TAGs, which would not be observed in GC because routine GC analysis of fatty acids requires methylation to eliminate the glycerol backbone. HPLC-UV is particularly selective and sensitive to fatty acids with conjugated double bonds, but its applications are limited to these structures. For structures without conjugated double bonds, an ELSD or an ESI-MS can provide quantitation. ESI-MS has been tested for quantification of intact TAGs, but most previous studies using ESI have reported percentage contents instead of absolute contents.

A major advantage of ^{1}H NMR techniques over the chromatographic methods is that they allow rapid analysis of the entire range of the functional groups present in a single run by simply monitoring the corresponding chemical shifts. It can also analyze any lipid sample without derivatization, resulting in less loss of unstable structures, such as hydroperoxydienes. However, its poor sensitivity compared to GC and HPLC methods

has limited its use for quantification of oxidation products to more extensively oxidized lipids.

Since SPE enables removal of non-altered FAMEs and concentration of oxidized FAMEs for GC analysis, this technique can be potentially used with IR and NMR, or HPLC-ELSD and would be expected to substantially improve sensitivity. Traditional SPE techniques are relatively time-consuming and may negate the advantages of speed and convenience associated with those instrumental techniques; therefore, commercial SPE cartridges and vacuum manifolds are preferred for batch analyses.

In conclusion, traditional chromatography methods (GC and HPLC) have exhibited excellent sensitivity over NMR and IR methods, but they require extra work with sample preparation and instrumental optimization. Comprehensive chromatography methods have great potential in studies of the minor products in lipid oxidation since they provide better resolution in complex media. GC-MS, a powerful tool to determine structures of minor components, should be well utilized for characterization of oxidized fatty acids and breakdown products. Based on the findings and rules reported in previous studies, identification of unknown peaks can be attempted. Future studies should focus on the development of a routine quantitative method, which is both selective and sensitive. Easily accessed instrumentation, such as GC-FID, should be considered in developing a routine method. HPLC seems to be more useful in analysis of intact TAGs and non-volatile products. Here, ESI and subsequent fragmentation can be applied for both qualitative and quantitative purposes. However, due to the soft ionization used with ESI, LC-MS gives mostly molecular weights rather than detailed structures of compounds.

Sound quantitative methods using HPLC-MS analysis of lipid oxidation products need to be developed and validated.

CHAPTER 3 ^{1}H NMR CHARACTERIZATION OF EPOXIDES DERIVED FROM POLYUNSATURATED FATTY ACIDS

3.1 Publication Information

This chapter is a modified version of a manuscript, reworked here for consistency, originally published as:

Xia, W., Budge, S. M., & Lumsden, M. D. (2016). ^{1}H-NMR Characterization of Epoxides Derived from Polyunsaturated Fatty Acids. *Journal of the American Oil Chemists' Society*, *93*(4), 467-478.

3.2 Abstract

In recent years, ^{1}H NMR has been used to study epoxides in lipid oxidation and industrial processes, but the peak assignments reported for monoepoxides and diepoxides have been inconsistent. Lack of clear assignments for chemical shifts of epoxides derived from PUFAs has also limited the use of ^{1}H NMR in detecting and quantifying these products during both oxidative degradation and industrial epoxidation. In this study, ^{1}H NMR was used to characterize the epoxides synthesized from trilinolein, trilinolenin, canola oil, and fish oils by reaction with formic acid and hydrogen peroxide. Assignments for epoxides derived from PUFAs in canola oil and fish oil were between 2.90 - 3.23 ppm and 2.90 - 3.28 ppm, distinct from other chemical groups in these oils. Chemical shifts of epoxy groups moved downfield with increasing number of epoxy groups in the fatty acid chain. Hence, peaks for diepoxides appeared at 3.00, 3.09, and 3.14 ppm and for triepoxides at 3.00, 3.16, and 3.21 ppm. Results also suggested that stereoisomers of diepoxides and

triepoxides were formed during the epoxidation process under the conditions of this study. These new assignments for di- and tri-epoxide stereoisomers were supported by GC-MS analysis of their methyl esters, H-H correlation spectroscopy (COSY) experiments, and a re-evaluation of several previous epoxide-related studies.

3.3 Introduction

Epoxides contain at least one epoxy group, which is a three-member cyclic ether. In industrial processes, epoxides are synthesized from vegetable oils and widely used as intermediates for a number of products, including lubricants (Sharma, Adhvaryu, Liu, & Erhan, 2006), plasticizers and chemical feedstocks (Mungroo, Pradhan, Goud, & Dalai, 2008). Epoxides are also intermediates of lipid oxidation (Schaich, 2012), which occurs in lipid-containing foods, such as oils. They are thought to be formed during free radical lipid peroxidation (Frankel, 1984; Yin, Xu, & Porter, 2011).

Several approaches have been reported previously for the determination of epoxides in oil samples, including methods utilizing hydrogen bromide (HBr) (Firestone, 2009b), 4-(*p*-nitrobenzyl) pyridine (NBP) (Hammock, Hammock, & Casida, 1974), N, N-diethyldithiocarbamate (DTC) (Dupard-Julien, Kandlakunta, & Uppu, 2007), and GC-FID (Mubiru et al., 2014). The HBr method is the most commonly used method to determine epoxides in industrial applications where they are important intermediates. However, secondary lipid oxidation products, such as conjugated dienes and α,β-unsaturated carbonyls, interfere with the HBr method, thereby causing the overestimation of epoxide content in oxidized oils (Firestone, 2009b). The NBP, DTC, and GC-FID

methods require derivatization and are relatively time-consuming (Dupard-Julien et al., 2007; Hammock et al., 1974; Mubiru et al., 2014).

NMR has been applied to the study of epoxides in both oxidized and epoxidized oils. For example, it has been used to analyze mono- and diepoxides in oxidized sunflower oil (Goicoechea & Guillen, 2010) and to study epoxidized vegetable oils (Hwang & Erhan, 2006; Mungroo et al., 2008). Without requiring preparation of derivatives, NMR can be a fast, direct, alternative method for quantification of epoxides, if the chemical shifts of epoxides are known.

An earlier study of monoepoxides had shown the chemical shift of the epoxy protons to be 2.9 ppm, while diepoxides were reported to give signals at 2.9 and 3.1 ppm (Aerts & Jacobs, 2004). However, these findings were not consistent with a number of more recent studies on epoxidized vegetable oils using ^{1}H NMR (Hwang & Erhan, 2006; Lathi & Mattiasson, 2007; Sammaiah, Padmaja, & Prasad, 2014; Sharma et al., 2006). For instance, epoxidized jatropha oil and soybean oil, both of which contain monoepoxides and diepoxides derived from oleic acid and linoleic acid, gave four signals between 2.8-3.2 ppm (Hwang & Erhan, 2006; Lathi & Mattiasson, 2007; Sammaiah et al., 2014; Sharma et al., 2006). Based on the previously established assignments for monoepoxides and diepoxides (Aerts & Jacobs, 2004), only three of the signals can be explained. Therefore, the currently used assignments may not be appropriate. Furthermore, the applications of ^{1}H NMR to the quantification of epoxides have been limited to the measurements of monoepoxides and diepoxides (Martínez-Yusta et al., 2014). For epoxidized oils which may contain triepoxides, including epoxidized canola oil (Campanella, Rustoy, Baldessari, & Baltanás, 2010; Mungroo et al., 2008), it is

difficult to make assignments because little research has been conducted on ^{1}H NMR characterization of triepoxides.

The objectives of this study were to interpret the ^{1}H NMR spectra of diepoxides, triepoxides, and epoxidized oils and to evaluate their potential use in quantitative studies of epoxides in oils. Epoxides were chemically synthesized and the reaction progress was followed by observing the changes in the ^{1}H NMR spectra. Specifically, we examined epoxides of trilinolein (two double bonds in each fatty acid) and trilinolenin (three double bonds in each fatty acid), and epoxides derived from unsaturated fatty acids in canola oil (zero to three double bonds in each fatty acid) and fish oil (zero to six double bonds in each fatty acid). This information is necessary for both identification and quantification of mixed epoxides in any epoxide-related studies.

3.4 Experimental Procedures

3.4.1 Materials

Trilinolein (C18:2, *cis*-9, *cis*-12), trilinolenin (C18:3, *cis*-9, *cis*-12, *cis*-15), and conjugated methyl linoleate (C18:2, *cis*-9, *trans*-11) were purchased from Nu-Chek Prep Inc. (Elysian, United States). Commercial canola oil was obtained from Loblaws Inc. (Toronto, Canada). Fish oil TAG (containing tocopherol as antioxidant), stripped fish oil TAG (without tocopherol), and fish oil ethyl esters (containing tocopherol as antioxidant) were kindly provided by Ascenta Health Ltd. (Dartmouth, Canada). Deuterated chloroform was purchased from Sigma-Aldrich Co. (St. Louis, United States). All chemicals were of analytical grade.

3.4.2 GC analysis

Both oils were analyzed for their fatty acid profiles via AutoSystem GC-FID (PerkinElmer Inc.). A modified version of the Global Organization for EPA and DHA (GOED) Voluntary Monograph for Omega-3 was followed to convert TAGs to FAMEs (GOED, 2012). GC conditions were employed from a previous study (Sullivan, Budge, & St-Onge, 2010) and FAMEs were separated on a DB-23 column ((50%-cyanopropyl)-methylpolysiloxane, 30 m, 0.25 mm ID, 0.25 μm thickness) using helium as the carrier gas. The initial temperature of the oven was held at 153 ºC for 2 min and increased to 205 ºC at 2.3 ºC/min and held for 8.3 min. The FID was set to 270 ºC while the injector was operated in split mode at 250 ºC (Sullivan et al., 2010). Commercially available reference standards GLC 682 and GLC 455 were obtained from Nu-Chek Prep, Inc. (Elysian, United States) and used to identify the fatty acids. Additionally, fatty acids which are not commercially available were first identified in a menhaden FAME sample by GC-MS; the sample was then used as a reference for retention times in GC-FID. The fatty acid profiles were reported as percent of total mass (Table 3.1).

Table 3.1 The composition of the major fatty acids in canola oil and fish oil expressed as mass % of total fatty acid mass, obtained from GC analysis.

Fatty acid	Canola oil	Fish oil
C14:0	/	7.47±0.05
C16:0	3.84±0.04	15.51±0.10
C18:0	1.77±0.04	2.86±0.03
C16:1n-7	/	9.16±0.06
C18:1n-7	3.42±0.05	2.93±0.02
C18:1n-9	59.96±0.13	8.93±0.04
C16:2n-4	/	1.39±0.01
C18:2n-6	18.89±0.11	1.02±0.01
C18:3n-3	9.63±0.07	0.72±0.01
C16:3n-4	/	2.02±0.01
C16:4n-1	/	3.02±0.02
C18:4n-3	/	2.66±0.02
C20:5n-3	/	21.20±0.18
C22:5n-3	/	2.34±0.03
C22:6n-3	/	8.37±0.08

3.4.3 Calculation of number of double bonds

The concentrations of double bonds in fatty acid standards and oils were calculated using molecular weight, structure and, if necessary, the fatty acid proportional data obtained from GC analysis. This information was used to calculate the amount of reagent necessary to prepare fully epoxidized fatty acid glycerol esters and oils. The concentrations of double bonds were estimated to be 6.82 mol/kg, 10.30 mol/kg, and 6.79 mol/kg for trilinolein, trilinolenin, and conjugated methyl linoleate, respectively. For canola and fish oils, the concentrations of double bonds were estimated from the fatty

acid proportional data obtained from GC analysis (Table 3.1) and found to be 4.46 mol/kg and 7.96 mol/kg, respectively.

3.4.4 Epoxidation

A molar ratio of 1: 0.5: 1.5 (double bonds: formic acid: hydrogen peroxide) was applied for epoxidation of the standards and oils following the procedures described in a previous work (Anuar, Zhao, Mugo, & Curtis, 2012). Moles of 50% and 30% hydrogen peroxide were calculated by taking into consideration their concentrations in solution. In oxidized oils, epoxy groups are often encountered with adjacent double bonds; thus, the ^{1}H NMR characterizations of the epoxides with remaining double bonds nearby are of great interests. To make partially epoxidized trilinolein, the amount of hydrogen peroxide was reduced to only 0.75 mol for each mol of double bonds.

In most cases, the three reagents were simply mixed together and heated at 38 ºC for 24 hours to allow epoxidation to occur (Dinda, Patwardhan, Goud, & Pradhan, 2008; Mudhaffar & Salimon, 2010). However, the process using 50% H_2O_2 with trilinolenin resulted in polymerization. To avoid this, trilinolenin was reacted with 30% H_2O_2 in two successive incubations at 38 ºC. Partially-epoxidized trilinolenin was recovered after incubating for 24 hours and analyzed for epoxides by ^{1}H NMR. The recovered product was then mixed with fresh 30% H_2O_2 /formic acid and reacted for an additional two hours to generate fully-epoxidized products. Epoxidized oils were extracted by dissolving the reaction mixtures in ethyl acetate, washing in sodium chloride, separating phases by centrifugation, and drying the oil phase under nitrogen according to published procedures (Anuar et al., 2012). The remaining oil was collected as the epoxidized sample.

The purity of the epoxidized products was verified by thin layer chromatography (TLC) using a plate of silica gel 60 (20cm×20cm, 250 μm thickness), obtained from EMD Millipore (Darmstadt, Germany), with hexane: ethyl ether (80:20, v/v) and chloroform: methanol (95:5, v/v) as developing solvents and visualization under ultraviolet light (254 nm). The epoxidized products were recovered from the plates with chloroform: methanol (90:10, v/v) and reanalyzed by ^{1}H NMR for comparison with the epoxidized products without TLC purification.

3.4.5 GC-MS

A Trace GC Ultra gas chromatography coupled with a PolarisQ mass spectrometer (Thermo Fisher Scientific Inc.) was used for analysis. The epoxidized trilinolein and trilinolenin were converted into their methyl esters following a base-catalyzed with sodium methoxide (Marmesat et al., 2008) and dissolved in hexane for GC-MS analysis. The analysis was performed in both EI and CI modes. The concentrations of the samples were 0.5 mg/ml for EI and 2 mg/ml for CI. Splitless-injection at 250 ºC was used. Separation was performed on a DB-23 capillary column (30 m×0.25 mm i.d.) with helium as the carrier gas at 1.2 ml/min. The oven temperature program was initially held at 60 ºC for 2 min, then increased to 235 ºC at 12 ºC/min and held for 40 min. The ionization energy was 70 eV, with multiplier voltage of 1643 V, source temperature at 200 ºC, and transfer line at 235 ºC. Spectral data were acquired over a mass range of *m/z* 60–600 in EI mode and *m/z* 60-400 in CI mode. The emission current was 250 μA for EI and 100 μA for CI. In CI mode, ammonia was used as the reagent gas at a flow rate of 1.3 ml/min.

3.4.6 ^{1}H NMR analysis

All standards and oil samples were prepared for ^{1}H NMR analysis by dissolving 0.1 g of sample in 2.0 mL of deuterated chloroform and placing 750 µL of the solution in a 5 mm diameter NMR tube. The acquisition parameters were modified from a reference (Guillén & Ruiz, 2004): spectral width 10080 Hz, relaxation delay 3 s, number of scans 64, acquisition time 3.25 s, pulse width 90°. The total acquisition time was 6.9 min. The ^{1}H NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer. The signal of non-deuterated chloroform present in the deuterated chloroform was used as a reference for chemical shifts. The assignment of the signals was made taking into consideration previous published assignments (Aerts & Jacobs, 2004). The ^{1}H NMR analysis of the epoxidized standards was conducted in duplicate, but the spectra of the epoxidized standards indicated that there was no difference between the two runs of the same standard. Thus, the ^{1}H NMR shifts were determined from a single analysis of each oil sample. To verify the ^{1}H NMR assignments, H-H COSY experiments were conducted with fully-epoxidized trilinolein and trilinolenin using 8 scans, 16 dummy scans, 7.6432 ppm of spectra width, and 1024×128 time domain data points (F2×F1).

3.4.7 Identification of an internal standard

Vegetable oils primarily consist of TAGs. The fish oil used in this study was also TAG oil. Because the glycerol backbone of these lipids is expected to be stable during oxidation as moisture is not present and hydrolysis of TAGs does not take place (Choe & Min, 2007); therefore, glycerol protons may be good candidates for internal standards. To

test their stability after the epoxidation process, the area ratios of signals of glycerol protons to methyl protons were calculated for both original oils and epoxidized oils. Stripped fish oil TAGs (without tocopherol) and fish oil ethyl esters (containing tocopherol as antioxidant) were epoxidized for comparison.

3.5 Results and Discussion

3.5.1 Epoxidation of the fatty acids and oils

The epoxidation process of canola oil was previously monitored by non-aqueous reversed phase liquid chromatography/mass spectrometry (NARP-LC/MS) where partially-epoxidized and fully-epoxidized products were reported to form during the reaction (Anuar et al., 2012). In the current study, formic acid with H_2O_2 successfully epoxidized trilinolein, trilinolenin, canola oil, and fish oils. The progress of epoxidation of trilinolein and trilinolenin was followed by the loss of double bonds and the appearance of epoxide peaks in the ^{1}H NMR spectra.

Original trilinolein and trilinolenin did not contain epoxy groups (Figure 3.1a and 3.2a). In the partially-epoxidized trilinolein and trilinolenin (Figure 3.1b and 3.2b, respectively), the signals appearing ~3.00 ppm indicated the formation of epoxides, while the remaining double bonds gave signals ~5.50 ppm. In the fully epoxidized trilinolein and trilinolenin, all the double bonds were converted into epoxy groups (Figure 3.1c and 3.2c). This is clear in the ^{1}H NMR spectra with the disappearance of the signals due to double bonds (~5.50 ppm) and methylene groups adjacent to the double bonds (~2.10 ppm), and the appearance of the signals due to epoxy groups (~3.00 ppm) and methylene

groups adjacent to these epoxy groups (~1.50 ppm). In both epoxidation processes, the signals associated with the glycerol backbone remained constant.

In epoxidized trilinolein, three distinct groups of signals (3.00, 3.09, 3.14 ppm) were found for the epoxy groups (Table 3.2). This result was different from a previous study, which only reported two signals (2.90, 3.10 ppm) for diepoxides derived from methyl linoleate (Aerts & Jacobs, 2004). The difference was likely due to the presence of stereoisomers of diepoxides, as discussed in detail in the next section. The chemical shifts of epoxidized trilinolenin (Figure 3.2) were similar to epoxidized trilinolein, with three signals for the epoxy groups (3.00, 3.16, 3.21 ppm) (Table 3.3). We also noted a clear change in the chemical shift of the signal originally at 1.00 ppm in untreated trilinolenin (Figure 3.2). The methyl groups in ω-3 fatty acids show a distinctive signal at 1.00 ppm which differentiates them from the methyl groups (0.88 ppm) in other fatty acids (Miyake, Yokomizo, & Matsuzaki, 1998). After epoxidation, the chemical shifts of the methyl groups in trilinolenin moved downfield from 1.00 ppm to 1.08 ppm, suggesting that the epoxy group on the third carbon from the end had a significant influence on the chemical shift of the methyl group. This change was not observed in the epoxidized trilinolein. The signal between 1.25-1.45 ppm represented the saturated acyl C4-C7 methylene groups in the fatty acid chain. A slight change in peak shape was observed after epoxidation because of the replacement of double bonds by epoxy groups; however, their chemical shifts remained in the same range.

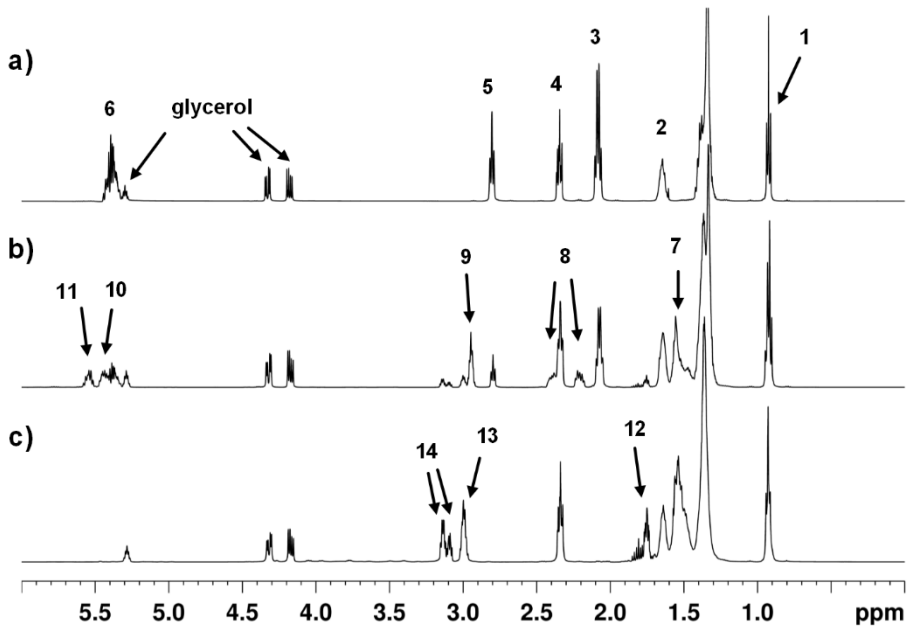


Figure 3.1 ^{1}H NMR spectrum of a) trilinolein, b) partially-epoxidized trilinolein and c) fully-epoxidized trilinolein. The corresponding chemical shift assignments are in Table 3.2.

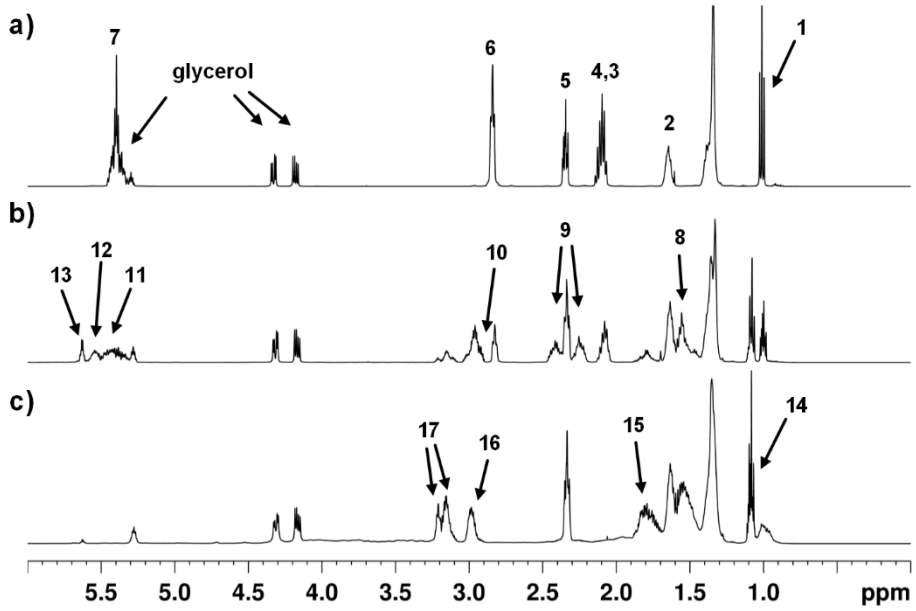
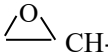


Figure 3.2 ^{1}H NMR spectrum of a) trilinolenin, b) partially-epoxidized trilinolenin and c) fully-epoxidized trilinolenin. The corresponding chemical shift assignments are in Table 3.3.

Table 3.2 ^{1}H NMR chemical shifts observed from the epoxidation of trilinolein.

spectrum[a]	signal	chemical shift[b] (ppm)	protons	functional groups
a)	1	0.88 (t)	C**H**$_3$-CH$_2$-CH$_2$-	methyl group
	2	1.65 (m)	-C**H**$_2$-CH$_2$-CO-O-	methylene group beta to acyl group
	3	2.08 (m)	-CH$_2$-C**H**$_2$-CH=CH-	allylic methylene group
	4	2.34 (t)	-CH$_2$-C**H**$_2$-CO-O-	methylene group alpha to acyl group
	5	2.80 (t)	-CH=CH-C**H**$_2$-CH=CH-	diallylic methylene group
	6	5.38 (m)	-C**H**=C**H**-	olefinic group
b)	7	1.45-1.60(m)	-CH$_2$-C**H**$_2$-CH(O)CH-	methylene group alpha to epoxy group
	8	2.20 (m), 2.40 [c]	-CH(O)CH-C**H**$_2$-CH=CH-	methylene groups between an epoxy group and an olefinic group
	9	2.95(m)	-C**H**(O)C**H**-CH$_2$-CH=CH-	epoxy group beta to olefinic group
	10	5.35-5.50 (m)	-CH(O)CH-CH$_2$-CH=C**H**-	olefinic group beta to epoxy group
	11	5.50-5.60 (m)	-CH(O)CH-CH$_2$-C**H**=CH-	

spectrum[a]	signal	chemical shift[b] (ppm)	protons	functional groups
c)	12	1.70-1.85 (m)	-CH  CH-C**H**$_2$-CH CH-	methylene group between two epoxy groups
	13	3.00 (m)	-C**H**  CH-CH$_2$-CH C**H-**	epoxy groups separated by a methylene group
	14	3.09 (m), 3.14 (m)	-CH  C**H**-CH$_2$-C**H** CH-	

[a] Here, a), b), and c) corresponded to the ^{1}H NMR spectrum a), b), and c) in Figure 3.1.
[b] Abbreviations used: t, triplet; m, multiplet.
[c] This signal overlapped with the signal at 2.34 ppm (t, -C**H**$_2$-CO-O-).

Table 3.3 ^{1}H NMR chemical shifts observed from the epoxidation of trilinolenin.

spectrum[a]	signal	chemical shift[b] (ppm)	protons	functional groups
a)	1	1.00 (t)	C**H**$_3$-CH$_2$-CH=CH-	methyl group in ω-3 fatty acid
	2	1.65 (m)	-C**H**$_2$-CH$_2$-CO-O-	methylene group beta to acyl group
	3	2.08 [c]	-CH$_2$-C**H**$_2$-CH=CH-	allylic methylene group
	4	2.11 [c]	CH$_3$-C**H**$_2$-CH=CH-	allylic methylene group
	5	2.34 (t)	-CH$_2$-C**H**$_2$-CO-O-	methylene group alpha to acyl group
	6	2.84 (t)	-CH=CH-C**H**$_2$-CH=CH-C**H**$_2$-CH=CH-	diallylic methylene group
	7	5.40 (m)	-C**H**=C**H-**	olefinic group

spectrum[a]	signal	chemical shift[b] (ppm)	protons	functional groups
b)	8	1.45-1.60 (m)	-CH_2-C**H**$_2$-CH(O)CH-	methylene group alpha to epoxy group
	9	2.25 (m), 2.40 [d]	-CH(O)CH-C**H**$_2$-CH=CH-	methylene groups between an epoxy group and an olefinic group
	10	2.95[e]	-C**H**(O)C**H**-CH_2-CH=CH-	epoxy group beta to olefinic group
	11	5.35-5.50 (m)	-CH(O)CH-CH_2-CH=C**H**-	olefinic group beta to epoxy group
	12	5.50-5.60 (m)	-CH(O)CH-CH_2-C**H**=CH-	
	13	5.63 (m)	-CH(O)CH-CH_2-C**H**=C**H**-CH_2-CH(O)CH-	
c)	14	1.08 (m)	C**H**$_3$$CH_2$-CH(O)CH-	methyl group in ω-3 fatty acid epoxide
	15	1.70-1.85 (m)	-CH(O)CH-C**H**$_2$-CH(O)CH-	methylene group between two epoxy groups
	16	3.00 (m)	-C**H**(O)CH-CH_2-CH(O)CH-CH_2-CH(O)C**H**-	epoxy groups separated by a methylene group
	17	3.16 (m), 3.21(m)	-CH(O)C**H**-CH_2-C**H**(O)C**H**-CH_2-C**H**(O)CH-	

[a] Here, a), b), and c) corresponded to the ^{1}H NMR Spectrum a), b), and c) in Figure 3.2. [b] Abbreviations used: t, triplet; m, multiplet.
[c] These two signals overlapped with each other. [d] This signal overlapped with the signal at 2.34 ppm (t, -C**H**$_2$-CO-O-).
[e] This signal overlapped with signal at 3.00 ppm (m, -C**H**OCH-CH_2-CHOCH-CH_2-CHOC**H**-).

The purity of the fully-epoxidized products were verified by TLC. Using hexane:diethyl ether (80:20, v/v) as developing solvent, only one band with R_f=0 was observed for each of the epoxidized products, while R_f values of trilinolein, trilinolenin, canola oil, and fish oil were 0.39, 0.35, 0.42, and 0.39, respectively. The absence of the band due to the substrates for all the epoxidized samples indicated that the TAGs had fully reacted. Use of chloroform: methanol (95:5, v/v) as developing solvent did not allow the separation of substrates (R_f range of 0.62-0.76) and epoxidized products (R_f range of 0.5-0.8). In this solvent, trace amounts of polar compounds with R_f=0 were found for epoxidized trilinolenin, while only one broad band (R_f range of 0.5-0.8) was observed for each of epoxidized trilinolein, epoxidized canola oil, and epoxidized fish oil. The products with R_f=0 were more polar than fully-epoxidized TAGs, and were likely due to ring-opened by-products formed during the epoxidation process (Mungroo et al., 2008). Most importantly, the epoxidized products purified by TLC gave ^{1}H NMR signals that were identical to those of the epoxidized products without TLC purification, indicating that the polar band (R_f=0) would not affect the ^{1}H NMR assignments of the epoxidized trilinolenin.

GC-MS enabled further confirmation of the structure of the reaction products (Figure A-1 and A-2 in Appendix A). The FAMEs derived from fully-epoxidized trilinolein and trilinolenin were expected to be methyl 9,10-12,13-diepoxyoctadecanoate *(cis/cis)* and methyl 9,10-12,13-15,16-triepoxyoctadecanoate *(cis/cis/cis),* with the configuration of the epoxy ring retained from the original double bond (Fankhauser-Noti et al., 2006). GC-MS analysis of the methyl esters of epoxidized trilinolein yielded two peaks with retention times of 22.6 and 24.1 min, and identical EI spectra (% relative

intensity): *m/z* 308(2), 295(2), 277(4), 187(7), 165 (12), 155 (50), 137 (20), 109 (62), 67 (100). Their EI spectra were consistent with that of methyl 9,10-12,13-diepoxyoctadecanoate reported in a previous study (Piazza et al., 2003). In PCI mode, a base peak of *m/z* 344 [M+18, addition of NH_4^+] and *m/z* 327 [M+1], 309 [M-17], 295 [M-31], 291 [M-35], and 277 [M-49] were identified for both stereoisomers of methyl 9,10-12,13-diepoxyoctadecanoate, but the spectra were not identical. The first eluted stereoisomer had a more intense *m/z* 291 than *m/z* 295, consistent with a previous study (Biedermann-Brem et al., 2007); the later eluting stereoisomer had a more intense *m/z* 295 than *m/z* 291.

The methyl esters of epoxidized trilinolenin had retention times of 38.8, 41.3, 42.2, and 47.1 min, and identical spectra in EI-MS (% relative intensity): *m/z* 155 (24), 109 (35), 67 (100). The EI spectra of the products were difficult to interpret, although *m/z* 155 was evidence of the presence of a C9,10 epoxy ring, formed from loss of CH_3OH from $[HO{=}CH(CH_2)_7COOCH_3]^+$ (Piazza et al., 2003). In PCI, the proton adduct of the molecular ion *m/z* 341 [M+1] was small; however, characteristic peaks of *m/z* 358 [M+18, addition of NH_4^+], 323 [M-17], 305 [M-35], and 291 [M-49] were abundant. As little description of MS spectra of triepoxides is available in the literature, comparison with other studies was impossible. However, the base peak *m/z* 358 [M+18] and the fragmentation in PCI, and the relatively long retention times suggested that the four stereoisomers of triepoxides were unlikely to be short-chain epoxide monomers or terminal epoxides; methyl 9,10-12,13-15,16-triepoxyoctadecanoate *(cis/cis/cis)* was a much more likely identity of the four stereoisomers.

Impurities in the FAMEs derived from fully-epoxidized trilinolein and trilinolenin found at 12.6 min and 12.7 min by GC-MS had convincing MS library spectral matches with methyl 9-oxo-nonanoate and nonanedioic acid dimethyl ester. These were estimated at < 1% in methyl diepoxyoctadecanoate (cis/cis) and < 8% in methyl triepoxyoctadecanoate (cis/cis/cis). No other epoxy compounds were observed in GC-MS. Therefore, it was concluded that all the peaks identified for diepoxides and triepoxides in ^{1}H NMR were internal epoxides.

The epoxidation method was unsuccessful when applied to conjugated methyl linoleate. Signals found ~ 3.00 ppm (Figure A-3) could be due to the partial generation of epoxides. The signals between 5.00 - 6.00 ppm indicated that some double bonds were not converted, while signals that occurred between 2.80 - 4.40 ppm and 8.00 - 9.50 ppm suggested that other products were formed in addition to epoxides (Figure A-3). It was assumed that, like the other standards, conjugated methyl linoleate would be readily epoxidized by hydrogen peroxide and formic acid; however, the combination of formic acid with both 30% and 50% hydrogen peroxide was found to be ineffective, with the products of the reactions using both concentrations of reagents giving identical ^{1}H NMR spectra. The signals were indistinct, so it was difficult to assign the signals to exact protons.

Epoxidation of the conjugated fatty acids may have been impossible because the structure does not allow required electron migration. The current epoxidation method utilized the peracid (RCO_3H) formed from the reaction of formic acid and hydrogen peroxide. The intramolecular hydrogen bond of the peracid results in an electrophilic oxygen atom that can be added to the double bond (Clayden, Greeves, & Warren, 2012).

The addition of oxygen to the double bond is accompanied by the migration of a pair of electrons from the π bond of the original double bond to the new C-O bond, which results in a carbocation adjacent to the double bond in the transition state (Bach, Canepa, Winter, & Blanchette, 1997). Normally, the carbocation will form another carbon-oxygen bond (-C-O-) with the oxygen, so an epoxy group is formed. However, when the transition state applies to a conjugated diene, the carbocation is conjugated to the adjacent double bond, which generates an allylic carbocation and causes the double bond to shift. This may isomerize with double bond migration, preventing the formation of the other C-O bond. In addition, the formation of an epoxy group in a conjugated diene will reduce the nucleophilicity of the remaining double bond, making the partially epoxidized structure less likely to undergo further epoxidation (Clayden et al., 2012). Compared to the ^{1}H NMR spectrum of the original conjugated methyl linoleate, hydroperoxides (~8.10 ppm) and unsaturated alcohols or cyclic peroxides (3.40 - 4.50 ppm) were found in the reaction product (Falch, Anthonsen, Axelson, & Aursand, 2004), which may be due to the shifting of the carbocation during the reaction. Another work (Marks & Larock, 2002) found a similar effect when epoxidizing oils using methyltrioxorhenium (MTO), pyridine, methylene chloride, and hydrogen peroxide. Their method was successful in epoxidizing fish oil ethyl esters but ineffective when applied to conjugated fish oil ethyl ester derived from the same fish oil ethyl ester. Since no information about the signals in ^{1}H NMR spectra was provided in that study (Marks & Larock, 2002), it is impossible to compare the products epoxidized by the MTO method and the method used here.

3.5.2 Stereoisomers of diepoxides and triepoxides

Unlike the previous assignments of diepoxides (Aerts & Jacobs, 2004), three signals were found for diepoxides derived from trilinolein. This difference may be due to the different reagents used for epoxidation. In a previous work, 3-Chloroperoxybenzoic acid (mCPBA) was used as the epoxidation reagent (Aerts & Jacobs, 2004) and it may have had a steric effect, leading to a stereospecific reaction. When formic acid was employed for epoxidation, the steric effect may have been inconsequential due to the relatively small size of the formic acid molecule, leading to the formation of both *cis/cis* diepoxide stereoisomers (Figure 3.3). Stereoisomers of tetrahydroindene diepoxide have been distinguished using ^{1}H NMR (Okovytyy et al., 2005) and stereoisomers derived from methyl linoleate using peracetic acid have also been discussed (Maerker, Haeberer, & Herb, 1966). Diepoxide stereoisomers have been verified using HPLC-MS (Piazza et al., 2003), GC-FID (Emken, 1971) and GC-MS (Fankhauser-Noti et al., 2006) but remained un-reported by ^{1}H NMR.

The methyl esters of epoxidized trilinolein gave two peaks in GC-MS with the same EI spectra, and were identified as the two stereoisomers of methyl 9,10-12,13-diepoxyoctadecanoate. The peak area ratio of the two peaks (RT 22.6 and 24.1 min) was 1.6:1, in agreement with the peak area ratio of the signals observed at 3.14 and 3.09 ppm in ^{1}H NMR (Figure 3.1c). For methyl esters of epoxidized trilinolenin, four potential stereoisomers were observed in GC-MS analysis. The peak area ratio of the four triepoxide stereoisomers was determined to be ~2.9:2:1.9:1 (RT 38.8, 41.3, 42.2, and 47.1 min) in GC-MS, potentially representing four different combinations of the three epoxy groups facing the same and opposite sides. In the ^{1}H NMR spectrum of epoxidized

trilinolenin (Figure 3.2c), the peak area ratio of the two signals at 3.16 ppm and 3.21 ppm was approximately 2:1. Further interpretation is difficult due to a lack of information about these stereoisomers in the literature. However, the methyl esters of epoxidized trilinolein and trilinolenin were analyzed by ^{1}H NMR. Identical signals were confirmed for diepoxides and triepoxides in their TAG and methyl ester forms, suggesting little influence of the glycerol backbone.

A mixture of stereoisomers could yield the three signals (3.00, 3.09, and 3.14 ppm) detected here for diepoxides in linoleic acid (Figure 3.1). The signal at 3.00 ppm (m, -C**H**OCH-CH_2-CHOC**H**-) can be assigned to the outer pairs of CH protons while the two signals at 3.09 ppm (m, -CHOC**H**-CH_2-C**H**OCH-) and 3.14 ppm (m, -CHOC**H**-CH_2-C**H**OCH-) can be assigned to the inner pairs of CH protons (Figure 3.3). The two oxirane oxygens could be located on either the same or opposite sides, causing different shielding and slightly different chemical shifts of the inner protons (Lambert, 1987).

Similarly, the three distinct groups of signals found for the triepoxides in linolenic acid at 3.00 ppm (m), 3.16 ppm (m), and 3.21 ppm (m) (Figure 3.2) were associated with the three epoxy groups derived from the original double bonds on each fatty acid chain of epoxidized trilinolenin. The signal at 3.00 ppm can be assigned to the outer pair of protons of the triepoxides (m, -C**H**OCH-CH_2-CHOCH-CH_2-CHOC**H**-), while the signals at 3.16 ppm and 3.21 ppm could be assigned to the inner protons (m, -CHOC**H**-CH_2-C**H**OC**H**-CH_2-C**H**OCH-), with the two different resonances due to the relative positions of the two oxirane oxygens separated by a methylene group (Table 3.3). The relative positions of the two adjacent oxirane oxygens (separated by a methylene group) produce two slightly different electronic environments for the inner protons. The protons with

higher electro-negativities could result in higher frequencies of resonances because of less shielding, generating a slightly greater chemical shift.

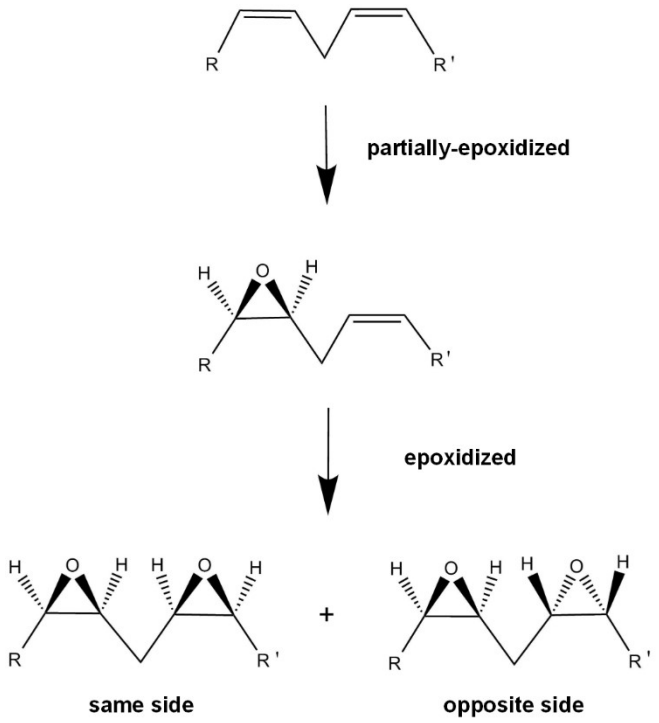


Figure 3.3 Stereoisomers of diepoxides derived from methylene-interrupted dienes differed in the relative positions of the two oxygens. The adjacent oxygen atoms can be located either on the same or opposite sides.

The ^{1}H NMR assignments were further confirmed using the H-H COSY experiments with fully-epoxidized trilinolein and trilinolenin (Figure A-4 and A-5). In their COSY spectra, the outer pair of the epoxy protons (3.00 ppm, -C**H**OCH-CH_2-CHOC**H**-) were coupling with the methylene protons that were only adjacent to one epoxy group (1.45-1.60 ppm, -CH_2-C**H**$_2$-CHOCH-). The inner pair of the epoxy protons (3.09, 3.14 ppm for epoxidized trilinolein, -CHOC**H**-CH_2-C**H**OCH-; 3.16, 3.21 ppm for epoxidized trilinolenin, -CHOC**H**-CH_2-C**H**OC**H**-CH_2-C**H**OCH-) were coupling with the

methylene protons between two epoxy groups (1.70-1.85 ppm, -CHOCH-C**H**$_2$-CHOCH-).

These new observations of stereoisomers are supported by re-examination of previous ^{1}H NMR analyses of epoxidized soybean oil (Hwang & Erhan, 2006; Lathi & Mattiasson, 2007; Sharma et al., 2006). In those studies, four clear signals were found for epoxides. Soybean oil typically contains 50-60% linoleic acid and 20% oleic acid, with only 7% linolenic acid. The amount of epoxidized linolenic acid may have been so low that the signals for the triepoxides were not clear. Based on the assignments made in this study, the four intense signals could be assigned to the monoepoxides (one signal, 2.90 ppm) and the diepoxides (three signals, 2.95 - 3.15 ppm). The shapes of the three signals associated with diepoxides in our study were similar to those signals assigned for diepoxides in the previous studies (Hwang & Erhan, 2006; Lathi & Mattiasson, 2007; Sharma et al., 2006).

3.5.3 ^{1}H NMR interpretation of epoxidized oils

In this study, the signals associated with epoxides appeared between 2.90-3.23 ppm for epoxidized canola oil (Figure 3.4), and between 2.90 - 3.28 ppm for epoxidized fish oil (Figure 3.5), while the shifts due to protons associated with the glycerol backbone remained at 4.18 ppm, 4.33 ppm (dd, -C**H**$_2$-O-CO-R) and 5.30 ppm (m, >C**H**-O-CO-R) for both oils. Unlike the lipid standards (trilinolein and trilinolenin), two signals (0.88, 1.00 ppm) were observed for methyl groups in fresh canola oil and fresh fish oil. The signal associated with the methyl groups in ω-3 fatty acids moved from 1.00 ppm to 1.08

ppm after epoxidation, but the signal associated with methyl groups in other fatty acids remained the same at 0.88 ppm.

By examining the spectrum of the epoxidized canola oil, it was clear that almost all the signals due to the double bonds disappeared (Figure 3.4). Only a small peak was detected at 5.60 ppm which might be due to the very few unconverted double bonds remaining. Assuming that all the double bonds in the canola oil had been converted into epoxy groups, it was expected that the ratio of the signals due to the monoepoxides, diepoxides, and triepoxides in the epoxidized canola oil (Figure 3.4) would be similar to the ratio of the content of oleic acid, linoleic acid, and linolenic acid in the canola oil (Table 3.1). The spectrum supports this (Figure 3.4), showing the signal associated with monoepoxides (2.92 ppm) as the most intense among the signals between 2.90 - 3.23 ppm, because the canola oil used in this study contained 59.96% oleic acid (Table 3.1). The three signals associated with diepoxides (3.00, 3.09, 3.14 ppm) were less intense, as the canola oil contained 18.89% linoleic acid. The signals associated with triepoxides (3.00, 3.16, 3.21 ppm) were the least intense because the canola oil contained only 9.63% linolenic acid and the signals of triepoxides coincided with those of diepoxides. The ^{1}H NMR spectrum of the epoxidized canola oil obtained in this experiment was consistent with a previous study (Mungroo et al., 2008). Therefore, epoxidized canola oil can be successfully interpreted by the assignments made in this work.

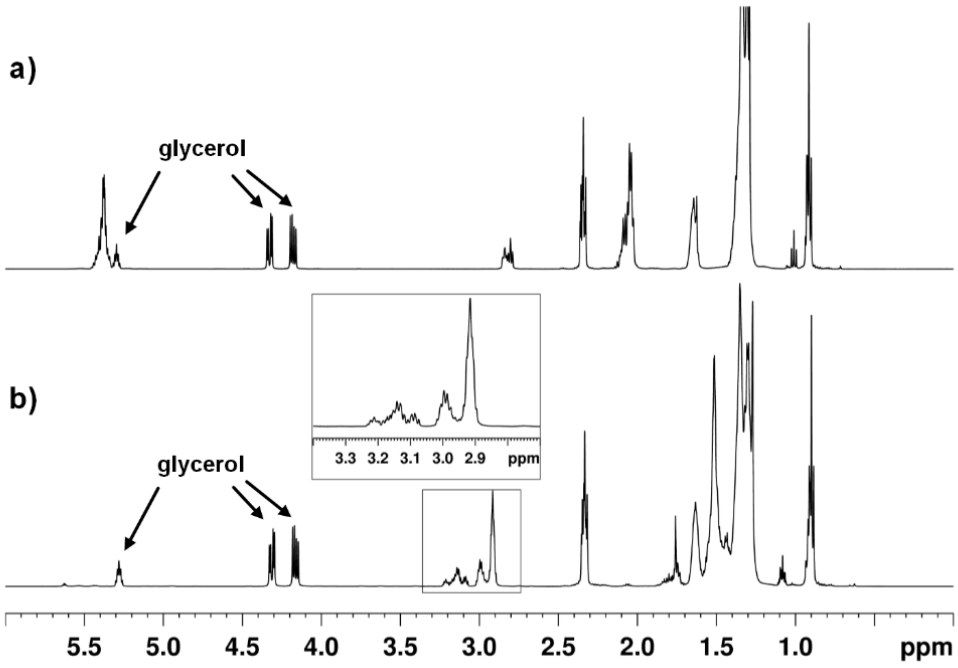


Figure 3.4 ^{1}H NMR spectrum of a) canola oil (top) and b) epoxidized canola oil (bottom). The signals between 2.90-3.23 ppm are due to epoxides.

The signals assigned to the mixed PUFA epoxides in fish oil appeared between 2.90 - 3.28 ppm, which differed from the signals of other chemical groups in fish oil TAGs (Figure 3.5). Based on the fatty acid composition of fish oil (Table 3.1) and the assignments of the signals given in Table 3.2 and 3.3, the sharp peak at 2.92 ppm in epoxidized fish oil was assigned to monoepoxides derived from monounsaturated fatty acids, while the signals between 2.95 - 3.28 ppm were likely due to the epoxides derived from PUFAs. These are the first presented ^{1}H NMR spectra of mixed epoxides derived from PUFAs in fish oil TAGs. For fish oil ethyl esters, the signals associated with the methylene protons of the ethyl group were ~ 4.20 ppm (m, CH_3-C**H**$_2$-O-CO-R), while the chemical shift of the methyl protons of the ethyl group was 1.30 ppm (t, C**H**$_3$-CH_2-O-CO-

R) (Gunstone et al., 2007; Marks, Li, Pacha, & Larock, 2001). Hence, the signals appearing between 2.90 - 3.28 ppm could be used for identification of epoxides in both fish oil TAGs and ethyl esters.

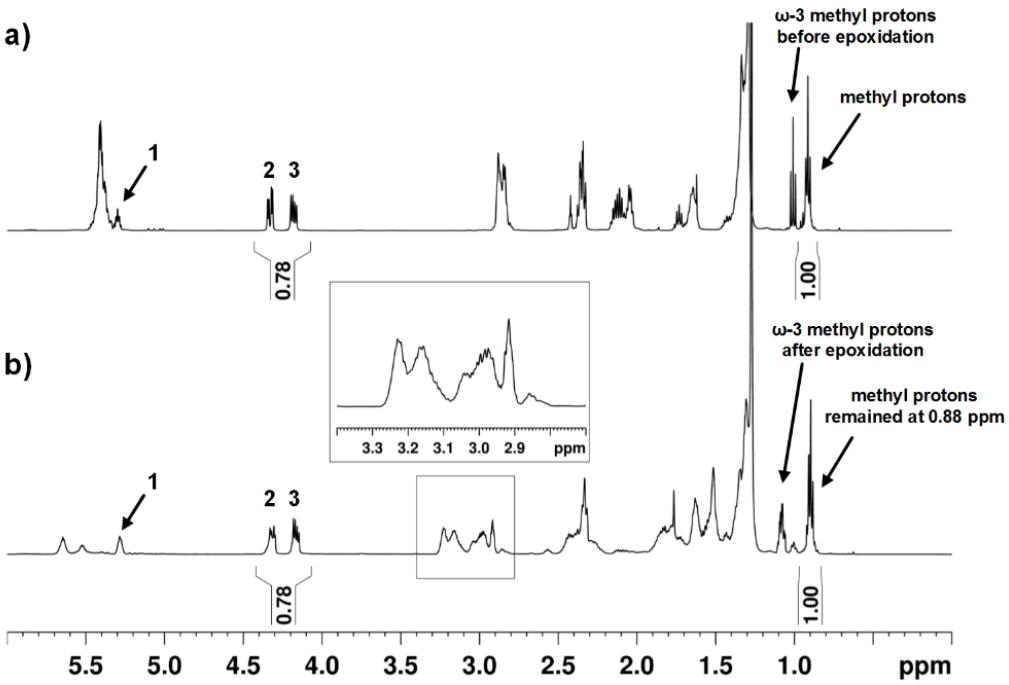


Figure 3.5 ^{1}H NMR spectrum of a) fish oil (top) and b) epoxidized fish oil (bottom) with integration. The signals between 2.90-3.28 ppm are due to epoxides. Signals 1, 2, and 3 are associated with the esterified glycerols in oil. Signals 2 and 3 are assigned to the *sn*-1 and *sn*-3 protons of esterified glycerol which have been integrated before (a) and after (b) epoxidation by taking the peak of methyl protons at 0.88 ppm, which does not include methyl protons in ω-3 fatty acids, as an internal reference.

When comparing the epoxidized fish oil to the epoxidized canola oil, it was noted that the signals associated with the mixed epoxides moved slightly downfield in fish oil. This might be because more epoxy groups in a fatty acid chain were present in epoxidized fish oil than in epoxidized canola oil. The involvement of more epoxy groups might lead to less shielding of the protons associated with the epoxy groups, which may

contribute to a slightly higher frequency of proton resonance. This information may be useful for identification of the compounds containing more than three epoxy groups.

In this study, epoxide generation in trilinolein and trilinolenin was a model system for synthesis of epoxidized oils. Thus, the ^{1}H NMR assignments made in this study may be useful for identification of epoxy products and can be used to follow industrial synthesis or test the degree of epoxidation in an unknown oil. It should be noted that during the oxidation of edible oils, the generation of epoxides and their specific structures depend on both the oil composition and the oxidation conditions (Schaich, 2005). Not all of the epoxides observed in this work may necessarily appear in oxidized edible oils. Further, epoxides are present along with other oxidation products in oxidized lipids. In our study, the chemical shifts of epoxides derived from these oils did not coincide with other chemical groups, a necessary requirement if ^{1}H NMR is to be used to quantify epoxides in canola oil and fish oil. Their signals were also distinct from those of primary lipid oxidation products, such as conjugated dienes (5.40 - 6.58 ppm) and hydroperoxides (8.30 - 8.90 ppm), and other secondary/further lipid oxidation products which gave signals at 3.43 - 3.62 ppm (alcohols), 6.08 - 6.82 ppm (double bond conjugated with a keto group), and 9.49 - 9.79 ppm (aldehydes) (Martínez-Yusta et al., 2014). Therefore, a significant advantage of ^{1}H NMR is the ability to simultaneously detect epoxides and other oxidation products in oxidized oils.

3.5.4 Identification of an internal standard

One of the objectives of this study was to investigate the potential of applying ^{1}H NMR to quantitative studies of epoxides produced during lipid oxidation. It was thus important

to identify an internal standard for quantification. In a previous study, the signal at 0.88 ppm due to methyl groups was taken as the internal standard for quantification in oil samples (Aerts & Jacobs, 2004). However, in oxidized oils, carbonyls and alkanes of shorter chain length are produced by the secondary scissions of the intermediate products of lipid oxidation, especially at high temperatures (Schaich, 2005). That is, the breakdown of hydroperoxides could result in the generation of more methyl groups during lipid oxidation. Thus, the signal at 0.88 ppm does not remain constant as oxidation proceeds and it may not be a good internal standard for quantitative studies with oxidized oils. As an alternative, the signals associated with the esterified glycerol backbone of TAGs were considered. In the ^{1}H NMR spectrum of fish oil, they are clear both before and after epoxidation (Figure 3.5) as Signals 1, 2, and 3. Specifically, the signals associated with the protons of *sn*-1 and *sn*-3 esterified glycerols (4.18 and 4.33 ppm) are good candidates as internal standards for TAG oils as they remain constant as long as no hydrolysis occurs.

It is important to note that a change in the shape of the glycerol signals occurred in the ^{1}H NMR spectra of epoxidized fish oil compared to the original fish oil. This change was not observed for the epoxidized canola oil. Such a modification might indicate a change in structure, making those signals at 4.18 ppm and 4.33 ppm (dd, -C**H**$_2$-O-CO-R) associated with glycerol inappropriate as internal standards. However, as the *sn* -1, 3 glycerol protons were well separated from those signals associated with epoxides and double bonds, the distortion of the peak shape could not be due to their overlap with signals of epoxy protons and double bonds. To investigate the stability of the glycerol protons, Signal 2 and Signal 3 (Figure 3.5) were integrated before and after epoxidation

by taking the signal of methyl protons (not including methyl groups in ω-3 fatty acids) at 0.88 ppm as an internal reference. Because epoxidation, rather than oxidation, had taken place, these methyl protons should remain stable, allowing us to use their signal as an internal reference to verify the stability of the esterified glycerol protons. The ratio of the total area of Signal 2 and Signal 3 to the area of methyl groups was found to be ~0.78 for both fresh fish oil and epoxidized fish oil. Thus, it can be inferred that the area of signals due to glycerol remains relatively constant during epoxidation, allowing the use of the glycerol signals as an internal reference for quantification of epoxides despite the change in shape of the signal.

The proposed method of epoxidation was also conducted on stripped fish oil (without tocopherol) as well as fish oil ethyl esters (containing tocopherol), but the same change was observed in the shape of the signals associated with esterified glycerol and ethyl groups after epoxidation. The change in the shape was not observed for fish oils with lower concentrations of epoxides (< 30% of double bonds converted into epoxy groups). It is likely that the change in the shape occurs when the concentration of epoxides reaches some threshold level. In fish oils, the double bonds can be located either close or distant to the ester groups. When the original double bonds were converted into epoxy groups, the hybridization of the carbon atoms changed from sp^2 to sp^3, which would allow rotation about the single bond. The presence of epoxides derived from Δ4 unsaturated fatty acids coupled with such rotation might allow the epoxy groups to be closer to the protons of the glycerol backbone than those in canola oil. This would have an influence on the protons in the esterified glycerol or ethyl groups and offers an explanation for the change in shape of the signals after epoxidation of fish oils.

3.6 Conclusion

In this study, epoxidation of trilinolein, trilinolenin, canola oil, and fish oils by formic acid and hydrogen peroxide was followed by ^{1}H NMR to determine epoxide peak patterns and chemical shifts. Consistent with previous studies, monoepoxides gave a signal at 2.90 ppm. New ^{1}H NMR assignments were made for diepoxides (3.00, 3.09, 3.14 ppm) and triepoxides (3.00, 3.16, 3.21 ppm). These assignments for epoxides have been successfully applied to evaluate the ^{1}H NMR spectra of epoxidized oils in several previous studies, as well as in this work. The signals associated with the mixed epoxides in epoxidized canola oil and fish oils were consistent with the standards and were located between 2.90 - 3.23 ppm and 2.90 - 3.28 ppm, respectively. The chemical shifts of epoxides were distinct from hydroperoxides, alcohols, acids, and aldehydes, so it is possible to distinguish multiple lipid oxidation products in a single analysis using ^{1}H NMR. This information will facilitate both identification and quantification of epoxides in oil samples. The observation that the chemical shifts of epoxy groups move downfield when more epoxy groups are present in a hydrocarbon chain will also be useful in determining the structure of oxirane-containing products. The current study was conducted with epoxide concentrations at 50 mM. Signals associated with the protons of *sn*-1 and *sn*-3 esterified glycerols (4.18 and 4.33 ppm) were proposed as internal standards for quantification of epoxides in TAG oils. Further studies are needed to validate this approach and determine the lower detection limits of epoxides by ^{1}H NMR.

CHAPTER 4 A NEW ^{1}H NMR-BASED TECHNIQUE TO DETERMINE EPOXIDE CONCENTRATIONS IN OXIDIZED OIL

4.1 Publication Information

This chapter is a modified version of a manuscript, reworked here for consistency, with permission from Xia, W., Budge, S. M., & Lumsden, M. D. (2015). New ^{1}H NMR-based technique to determine epoxide concentrations in oxidized oil. *Journal of Agricultural and Food Chemistry,* 63(24), 5780-5786. Copyright 2015 American Chemical Society.

4.2 Abstract

A new method using ^{1}H NMR to determine epoxide concentrations in oxidized oils was developed and validated. Epoxides derived from lipid oxidation gave signals between 2.90 and 3.24 ppm, well separated from the signals of other lipid oxidation products. To calibrate, soybean oils with a range of epoxide concentrations were synthesized and analyzed using ^{1}H NMR by taking the *sn* -1, 3 glycerol protons (4.18, 4.33 ppm) as internal references. The ^{1}H NMR signals were compared to the epoxide content determined by titration with hydrogen bromide (HBr)-acetic acid solution. As expected, the signal response increased with concentration linearly (R^2 = 99.96%) and validation of the method gave results comparable to the HBr method. A study of the oxidative stability of soybean oil was performed by applying this method to monitor epoxides during thermal lipid oxidation. The epoxide content increased over time and showed a different trend compared to PV.

4.3 Introduction

Oils containing unsaturated fatty acids are susceptible to lipid oxidation, which brings about off-flavours and toxicity. In the study of lipid oxidation, much attention has been given to the measurement of primary oxidation products, such as hydroperoxides, because they are considered to be important intermediates in the oxidation process. Several methods have been developed to measure hydroperoxides (Barriuso et al., 2013), including the commonly used method, PV, which is easy to perform, making it the most popular indication of lipid oxidation (Firestone, 2009c). In PUFAs, the formation of hydroperoxides is accompanied by the rearrangement of methylene-interrupted double bonds, which generates conjugated dienes (Frankel, 2005). Conjugated dienes can be determined by measuring the absorption of ultraviolet-visible (UV-Vis) light (AOCS Method 7-58) (Firestone, 2009d) and this technique has also been applied extensively for the purpose of studying oxidative stability. The decomposition of hydroperoxides generates more active radicals and gives rise to the formation of secondary lipid oxidation products, including unsaturated aldehydes and short-chain volatiles. These products are most commonly followed by headspace GC-MS to provide a measure of progression of lipid oxidation to secondary stages (Barriuso et al., 2013). Conjugated dienes, hydroperoxides, and volatile products have become routine analyses of lipid oxidation applied in research and quality control. However, even together they provide an incomplete picture of lipid oxidation.

Epoxides have been reported to form from the rearrangement of the alkoxyl radicals and thus were initially recognized as secondary oxidation products (Frankel, 1984). More recently, epoxides have been considered as important intermediates

generated from peroxyl radical addition to double bonds, thus bypassing hydroperoxide formation (Schaich, 2012). However, they have been rarely monitored during oxidative stability studies of oils. Thus, much less information is available about their formation. Not accounting for epoxides may lead to misunderstandings or underestimation of the extent of lipid oxidation (Schaich, 2012). Monitoring epoxides should also provide a more comprehensive understanding of lipid oxidation mechanisms and provide explanations for unexpected products.

A number of methods have been developed to measure epoxides in oxidized oils, although all have associated challenges. The most widely used method to determine epoxide concentrations in epoxized oil is the HBr method, involving direct titration of oil with HBr - acetic acid solution (AOCS Method Cd 9-57) (Firestone, 2009b). However, this method is not applicable to oxidized oils, because α-, β- unsaturated carbonyls and conjugated dienes formed during lipid oxidation can also react with HBr, resulting in overestimations of epoxide content. A number of other methods have been introduced recently to measure epoxides in oils, including utilization of NBP (Hammock et al., 1974), DTC (Dupard-Julien et al., 2007), and transmethylation (Velasco et al., 2004). However, while these methods are sensitive, they require derivatization of epoxides before analysis, and the extra procedures for pretreatment are time-consuming.

^{1}H nuclear magnetic resonance (NMR) was recently applied to measure epoxides in oils by identifying the chemical shifts of epoxide groups, where a variety of internal standards were used (Aerts & Jacobs, 2004; Goicoechea & Guillen, 2010). In one of these studies (Goicoechea & Guillen, 2010), the non-deuterated chloroform present in the deuterated chloroform was used as an internal reference for quantitative purposes.

Generally, the main benefit of using the residual solvent signal as an internal standard is that it allows the sample to be recovered when the solvents are removed. However, the non-deuterated solvent content was found to vary from batch to batch so its use for quantitative purposes required external calibrants to determine its content in the deuterated solvent (Pauli, Gödecke, Jaki, & Lankin, 2012). Moreover, since chloroform is easily evaporated, when it is used as an internal standard for quantification, special care must be taken in handling the samples to avoid loss of solvent leading to changes in analyte concentrations. Aerts and Jacobs (2004) used signals from methyl groups as the internal standard for quantification, but the applicability of this method to oxidized oils needs to be verified, because alkanes can be formed when oils undergo lipid oxidation (Schaich, 2005), especially at high temperatures. As an alternative, Guillén & Uriarte (2009) found that the signals from glycerol protons were constant during oxidation in the absence of water so could be used reproducibly as alternative internal standards for ^{1}H NMR quantification, facilitating sample preparation.

In this study, a quantitative NMR method of measuring epoxides was developed and validated using soybean oil. This method represents significant improvement over other methods (Aerts & Jacobs, 2004; Goicoechea & Guillen, 2010; Velasco et al., 2004) in that partially-epoxidized oils are synthesized and used for quantification, instead of using epoxy fatty acid standards, so that signals are generated to mimic those that would be encountered in oxidized oils. Specifically, synthetic epoxidized soybean oils were made to establish a calibration curve, and used to validate the proposed method. The one-step sample preparation allowed rapid determination of epoxides without any addition of external references. The proposed method was applied to determine epoxides in soybean

oils thermally oxidized under accelerated shelf-life conditions.

4.4 Materials and Methods

4.4.1 Materials

Commercial soybean oil was obtained from Clic International Inc. (Montreal, Canada). Hydrogen bromide-acetic acid solution (33 wt. % in acetic acid) and deuterated chloroform were purchased from Sigma-Aldrich Co. (St. Louis, United States). Hydrogen peroxide (50%) was from Fisher Scientific (Ottawa, Canada). All the chemicals were of analytical grade.

4.4.2 GC analysis of fresh soybean oil

The fatty acid profile of soybean oil was analyzed using GC-FID. A modified version of the GOED Voluntary Monograph was followed to convert TAGs into FAMEs (GOED, 2012). FAMEs were separated on a DB-23 column ((50%-cyanopropyl)-methylpolysiloxane, 30 m× 0.25 mm ID, 0.25 μm thickness) using helium as the carrier gas. The GC conditions were as follows: the initial temperature of the oven was held at 153 ºC for 2 min and increased to 205 ºC at 2.3 ºC/min and held for 8.3 min; the FID was set to 270 ºC while the injector was operated in split mode (100:1) at 250 ºC (Sullivan et al., 2010). The fatty acid profile was reported as percent of total mass.

4.4.3 Epoxidation

The concentration of double bonds in the commercial soybean oil was calculated using fatty acid proportional data obtained by GC-FID (described above). To prepare

epoxidized soybean oil, a mixture of formic acid, hydrogen peroxide, and soybean oil in a molar ratio of 1: 0.5: 1.5 (double bonds: formic acid: hydrogen peroxide) was heated at 38ºC for 6 hours (Dinda et al., 2008; Mudhaffar & Salimon, 2010). To establish a calibration curve, epoxidized soybean oils with a range of different concentrations of epoxides were prepared by reducing the amount of formic acid and hydrogen peroxide while maintaining a constant molar ratios of formic acid and hydrogen peroxide at 0.5:1.5 (1:3). As the amount of epoxides generated from lipid oxidation is usually small, much lower amounts (<10% of the amount required to convert all double bonds into epoxy groups) of epoxidation reagents were used. For converting less than 2% double bonds in soybean oils to epoxy groups, samples were heated for 2 hr instead of 6 hr at 38 ºC. Epoxidized oil was recovered by extraction into ethyl acetate following the procedures in a previous study (Anuar et al., 2012), then dried with anhydrous sodium sulfate. The solvent in the oil layer was evaporated under nitrogen and the remaining oil was collected as the epoxidized soybean oil.

4.4.4 ^{1}H NMR analysis

The ^{1}H NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer. Oil samples were prepared for ^{1}H NMR analysis by dissolving 0.1 g of sample in 2.0 mL of deuterated chloroform and placing 750 µL of the solution in a NMR tube (5 mm diameter, 8” in length, Wilmad-LabGlass, US). The acquisition parameters were modified from a previous study (Guillén & Ruiz, 2004): spectral width 10080 Hz, relaxation delay 3 s, number of scans 64, acquisition time 3.25 s, with a total acquisition time of 6.9 min for each sample. The signal of non-deuterated chloroform present in the deuterated

chloroform was used as a reference for chemical shifts and the signals associated with the *sn* -1, 3 glycerol protons (4.18, 4.33 ppm) on the glycerol backbone were taken as an internal standard for quantification. All the integrations for quantitative NMR were conducted using Topspin 2.1.

4.4.5 Epoxide content determined by the HBr method

The concentration of epoxides in synthetic epoxidized soybean oil was determined by a modified AOCS Method Cd 9-57 using HBr (Firestone, 2009b). Diluted HBr-acetic acid solution and increased sample size were used to accurately determine the low epoxide concentrations. As HBr is sensitive to air and light, the HBr-acetic acid solution was standardized each time before use. Crystal violet solution (0.001 g/mL in acetic acid) was used as the indicator and the samples were titrated to a blue-green endpoint. This determination was performed in triplicate for each sample.

In order to facilitate comparison with PV, the concentration of epoxides was reported as millimole of epoxy groups per kilogram of oil (mmol/kg):

Epoxide content (mmol/kg) = (V×N×1000)/ M

where, V is the volume of HBr to titrate the sample in mL, N is the normality of the HBr solution in mol/L, and M is the mass of sample in g.

4.4.6 Characterization of epoxides by ^{1}H NMR

The fresh and the epoxidized soybean oils were analyzed by ^{1}H NMR for characterization of epoxides. The signals were assigned by comparison to the chemical shifts determined in a previous work (Goicoechea & Guillen, 2010).

4.4.7 Quantification of epoxides by ^{1}H NMR

A standard curve is usually established by analyzing a series of standard solutions and plotting the signal responses versus the concentrations. However, when using NMR, this may contribute to large deviations from a linear relationship over a variety of concentration, as observed in previous work (Mo, Harwood, & Raftery, 2010). Therefore, rather than establishing a standard curve by making serial dilutions of a stock solution, a calibration curve method was used to keep the NMR samples at the same total lipid concentration of ~50 mM but with varying epoxide contents.

A set of 20 synthetic epoxidized soybean oil samples were analyzed by ^{1}H NMR. The signals of the *sn*-1 and *sn*-3 glycerol protons (4.18, 4.33 ppm) were taken as an internal standard for quantification because they were well separated from other signals and remained constant during both epoxidation and oxidation. The area of the signal of epoxides was obtained by calibrating the area of the internal standard as 4.0000. The integrations were performed in triplicate. A calibration curve was established by plotting the epoxide peak area versus the epoxide concentration determined by HBr titration (AOCS Method Cd 9-57).

This quantitative NMR technique was tested for linearity, parameters of accuracy, and repeatability (Malz & Jancke, 2005) followig guidelines previously addressed by the International Union of Pure and Applied Chemistry (IUPAC) (Thompson, Ellison, & Wood, 2002). Statistical tests were conducted using Minitab 17. For the test of linearity, the regression and the residuals of the calibration curve were analyzed. For the estimation of accuracy and precision, four epoxidized samples were analyzed by ^{1}H NMR in five

replicates. The average epoxide content of each sample was compared to the value determined by AOCS Method Cd 9-57 for the test of trueness or bias, using a two-sample t-test (Table 4.1). The precision was described by the relative standard deviation (RSD) of the quintuplet results (Table 4.1) and characterized by the Horwitz equation (Boyer, Horwitz, & Albert, 1985). According to the Horwitz equation, an acceptable Horwitz RSD was calculated as:

$$\%RSD = \frac{2}{3} \times 2^{(1-0.5logC)}$$

where, C was the mass concentration of epoxides in decimal fraction. %RSD falling below this level indicated sufficient precision (Boyer et al., 1985).

To assess precision using the Horwitz equation (Boyer et al., 1985), the epoxide content was calculated as mass fraction of the oxirane oxygen in the oil (Firestone, 2009b):

Epoxide content = (V×N×0.016)/ M

where, V is the volume of HBr to titrate the sample in mL, N is the normality of the HBr solution in mol/L, and M is the mass of sample in g.

The precision of the method was determined in analyses of thermally oxidized soybean oils. Five soybean oil samples were incubated in open test tubes at 100 °C for 4, 8, 12, 16, and 20 days. Each sample was tested for its PV (AOCS Method Cd 8-53) (Firestone, 2009c) and epoxide content (the proposed method). The tests were conducted for each sample in triplicate (Table 4.2). The precision was described by the RSD of the triplicate results on individual samples.

4.4.8 Soybean oil oxidation under accelerated shelf-life conditions

To test the proposed methods, changes in PV and epoxide content were determined in soybean oil oxidized under accelerated shelf-life conditions. Samples of soybean oil (30 g) were incubated at 100 ºC in three open amber containers (125 mL), and every two days 2.5 g of oil from each container was analyzed for PV (AOCS Method Cd 8-53) and epoxides (the proposed method). The tests of each oil sample were performed in triplicate. PV and epoxide content were plotted over time for comparison.

4.5 Results and Discussion

4.5.1 Characterization of epoxides derived from soybean oil

The initial soybean oil contained 10.2 ± 0.1% palmitic acid (C16:0), 21.9 ± 0.3% oleic acid (C18:1n-9), 52.5 ± 0.8% linoleic acid (C18:2n-6), and 7.7 ± 0.2% linolenic acid (C18:3n-3). Therefore, the total unsaturation was estimated to be 5.46 mol double bonds per kg of oil. The signals of epoxides derived from soybean oil were between 2.90 - 3.24 ppm; as the size of those signals increased, the size of the signals due to double bonds (~ 5.40 ppm) decreased correspondingly (Figure 4.1, Table B-1 in Appendix B). Thus, the epoxidation method used here was successful in epoxidizing the commercial soybean oil, generating obvious signals of epoxides in the ^{1}H NMR spectra.

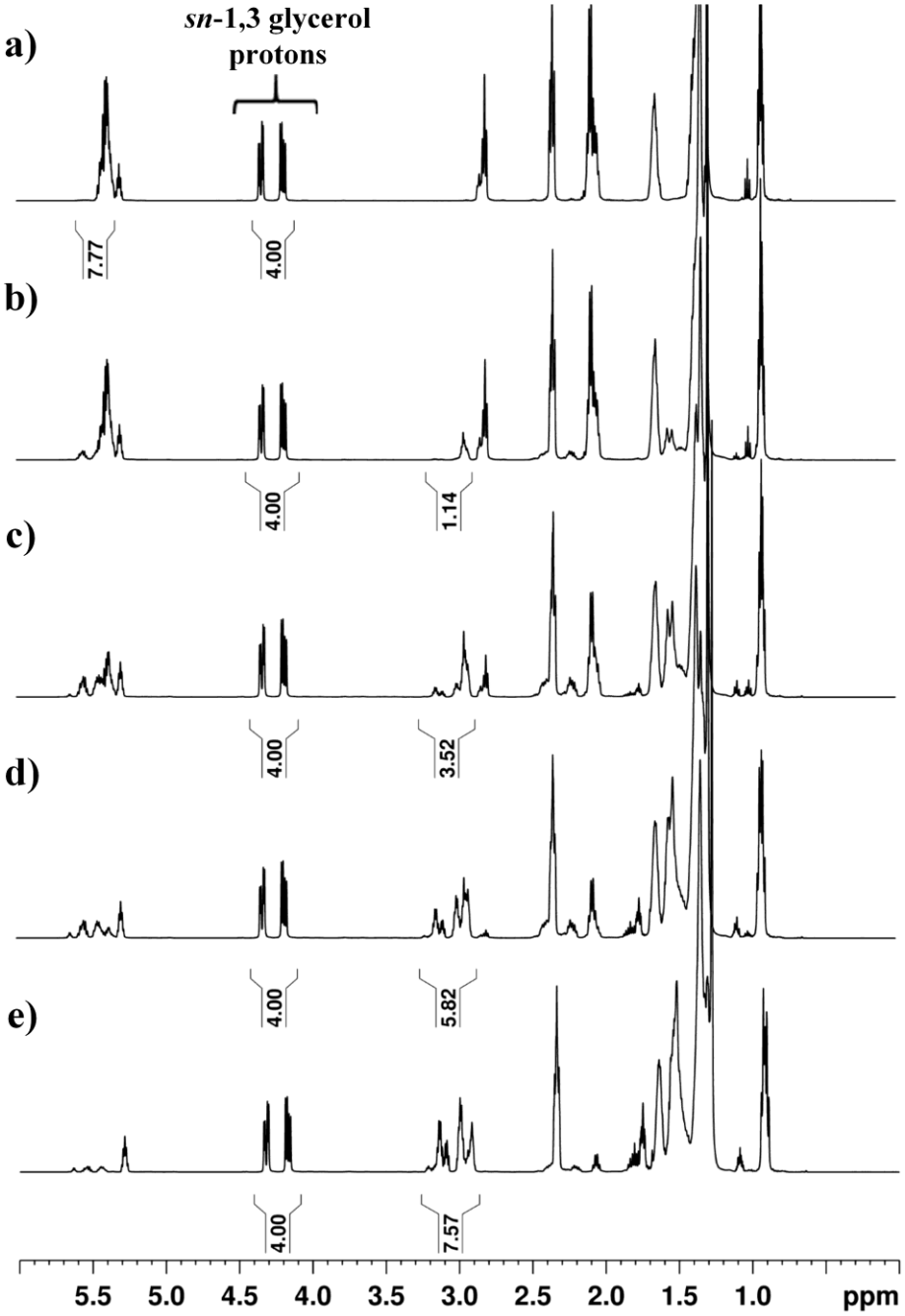


Figure 4.1 ^{1}H NMR spectrum of a) fresh soybean oil, and epoxidized soybean oils with epoxide content of b) 0.70 mol/kg, c) 2.06 mol/kg, d) 3.41 mol/kg, and e) 4.16mol/kg. The integrations of the double bonds (5.40 – 5.60 ppm) in a) and the epoxides (2.90 – 3.24 ppm) in b), c), d), and e) were made by taking the signal of *sn*-1 and *sn*-3 glycerol protons (4.18 and 4.33 ppm) as an internal standard. The area of the internal standard was calibrated as 4.00 as the number of *sn*-1 and *sn*-3 glycerol protons is 4.

Based on previous publications (Aerts & Jacobs, 2004; Goicoechea & Guillen, 2010) and our observation of the ^{1}H NMR spectra in Figure 4.1, we made the assignments as follows, where -CHOCH- represents an epoxy group: δ 1.45-1.60 (-C**H**$_2$-CHOCH-), 1.70-1.85 (-CHOCH-C**H**$_2$-CHOCH-), 2.00-2.10 (-C**H**$_2$-CH=CH-), 2.80-2.85 (-CH=CH-C**H**$_2$-CH=CH-), 2.90-2.95 (-C**H**OC**H**-), 2.90-2.95 (-C**H**OC**H**-CH$_2$-CH=CH-), 3.00 (-C**H**OCH-CH$_2$-CHOC**H**-), 3.06-3.24 (-CHOC**H**-CH$_2$-C**H**OCH-), 5.35-5.45 (-C**H**=C**H**-), 5.40-5.60 (-C**H**=C**H**-CH$_2$-CHOCH-), 5.60-5.65 (-CHOCH-CH$_2$-C**H**=C**H**-CH$_2$-CHOCH-) (Table B-1). The range of chemical shifts of epoxides increased with the concentration of epoxides in the oil (Figure 4.1). For oils with a low epoxide content, the signals of epoxides appeared around 2.90-2.95 ppm, which was likely related to the formation of the partially epoxidized linoleic acid and linolenic acid (-C**H**OC**H**-CH$_2$-CH=CH-), and overlapped with the less intense signal of epoxy oleic acid at 2.90-2.95 ppm (-CH$_2$-C**H**OC**H**-CH$_2$-). For oil samples with higher epoxide content (Figure 4.1c), the signals associated with diepoxides appeared ~ 3.00 ppm (-C**H**OCH-CH$_2$-CHOC**H**-) and 3.09 and 3.16 ppm (-CHOC**H**-CH$_2$-C**H**OCH-) and their intensities increased with increasing concentration. The signal which indicated the presence of triepoxides appeared at 3.20 ppm, when ~62% of the original double bonds had been converted into epoxy groups (Figure 4.1d). The epoxidized soybean oil gave five signals between 2.90 - 3.24 ppm with small signals due to double bonds remaining between 5.40 - 5.60 ppm (Figure 4.1e), which indicated that ~76% of the double bonds were converted into epoxy groups. The area ratio of the signals of mono-, di-, and tri-epoxides corresponded to the ratio of the original composition of the mono-, di-, and tri-unsaturated fatty acids.

Lipid oxidation is a relatively complex autoxidation process, so the structure of

epoxy products may vary with the conditions of oxidation. While the chromatography method employs common GC-FID instrumentation (Mubiru et al., 2014), making it an accessible technique to many analysts, it does rely on the use of epoxy FAME standards for identification of the signals on the chromatograms. Any epoxide structures which vary from the standards may be overlooked during identification and cause underestimations of epoxide concentration. In contrast, the selectivity of quantitative NMR did not depend on the structure of epoxides so it gave an overview of all the epoxy groups formed in the oil. The chemical shifts of epoxides (2.90 - 3.24 ppm) were distinct from other oxidation products including alcohols (3.43 - 3.62 ppm), conjugated dienes (5.40 - 6.58 ppm), double bonds conjugated with carbonyls (6.08 - 6.82 ppm), hydroperoxides (8.30 - 8.90 ppm), and aldehydes (9.49 - 9.79 ppm) (Martínez-Yusta et al., 2014). In summary, ^{1}H NMR can be used to detect epoxides in oxidized soybean oils with superior selectivity, which is a major advantage over the chromatography method.

4.5.2 Quantification of epoxides

Although fully epoxidized soybean oil gave signals between 2.90 - 3.24 ppm, oxidized oils with relatively low epoxide content gave signals between 2.90 - 3.10 ppm, mostly due to monoepoxides with or without adjacent double bonds (Table B-1). The epoxy protons were monitored for quantitative purposes because the size of the signals between 2.90 - 3.24 ppm was proportional to the amount of epoxy groups generated in the oil, regardless of the presence of saturated/unsaturated mono- and di-epoxides. Although the signals at 1.45-1.60 ppm (-C**H**$_2$-CHOCH-) and 2.00-2.10 ppm (-C**H**$_2$-CH=CH-) were related to the generation of epoxides (Figure 4.1), the change in size of the signals was

not proportional to the amount of epoxy groups; therefore, the epoxy protons were the best indicators of formation of epoxides.

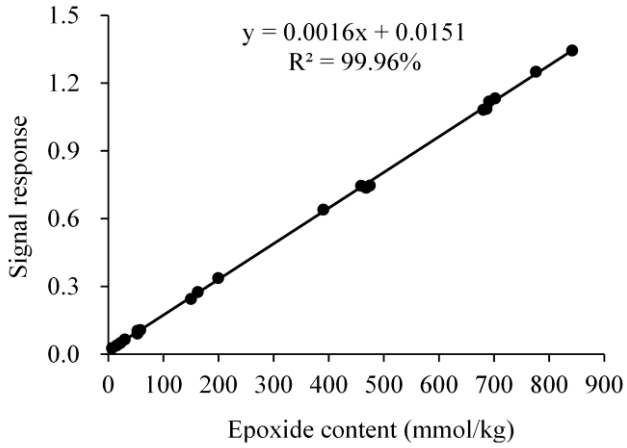


Figure 4.2 Fitted linear relationship by plotting signal response (y) versus the epoxide concentration (x) measured by the HBr method. Each point represents a single epoxidized soybean oil sample. The slope and intercept were $(1.579 \pm 0.004)\times10^{-3}$ and $(1.5 \pm 0.2)\times10^{-2}$, respectively.

By plotting signal response versus concentration, a calibration curve was established for epoxides in soybean oil with a good linear relationship. The regression equation was $y = 0.0151 + 0.0016x$ with a R^2 of 99.96%, where y represented the signal response and x was the concentration of epoxides (Figure 4.2). To our knowledge, this is the first demonstration of the agreement between the ^{1}H NMR method and the HBr method for epoxide concentrations. Residual analysis of all calibration points did not reveal any trends relating to the concentration of epoxides. The residuals passed the Anderson-Darling test and followed a normal distribution with a p-value of 0.148. Thus, the linear relationship between the signal response and the epoxide content did not vary with the concentration of epoxides in the experimental range of concentrations (6.7 – 841.6 mmol/kg). The area ratio of the signals of epoxides/internal reference increased

linearly with the epoxide content. Since epoxides were not found in the fresh soybean oil using the HBr method, the fresh soybean oil was considered a blank. The LOQ was determined to be 6.3 mmol/kg, by taking into account the standard deviation (σ) of the blank and the slope (s) of the calibration curve (LOQ = 10σ/s) using the method described by Shrivastava and Gupta (2011).

The testing of analytical accuracy was conducted on four epoxidized samples with different concentrations. As shown in Table 4.1, the proposed method gave results similar to those measured by HBr titration, with little bias. Two-sample t-tests showed no difference between the mean values determined by the HBr method and the proposed method for each sample (p-value>0.05). The precision of the proposed method (RSD 1.33 – 3.73%) was slightly poorer than that of the HBr method (RSD 0.71 – 2.26%), which was likely due to the errors introduced by manual integration of the ^{1}H NMR signals. The precision of both methods varied with concentration. Compared to the acceptable Horwitz RSD values (2.65 – 4.88%), the proposed method provided good precision (RSD 1.33 – 3.73%). Thus, quantitative NMR was shown to provide reliable results.

Table 4.1 Test of accuracy and precision of the proposed method, evaluated by the p-values of the two-sample t-test and the acceptable Horwitz RSDs, respectively.

Sample No.	epoxide content (HBr method)		epoxide content (^{1}H NMR)		two-sample t-test	acceptable Horwitz RSD
	mean±SD (mmol/kg)	RSD (%)	mean±SD (mmol/kg)	RSD (%)	p-value	RSD (%)
1	666.1±4.7	0.71	660.7±8.8	1.33	p>0.10	2.65
2	325.9±5.4	1.66	320.8±5.8	1.81	p>0.10	2.96
3	54.6±0.8	1.49	55.6±1.4	2.61	p>0.10	3.87
4	11.7±0.3	2.26	12.2±0.5	3.73	p>0.05	4.88

Table 4.2 PV and epoxide content measured for soybean oil heated at 100 ºC for the test of repeatability, expressed by RSDs.

Sample No.	peroxide value		epoxide content	
	mean±SD (mmol/kg)	RSD (%)	mean±SD (mmol/kg)	RSD (%)
1	47.2±0.3	0.61	8.4±0.4	4.15
2	48.3±1.4	2.80	17.2±0.6	3.58
3	89.1±0.7	0.79	29.2±1.1	3.85
4	87.6±0.6	0.64	55.7±1.5	2.69
5	60.0±0.6	1.02	90.7±2.0	2.20

The objective of this work was to develop a quantitative method to determine epoxide content in oxidized oils for the study of lipid oxidation. Therefore, it was important to verify the applicability of this method to thermally-oxidized oils. The test of repeatability of the method was performed on five thermally-oxidized soybean oil samples, which were heated at 100 ºC for 4, 8, 12, 16, and 20 days (Table 4.2). There were contrasting patterns of PV and epoxide content with Sample 1 and Sample 2 (Table 4.2) having similar PV (47.2 and 48.3 mmol/kg) but different epoxide contents (8.3 and 17.2 mmol/kg). Similarly, Sample 3 and Sample 4 (Table 4.2) had comparable PV (89.1 and 87.6 mmol/kg) but very different epoxide contents (29.2 and 55.7 mmol/kg). The differing epoxide concentrations indicate that the two samples had undergone different oxidation processes, an observation that would be overlooked with just examination of PV. Thus, epoxide content provided a different evaluation of the extent of lipid oxidation compared to PV.

4.5.3 ^{1}H NMR analysis of epoxide formation in soybean oil oxidized under accelerated shelf-life conditions

It was important to verify the applicability of this method to actual oxidized oils. For this, oils were oxidized under accelerated shelf life conditions (100 ºC) for 2, 4, 6, 8, 10, and 12 days. The curves presenting PV and epoxide content showed different patterns over time, although deviations were observed between the three samples determined at each test point (RSD between 1.62 – 10.63 % for PV, and 0.61 – 8.69 % for epoxide content; Figure 4.3). PV increased rapidly during the first eight days and then reached a peak, followed by a slow decline. In contrast, epoxide contents remained very low over the first

four days, then increased drastically and continuously after that, with no decreases during the experimental period. This pattern suggests that both peroxyl radical addition and alkoxyl radical rearrangement are active pathways generating epoxides, with perhaps stronger contributions from the latter. Another important point to note is that the epoxide concentrations approached levels of hydroperoxides at the end of the incubation period and probably would have exceeded PVs with longer reaction times, given the product formation curves. This observation verifies that epoxides are critically important products in lipid oxidation and should be monitored on a regular basis along with PV.

Lower amounts of epoxides were found in the soybean oil in this work than reported in a previous stability study of sunflower oil at 100 ºC (Goicoechea & Guillen, 2010). The two oils have very different fatty acid compositions which may influence the rate of formation and structure of lipid oxidation products. The surface area of oil during oxidation was also much greater in the previous work (Goicoechea & Guillen, 2010), likely leading to a faster oxidation process (McClements et al., 2008).

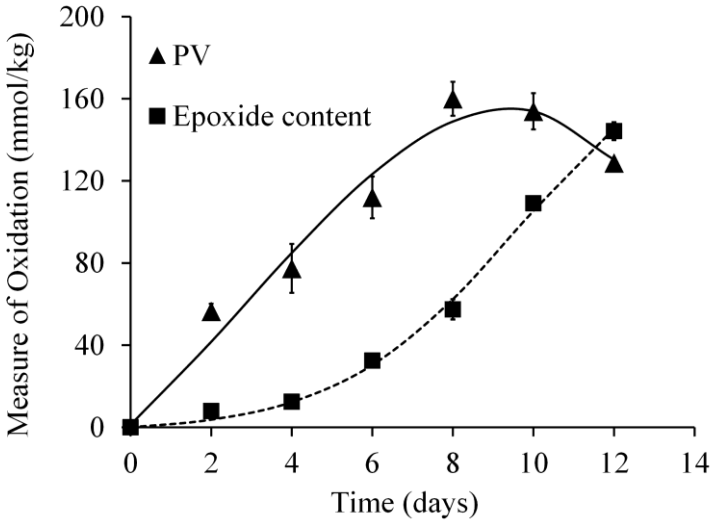


Figure 4.3 PV and epoxide content (mean +/- standard deviation, n=3 for each point) in the study of soybean oil oxidation performed at 100 ºC.

4.5.4 Comparison with other methods

The method described here is an original approach. Rather than spiking fully epoxidized soybean oil or epoxy fatty acid standards into samples, synthetic partially-epoxidized oils (<10% double bonds were converted into epoxy groups) were used to establish a calibration curve. The synthetic epoxidized oils with low epoxide levels gave similar signals (2.90 - 3.10 ppm) to those derived from the oxidation process, making it possible to establish a calibration curve mimicking the real situation. The use of synthetic epoxidized oils as calibration standards has a number of advantages. First, commercial unsaturated epoxy fatty acid standards and diepoxide standards are only available as a limited number of structures. Synthesizing partially-epoxidized oils with some double bonds remaining provides molecules with structures expected in test oils and avoids the limitation of the commercially available standards. Second, the extent of epoxidation can be controlled by the amount of reagents used, making it possible to adjust the range of the concentrations encompassed by the calibration curve for different oxidation conditions. When epoxides are expected at relatively low levels, epoxy groups are likely surrounded by double bonds, and only a small amount of reagents is needed. When epoxides are expected at a high level, diepoxides can be synthesized by increasing the amount of epoxidation reagents. Third, the current epoxidation method can be easily applied to all types of oils (Anuar et al., 2012; Dinda et al., 2008), so this method will have a wide range of applications.

In this study, the *sn*-1,3 glycerol protons were taken as the internal standards instead of using the signals due to the residual chloroform (Goicoechea & Guillen, 2010) or the methyl protons (Aerts & Jacobs, 2004). As described earlier, methyl protons have limited applications as internal standards for oxidized oils, while the use of the residual

solvent as standard may require external calibrants and special handling of the samples. Therefore, taking the signals related to the structure of TAGs as internal standards can overcome these problems and still allow the sample to be recovered in its pure form, because no extra standard material is added. Unfortunately, information describing LOQ, accuracy, or precision was not provided in those previous studies (Aerts & Jacobs, 2004; Goicoechea & Guillen, 2010), so it is impossible to compare the performance of the proposed method with the previous methods in those aspects.

Compared to the HBr method (Firestone, 2009), the proposed method overcomes the problem of the interferences caused by other lipid oxidation products (α-, β-unsaturated carbonyls and conjugated dienes) in the HBr method. Additionally, much less organic solvent is required for NMR analysis, because standardization of HBr solution is required before use of the reagent; HBr titrations are also time-consuming.

The limitation of the NMR technique is its low sensitivity compared to the HPLC and GC approaches. However, compared to those sensitive methods, such as the NBP (Hammock et al., 1974), DTC (Dupard-Julien et al., 2007), and the GC (Mubiru et al., 2013) methods, the major advantage of quantitative NMR is its convenience. No derivatization is needed for analysis of epoxy compounds, and the preparation of an NMR sample is simple and rapid, making NMR still a competitive approach to determine epoxides.

CHAPTER 5 GC-MS CHARACTERIZATION OF HYDROXY FATTY ACIDS GENERATED FROM LIPID OXIDATION IN VEGETABLE OILS

5.1 Abstract

Lipid oxidation has long been described as following a radical chain reaction mechanism, where hydrogen abstraction is considered the preferred pathway. Hydroxy compounds are, in theory, major products formed from hydrogen abstraction but their presence is rarely monitored. In this study, we describe a GC-MS technique to characterize hydroxy fatty acids formed during the oxidation of sunflower and canola oils. First, hydroxy fatty acids in oxidized oils were methylated and isolated from non-oxygenated structures using SPE. They were then converted into their TMS derivatives using a N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA)-pyridine method and identified by their EI and PCI spectra. Separation of most isomeric hydroxy fatty acids with very similar structures was accomplished using a DB-23 capillary column with (50%-cyanopropyl)-methylpolysiloxane phase. The fragmentation patterns of the TMS derivatives are discussed in detail and several easily applicable rules for spectral interpretations are presented. The major hydroxy fatty acids arising from oxidation of sunflower and canola oil were allylic and conjugated structures, specifically 8-, 9-, 10- and 11-hydroxyoctadecenoic acid (OH-C18:1) and 9- and 13-hydroxyoctadecadienoic acid (OH-C18:2).

5.2 Introduction

For decades, lipid autoxidation has been thought to follow a radical chain reaction mechanism, which includes the initiation, propagation, and termination stages (Schaich, 2012). In the initiation stage, radicals form from lipid molecules in the presence of initiators such as light, heat, and metals (Choe & Min, 2006). Oxygen is then added to lipid radicals ($L^{\bullet}$) to form peroxyl radicals ($LOO^{\bullet}$) in the propagation step. Peroxyl radicals can abstract hydrogen atoms from other lipid molecules, generating new lipid radicals and hydroperoxides. The new lipid radicals formed can then participate in further chain reactions. The hydroperoxides produced at this stage are considered primary oxidation products and important intermediates. However, they are only metastable and decompose to produce alkoxyl radicals ($LO^{\bullet}$) in the presence of heat, UV, light, or reducing metals. This reaction scheme maintains hydrogen abstraction as the preferred pathway in the chain reaction, so hydroxy compounds formed through hydrogen abstraction by alkoxyl radicals should also be major products. However, this reaction competes with other pathways such as cyclization and scission of alkoxyl radicals (Schaich, 2005), so alcohols are not the only product deriving from alkoxyl radicals.

Compared to the large number of studies on hydroperoxides, literature reports of the formation of hydroxy fatty acids are rare. Preliminary studies on the formation of hydroxy fatty acids were conducted through oxidation of fatty acid standards or hydroperoxide standards (Dix & Marnett, 1985; Frankel et al., 1977; Gardner et al., 1974). In more recent years, quantitative studies of hydroxy fatty acids in oxidized edible oils have been carried out with GC approaches which exhibited excellent accuracy and sensitivity (Marmesat et al., 2008; Wilson et al., 1997). Methylation of hydroxy groups

allowed their GC-MS determination as the formation of methoxy groups provided characteristic ions in the mass spectra that indicated the location of the original hydroxy groups (Wilson et al., 1997). In addition, the utilization of commercial standards and GC-MS techniques enabled the identification of GC peaks associated with underivatized hydroxy FAMEs (Marmesat et al., 2008). However, both earlier studies employed hydrogenation to simplify the chromatograms (Marmesat et al., 2008; Wilson et al., 1997), which eliminated any information regarding the position of double bonds that would be particularly important in the study of reaction mechanisms.

Here, we report our observations on the formation of hydroxy fatty acids during the oxidation of edible oils through a GC-MS approach. The objective of this work was to develop a GC-MS method with sufficient resolution to allow the characterization and quantification of unsaturated hydroxy fatty acids. We accomplished this through the use of methylation and TMS derivatization, fractionation with SPE, and application of EI and PCI techniques. Several EI mass spectra of TMS derivatives of hydroxy fatty acids have been reported in a previous study and their fragmentation rules were discussed (Kleiman & Spencer, 1973). Here, we followed those rules and present the first description of both saturated and unsaturated hydroxy fatty acids derived from canola oil and sunflower oil. PCI studies of hydroxy fatty acids have been limited in the literature, especially of allylic and conjugated structures. In this paper, we present the EI and methane PCI spectra of TMS derivatives of hydroxy fatty acids and characterize their mass spectra with diagnostic ions.

5.3 Materials and Methods

5.3.1 Chemicals and standards

N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) (>99%, for GC derivatization), sodium methoxide solution (0.5M CH_3ONa in methanol), methyl 12-hydroxystearate (>99%), and methyl ricinoleate (>99%, methyl 12-hydroxyoctadec-*cis*-9-enoate) were purchased from Sigma-Aldrich Inc (St. Louis, MO, US). (S)-13-HODE ((S)-13-hydroxyoctadeca-9,11-dienoic acid) methyl ester (>98%) and hydroxy linoleins (>98%, a mixture of OH-C18:2 isomers derived from trilinolein) were obtained from Cayman Chemical Company (Ann Arbor, Michigan, US). Si gel 40 (35-70 mesh) from Fluka Analytical (Switzerland) was used for SPE.

5.3.2 Oils and oxidation conditions

Sunflower and canola oils were obtained from local markets. Proportions of the major fatty acids in each oil (Table 5.1) were determined by transmethylation as described below. The oils (30 g) were weighed into 100 mL beakers and oxidized open in the dark at 70 ^{o}C for 7 days.

Table 5.1 Fatty acid composition (%, w/w) of the oils.

Fatty acid	Sunflower oil (mean ± SD)	Canola oil (mean ± SD)
C16:0	5.89±0.02	3.65±0.02
C18:0	3.28±0.00[a]	1.75±0.00[b]
C18:1n-7	0.60±0.01	3.10±0.02
C18:1n-9	34.34±0.02	59.76±0.02
C18:2n-6	53.87±0.01	18.39±0.01
C18:3n-3	0.08±0.00[c]	9.35±0.00[d]
others	1.94	4.00

[a] SD=0.004
[b] SD=0.004
[c] SD=0.001
[d] SD=0.001

5.3.3 Transmethylation

Transmethylation with sodium methoxide was carried out at room temperature following the procedures described in a previous study (Velasco et al., 2002). Aliquots of 100 mg of oil (or 50 µg of hydroxy linolein) were placed into 10 mL test tubes to which was added 1mL of *tert*-butyl methyl ether and 0.5 mL of 0.2 M sodium methoxide in methanol. The test tubes were vortexed for 1 min and left still for 2 min. Then 0.1 mL of 0.5 M sulfuric acid in water was added, followed by addition of 1.5 mL of water. After shaking for 10 s and centrifugation, the organic layer containing FAMEs were collected, and the solvent was evaporated under nitrogen.

5.3.4 SPE conditions

After transmethylation, 4-20 mg of FAMEs obtained in Section 5.3.3 were dissolved in 1 mL of 98:2 hexane: ethyl ether for SPE using a self-packed silica column. The silica column was prepared by filling a Pasteur pipette with 1 g of activated Si gel. A three-step

SPE approach was used for extraction of hydroxy FAMEs (Mubiru et al., 2013). The non-polar fraction was eluted using 15 mL of 98:2 hexane: ethyl ether. Then Polar Fraction 1, which contained epoxy FAMEs, was removed from the silica column using 15 mL of 90:10 hexane: ethyl ether. Polar Fraction 2, containing hydroxy FAMEs, was eluted last using 30 mL of ethyl ether.

5.3.5 TMS derivatization

Polar Fraction 2 collected in Section 5.3.4 was evaporated to dryness under nitrogen, mixed with 50 µL BSTFA and 50 µL pyridine, and left to sit for 30 min at room temperature. After the reaction, the sample was again evaporated to dryness and then dissolved in 200 µL hexane for GC analysis. To ensure that the reaction went to completion, the hydroxy FAME standards were carried through the process and examined by GC-MS; the absence of the original standards in the chromatograms indicated that the hydroxy FAMEs were fully converted into their corresponding TMS ethers.

5.3.6 GC-MS instrumentation

5.3.6.1 GC Conditions

Four GC capillary columns were used to test the separation of TMS derivatives of hydroxy fatty acids: DB-1ms (100% dimethylpolysiloxane, 30 m×0.25 mm, 0.25 µm), ZB-35HT (35%-phenyl-65%-dimethylpolysiloxane, 30 m×0.25 mm, 0.25 µm), DB-23 (50%-cyanopropyl-methylpolysiloxane, 30 m×0.25 mm, 0.25 µm), and Rtx-2330 (90% biscyanopropyl/10% phenylcyanopropyl polysiloxane, 105 m×0.25 mm, 0.2 µm). The temperature programs used for the above four GC columns were the following:

DB-1ms: started at 60 ºC and held for 2 min, increased to 180 ºC at 12 ºC/min and held for 5 min, increased to 225 ºC at 2 ºC/min and held for 10 min, increased to 300 ºC at 10 ºC/min and held for 10 min with a total time of 67 min. The carrier gas was helium at 1.2 mL/min, with the injector at 280 ºC in splitless mode and MS transfer line at 310 ºC.

ZB-35HT: started at 60 ºC and held for 2 min, increased to 180 ºC at 12 ºC/min, then increased to 215 ºC at 2.5 ºC/min and held for 20 min, increased to 300 ºC at 10 ºC/min and held for 10 min with a total time of 64.5 min. The carrier gas was helium at 1.4 mL/min, with the injector at 280 ºC in splitless mode and MS transfer line at 310 ºC.

DB-23: started at 50 ºC and held for 2 min, increased to 165 ºC at 15 ºC/min and held for 15 min, then increased to 205 ºC at 10 ºC/min and held for 10 min, increased to 230 ºC at 10 ºC/min and held for 10 min with a total time of 51.17 min. The carrier gas was helium at 1.2 mL/min, with the injector 250 ºC in splitless mode and MS transfer line at 235 ºC.

Rtx-2330: started at 50 ºC and held for 2 min, increased to 240 ºC at 15 ºC/min and held for 35 min, with a total time of 49.67 min. The carrier gas was helium at 1.0 mL/min, with the injector at 250 ºC in splitless mode and MS transfer line at 245 ºC.

5.3.6.2 MS Parameters

In EI mode, the ion source was kept at 200 ºC and emission current was at 250 μA, with a mass scan range of 60-500 and ionization voltage at 70 eV. For PCI analysis, methane was used as the reagent gas at 1.5 mL/min and the parameters remained the same as EI.

Methyl 12-hydroxystearate, methyl ricinoleate, and (S)-13-HODE methyl ester were used as references for the retention times and fragmentation patterns of OH-C18:0, OH-C18:1, and OH-C18:2. Transmethylation of the hydroxy linolein standard produced methyl esters of both 13-hydroxyoctadeca-9,11-dienoic acid and 9-hydroxyoctadeca-10,12-dienoic acid for the confirmation of the retention times and mass spectra of OH-C18:2 found in oxidized oils. Further assignments of the other peaks were made according to the rules reported in the previous study (Kleiman & Spencer, 1973).

5.4 Results and Discussion

5.4.1 GC column selection and patterns of hydroxy fatty acids

In this chapter, all hydroxy fatty acids are discussed as methyl esters in their TMS ether forms, where the original hydroxy groups were converted into OTMS groups. The following aspects were taken into consideration: 1) DB-1ms and ZB-HT35 were not able to resolve the TMS derivatives of hydroxy FAMEs from other unidentified non-TMS compounds; thus, the interferences from unknown peaks prevented conclusive identification and quantification of hydroxy FAMEs; 2) Rtx-2330 showed equivalency to DB-23 in separation of the hydroxy FAMEs from other polar FAMEs, but it did not allow the separation of 9-OH and 10-OH-C18:0 from the isomers of OH-C18:1; therefore, DB-23 was considered superior than Rtx-2330. However, none of the columns could completely resolve all positional isomers of allylic OH-C18:1 (C8, 9, 10, 11-substituted positional isomers). Therefore, DB-23 showed the best separation for quantitative purpose, although the allylic OH-C18:1 isomers co-eluted with each other.

Hydroxy fatty acids with the same structures and retention times were found in both fresh and oxidized canola and sunflower oils (TIC shown for canola oil in Figure 5.1, peak identities in Table 5.2), with the exception of 15-hydroxyoctadeca-9,12-dienoic acid that was not found in sunflower oil but was present in canola oil. The 15-hydroxyoctadeca-9,12-dienoic acid co-eluted with a 13-hydroxyoctadec-9,11-dienoic acid isomer and could not be separated using any of the four GC columns. The monounsaturated hydroxy fatty acids were present in both *cis* and *trans* forms; therefore, each OH-C18:1 positional isomer occurred as two geometric isomers. Also, both 13-hydroxyoctadec-9,11-dienoic acid and 9-hydroxyoctadec-10,12-dienoic acid had two geometric isomers and thus appeared as four peaks in chromatograms (Figure 5.1b). The two geometric isomers of OH-C18:2 were likely *cis/trans* and *trans/trans* structures, according to a previous study determining hydroxy dienes (Morales et al., 2010). Although it was inferred that the *trans* isomers would elute before their *cis* isomers on the GC column employed, further tests should be conducted to confirm the identities of these hydroxy fatty acids. The geometric isomers could not be differentiated by their EI or CI spectra.

In terms of the resolution of epoxy-hydroxy fatty acids, only DB-23 and Rtx-2330 were compared for their performances, with the Rtx-2330 having slightly better performance due to its highly polar phase. However, co-elution of peaks still occurred with Rtx-2330 (Figure 5.2a). The identification of the epoxy-hydroxy structures was based on the comparison with the mass spectra shown in a previous report of the homolytic decomposition of linoleic hydroperoxides (13-hydroperoxyoctadeca-*cis*-9,*trans*-11-dienoic acid and 9-hydroperoxyoctadeca-*cis*-12,*trans*-10-dienoic acid)

(Gardner et al., 1974). Several peaks had identical mass spectra, confirming the presence of epoxy-hydroxy stereoisomers. Possible geometric isomers would include *cis*/*trans* epoxy and *cis*/*trans* double bonds in their structures. Also, stereoisomers of 12,13-epoxy-11-hydroxyoctadec-9-enoic acid (*erythro*- and *threo*- configurations) have been identified as decomposition products from 13L-hydroperoxyoctadeca-9,11-dienoic acid in the presence of hemoglobin (Hamberg, 1975). The presence of such stereoisomers cannot be excluded. However, due to the co-elution of the epoxy-hydroxy isomers and interferences from other unknown compounds, it is difficult to determine the number of geometric isomers found for either of 9,10-epoxy-11-hydroxyoctadec-12-enoic acid and 12,13-epoxy-11-hydroxyoctadec-9-enoic acid. In future studies, an alternative method will be necessary to describe the number of geometric isomers of epoxy-hydroxy fatty acids derived from oils. GC-MS alone would not distinguish the geometric structures.

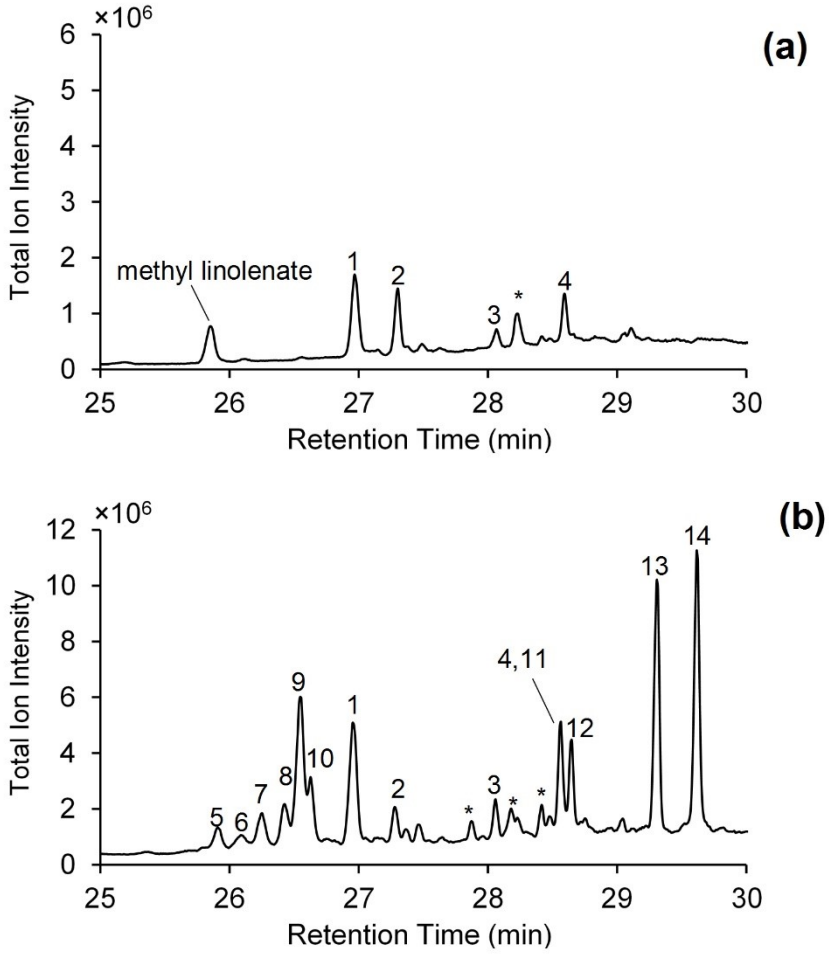


Figure 5.1 Total ion chromatograms of TMS ethers of hydroxy FAMEs derived from fresh canola oil (a) and oxidized canola oil (b), obtained using a DB-23 capillary column. Oxidation conditions: 70 ºC, 7 days, in dark. *represents unidentified hydroxy compounds.

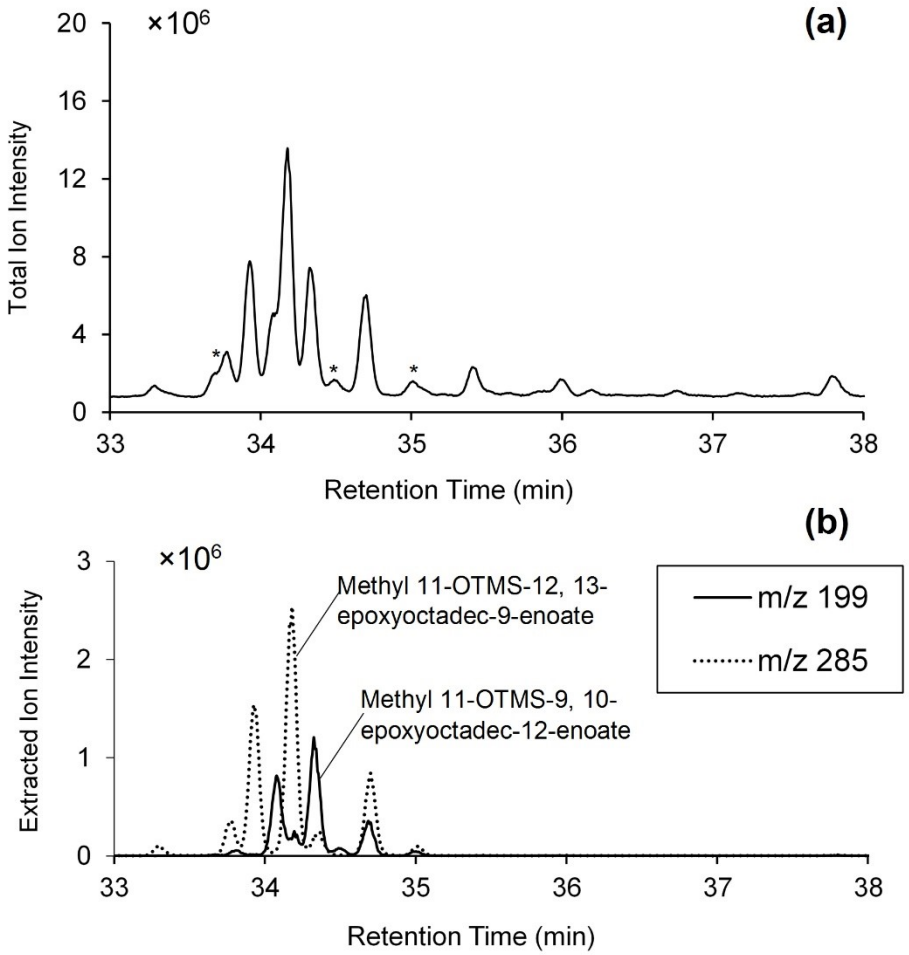


Figure 5.2 Total ion chromatogram (a) and extracted ion chromatogram (b) of TMS ethers of epoxy-hydroxy FAMEs derived from oxidized sunflower oil using an Rtx-2330 capillary column. The extracted ion chromatograms were plotted by monitoring *m/z* 199 and 285 for methyl 11-OTMS-9,10-epoxyoctadec-12-enoate and 11-OTMS-12,13-epoxyoctadec-9-enoate, respectively. *represents unknown peaks which caused interferences.

Table 5.2 Characteristic EI spectral ions corresponding to the identified compounds in Figure 5.1 and 5.2.

Compound (in TMS ether form)	Peak No.	Molecular weight	Characteristic EI spectral ions *m/z* (Relative abundance %)
OH-C18:0			
Methyl 9-hydroxyoctadecanoate	1[a]	386	371 (4), 339 (22), 259 (100), 229 (29), 155 (68)
Methyl 10-hydroxyoctadecanoate	1[a]	386	371 (4), 339 (12), 273 (100), 215 (28), 169 (30)
OH-C18:1			
Methyl 12-hydroxyoctadec-9-enoate	2	384	369 (6), 337 (20), 299 (14), 270 (54), 187 (100)
Methyl 8-hydroxyoctadec-9-enoate	5,8	384	369 (2), 337 (10), 241 (100), 129 (52)
Methyl 10-hydroxyoctadec-8-enoate	6[b],9[b]	384	369 (2), 337 (12), 271 (100), 149 (80)
Methyl 9-hydroxyoctadec-10-enoate	6[b],9[b]	384	369 (2), 337 (8), 227 (100), 129 (62)
Methyl 11-hydroxyoctadec-9-enoate	7,10	384	369 (2), 337 (7), 285 (100), 163 (50)
OH-C18:2			
Methyl 15-hydroxyoctadeca-9,12-dienoate	4[c]	382	335 (7), 310 (67), 145 (70)
Methyl 13-hydroxyoctadeca-9,11-dienoate	11[c],14	382	382 (M^+, 40), 367 (4), 335 (7), 311(100), 225 (40)
Methyl 9-hydroxyoctadeca-10,12-dienoate	12,13	382	382 (M^+, 42), 367 (2), 311(30), 225 (100)
diOH-C18:0			
Methyl 9,10-dihydroxyoctadecanoate	3	474	332 (28), 259 (100), 215 (30), 155 (83)

Compound (in TMS ether form)	Peak No.	Molecular weight	Characteristic EI spectral ions *m/z* (Relative abundance %)
Epoxy-OH-C18:1			
Methyl 9,10-epoxy-11-hydroxyoctadec-12-enoate	-[d]	398	383 (3), 241 (11), 199 (100)
Methyl 12,13-epoxy-11-hydroxyoctadec-9-enoate	-[d]	398	383 (2), 327 (10), 285 (100)

[a] Methyl 9-hydroxyoctadecanoate co-eluted with methyl 10-hydroxyoctadecanoate at Peak 1.
[b] The *trans/cis* isomer of methyl 10-hydroxyoctadec-8-enoate co-eluted with the *trans/cis* isomer of methyl 9-hydroxyoctadec-10-enoate at Peak 6 and Peak 9.
[c] Methyl 15-hydroxyoctadeca-9,12-dienoate (Peak 4) co-eluted with an isomer of methyl 13-hydroxyoctadeca-9,11-dienoate (Peak 11).
[d] The TMS ethers of methyl 9,10-epoxy-11-hydroxyoctadec-12-enoate and methyl 12,13-epoxy-11-hydroxyoctadec-9-enoate are labelled as 11-OTMS-9,10-epoxyoctadec-12-enoate and 11-OTMS-12,13-epoxyoctadec-9-enoate in Figure 5.2, respectively.

5.4.2 GC-MS characterization of hydroxy fatty acids

5.4.2.1 Saturated OH-FAME

In EI mass spectra, *m/z* 73 ($[(CH_3)_3Si]^+$) and *m/z* 75 ($[(CH_3)_2SiOH]^+$) are diagnostic ions for TMS ethers (Poole, 2013), indicating the presence of a hydroxy group in the original FAME and differentiating the identities of hydroxy FAMEs from other polar FAMEs. Molecular ions are not detectable; therefore, a $[M-15]^+$ ion, generated from a loss of a methyl moiety, is normally used as an indication of the molecular mass (Poole, 2013). In addition, a few intense ions in EI spectra can normally be used to determine the location of the OTMS which represents the hydroxy group in the fatty acid chain.

Specifically, the cleavage α to the OTMS moiety results in characteristic ions in EI spectra. For example, methyl 10-OTMS-octadecanoate, or 10-OH-C18:0, is identified by the two ions (*m/z* 215 and 273) resulting from cleavage of the bonds on either side of the OTMS carbon. However, it was also noticed that *m/z* 371, although being the $[M-15]^+$ ion, was weak in the spectrum (Figure 5.3a).

PCI spectra do not differentiate between structural isomerism. Therefore, methyl 9-OTMS, 10-OTMS, and 12-OTMS octadecanoate gave the same PCI spectra (Figure 5.3b), with the major ions being *m/z* 385 $[M-1]^+$, 371 $[M-15]^+$, 339 $[M+H-48]^+$, 325 $[M+C_2H_5-90]^+$, 297 $[M+H-90]^+$, 265 $[297-32]^+$, and 247 $[265-18]^+$. In the PCI spectra of saturated structures containing TMS ethers, the expected ion $[M+1]^+$ was not detectable; instead, $[M-1]^+$ at *m/z* 385 was present, while *m/z* 371, the $[M-15]^+$ ion, was abundant (21% of the base peak) and could be used to determine the molecular mass of the peak. The base peak in the PCI spectrum was *m/z* 297 $[M+H-90]^+$, produced by the loss of a $[(CH_3)_3SiOH]^+$ moiety from the molecule (Plattner, Gardner, & Kleiman, 1983). It should

be noted that, in the PCI spectra of the TMS ethers of OH-C18:0, the relative intensities of *m/z* 325, 297, 265, and 247 resembled those in the PCI spectrum of methyl oleate (Figure 5.3c).

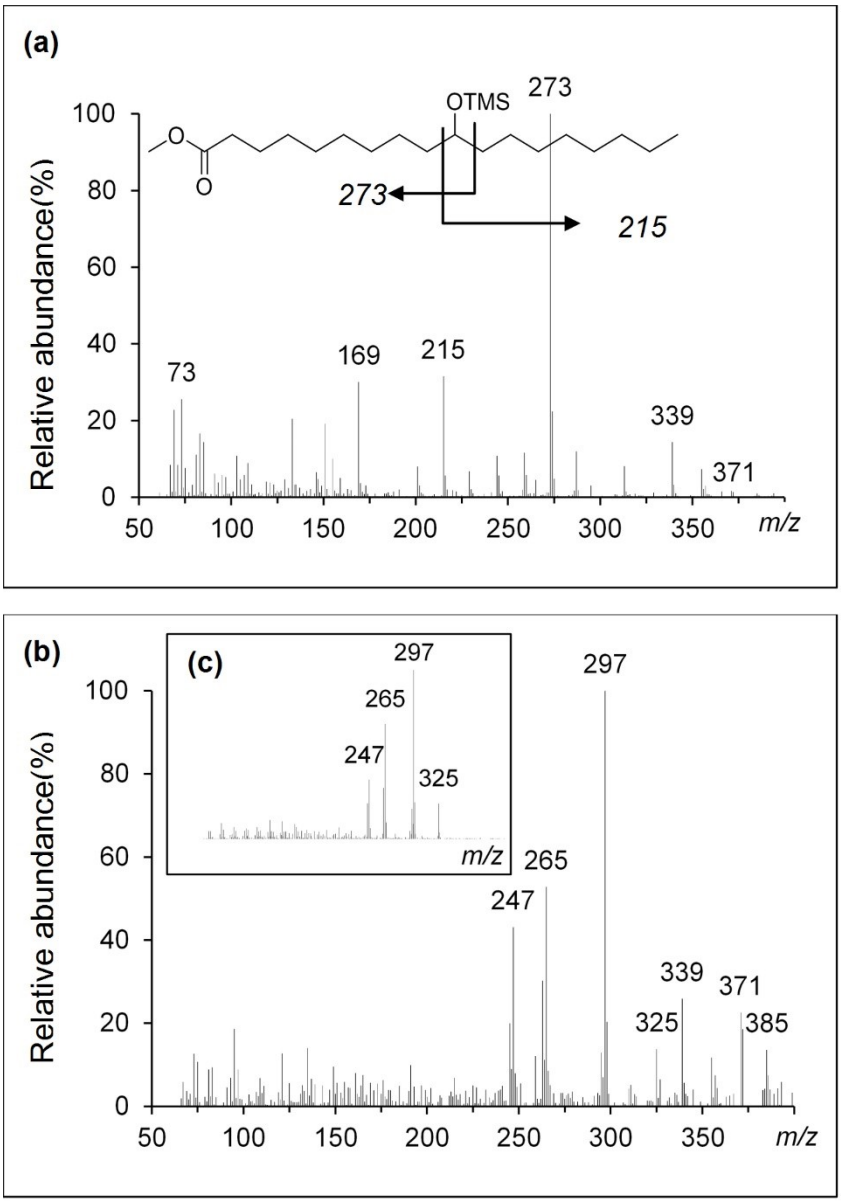


Figure 5.3 EI (a) and PCI (b) spectra of methyl 10-OTMS-octadecanoate (molecular mass: 386). PCI (c) spectrum of methyl oleate. CI reagent gas: methane at 1.5 mL/min.

The PCI spectrum of methyl 12-OTMS-octadecanoate has been reported using isobutane as reagent gas (Plattner et al., 1983), where weak signals were reported for $[M+H]^+$, $[M-H]^+$, $[M+H-15]^+$, and $[M-H-15]^+$, with all at ~3-5% of the base peak *m/z* 297 $[M+H-90]^+$. Its PCI spectrum with ammonia as reagent gas also gave an abundant *m/z* 297 $[M+H-90]^+$, with intense ions at *m/z* 314 $[M+NH_4-90]^+$, 387 $[M+H]^+$, and 404 $[M+NH_4]^+$ in the spectrum. Both of those studies identified the primarily protonated molecules $[M+H]^+$ arising from proton transfer and expected with PCI; however, in the present work, $[M+1]^+$ generated in the PCI spectrum of methyl 12-OTMS-octadecanoate with methane was less than 1% in relative intensity. Methane has the lowest proton affinity (PA) (546.0 kJ/mol for CH_5^+ and 684.1 kJ/mol for $C_2H_5^+$) among the three reagent gases discussed here (823.8 kJ/mol for isobutane reactant ion $C_4H_9^+$, 857.7 kJ/mol for ammonia reactant ions NH_4^+) (Westmore & Alauddin, 1986). The molecules protonated with methane (CH_5^+ and $C_2H_5^+$) had higher internal energy than those with isobutane and ammonia, which is determined by the difference of the PA between the molecules and the reagent gas (Sparkman, Penton, & Kitson, 2011), so PCI with methane produced more fragmentation than isobutane and ammonia, resulting in less intense $[M+H]^+$ ions. In addition, the high hydride ion affinities (HIA) of methane (1130 kJ/mol for CH_5^+ and 1138 kJ/mol for $C_2H_5^+$) and isobutane (967 kJ/mol for isobutane reactant ions $C_4H_9^+$), compared to ammonia (816 kJ/mol for ammonia reactant ions NH_4^+) (Westmore & Alauddin, 1986), facilitated the formation of deprotonated molecules and thus explained the presence of $[M-H]^+$ in the spectra. Therefore, the high HIA and low PA of methane produced a high degree of fragmentation, a relatively small $[M+H]^+$, and a visible $[M-H]^+$ in PCI spectra.

The collision-stabilized complexes ($[M+29]^+$ and $[M+41]^+$), which are normally seen in methane PCI spectra, were not observed for the TMS derivatives. However, *m/z* 325 $[M+29]^+$ was clear in the PCI spectrum of methyl oleate (Figure 5.3c) under the same CI-MS conditions.

5.4.2.2 Mono-unsaturated OH-FAME

5.4.2.2.1 Non-allylic structures

In the non-allylic structure, where the OTMS group is separated from a double bond by at least one carbon atom, the α-cleavage resembled that occurring in saturated OH-FAME. For example, α-cleavage in methyl 12-OTMS-octadec-9-enoate (TMS ether of methyl ricinoleate) resulted in *m/z* 187 and 299 (Figure 5.4a). However, an abundant ion of even numbered mass was also found at *m/z* 270; this is known as the 'migration ion' ($CH_3O(TMSO)C^+(CH_2)_7CH=CH-CH_2$) and is formed by transferring the TMS group to the ester group and cleaving between C11 and C12 (Kleiman & Spencer, 1973). Because of the even numbered mass, it is distinct from the other major ions in spectra.

The PCI spectrum of the same structure showed major ions at *m/z* 383 $[M-1]^+$, 369 $[M-15]^+$, 337 $[M+H-48]^+$, 295 $[M+H-90]^+$, 263, and 245 (Figure 5.4b). Compared to the PCI spectra of the saturated structures, the base peak shifted to $[M-15]^+$, providing an indication of the molecular weight.

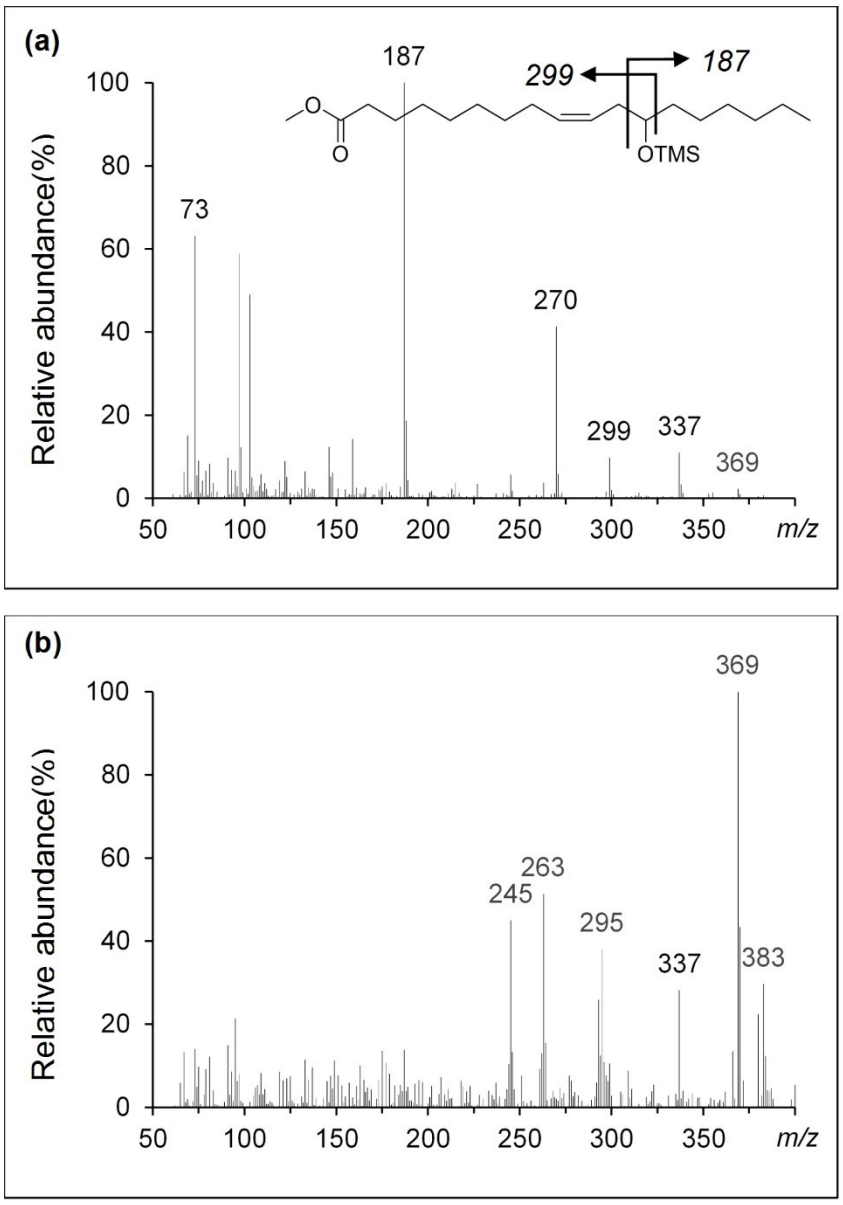


Figure 5.4 EI (a) and PCI (b) spectra of methyl 12-OTMS-octadec-9-enoate (molecular mass: 384). CI reagent gas: methane at 1.5 mL/min.

5.4.2.2.2 Allylic structures

When the OTMS group was bonded to an allylic carbon, the cleavage α to the carbon carrying the OTMS group still resulted in the base ion in EI spectra (Kleiman & Spencer, 1973); however, the α-cleavage between the OTMS group and the double bond was not

observed. For instance, *m/z* 241 was the base ion in the spectrum of 8-OTMS-octadec-9-enoate and the ion resulting from the cleavage between C8 and C9 was not detectable (Figure 5.5a). The ion *m/z* 369 $[M-15]^+$ was present but at less than 10% intensity.

Again, the positional isomers of TMS-derivatized allylic OH-C18:1 could not be differentiated from each other by their CI spectra. However, the base ion was *m/z* 263 (Figure 5.5b), different from the non-allylic structures (Figure 5.4b). It should be noted that the $[M-15]^+$ ion in the PCI spectra was much less intense for all of the allylic structures, while it was often the base ion for non-allylic structures. The PCI spectrum of 8-OTMS-octadec-9-enoate still gave an ion of *m/z* 295 $[M+H-90]^+$, but *m/z* 293 was more intense. The presence of *m/z* 293, 263, and 245 was similar to those shown in the PCI spectrum of methyl linoleate (Figure 5.5c).

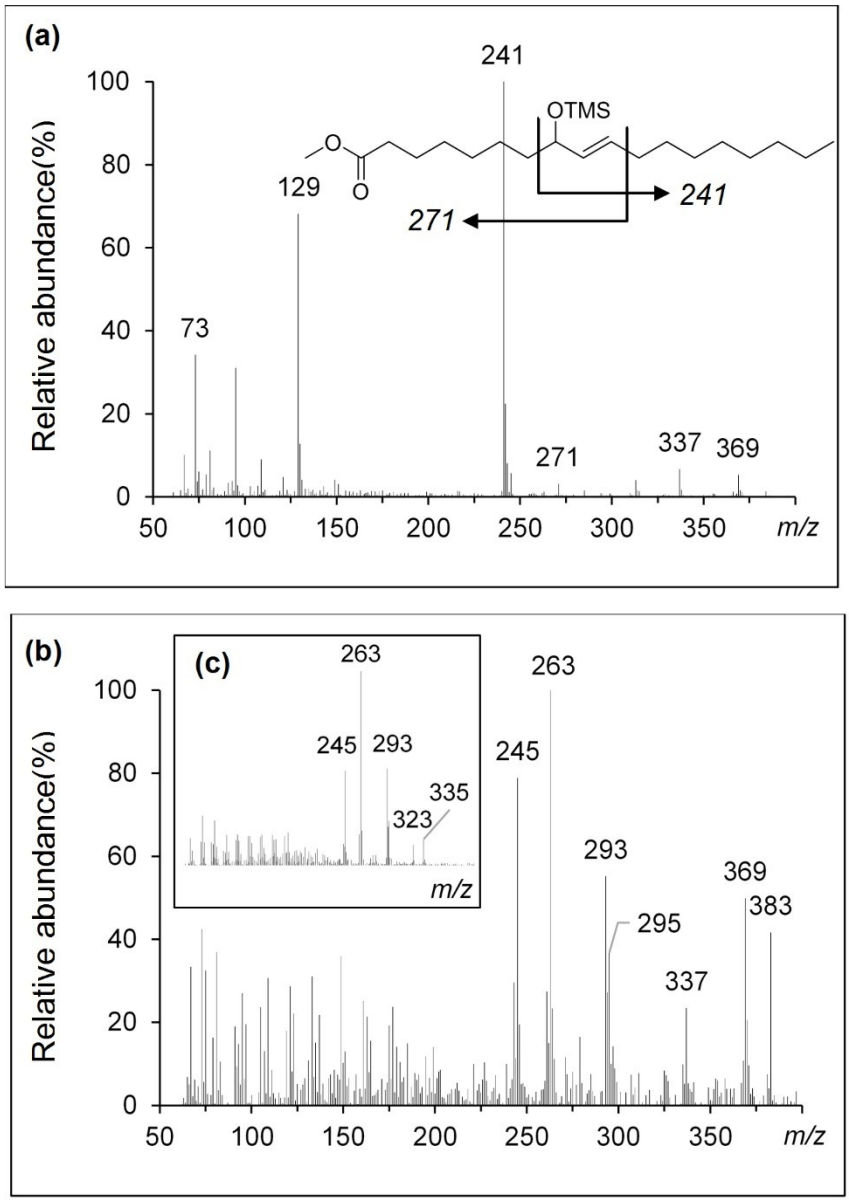


Figure 5.5 EI (a) and PCI (b) spectra of methyl 8-OTMS-octadec-9-enoate (molecular mass: 384). PCI (c) spectrum of methyl linoleate. CI reagent gas: methane at 1.5 mL/min.

5.4.2.3 Di-unsaturated OH-FAME

5.4.2.3.1 Non-conjugated structures

The only non-conjugated OH-C18:2 identified here was methyl 15-OTMS-octadeca-9,12-dienoate. Its EI spectrum showed two diagnostic ions, *m/z* 145 and 310, with no molecular ion observed (Figure 5.6a). The α-cleavage occurred on both sides of the OTMS group, producing *m/z* 145 and 339. As described above for non-allylic structures (methyl 12-OTMS-octadec-9-enoate), a migration ion can form from the fragment containing the ester group; therefore, *m/z* 310 was generated from a migration ion ($CH_3O(TMSO)C^+(CH_2)_7CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2$) with cleavage between C14 and C15. The PCI spectrum of methyl 15-OTMS-octadeca-9,12-dienoate gave a base peak of *m/z* 293 $[M+H-90]^+$, with other major ions found at *m/z* 381$[M-H]^+$, 367 $[M-15]^+$, 335 $[M-48]^+$, 261, and 243. The peaks of *m/z* 293, 261, and 243 were also found in the PCI spectrum of methyl linolenate. This pattern will be compared to that shown for methyl 9-OTMS-octadeca-10,12-dienoate in Section 5.4.2.3.2.

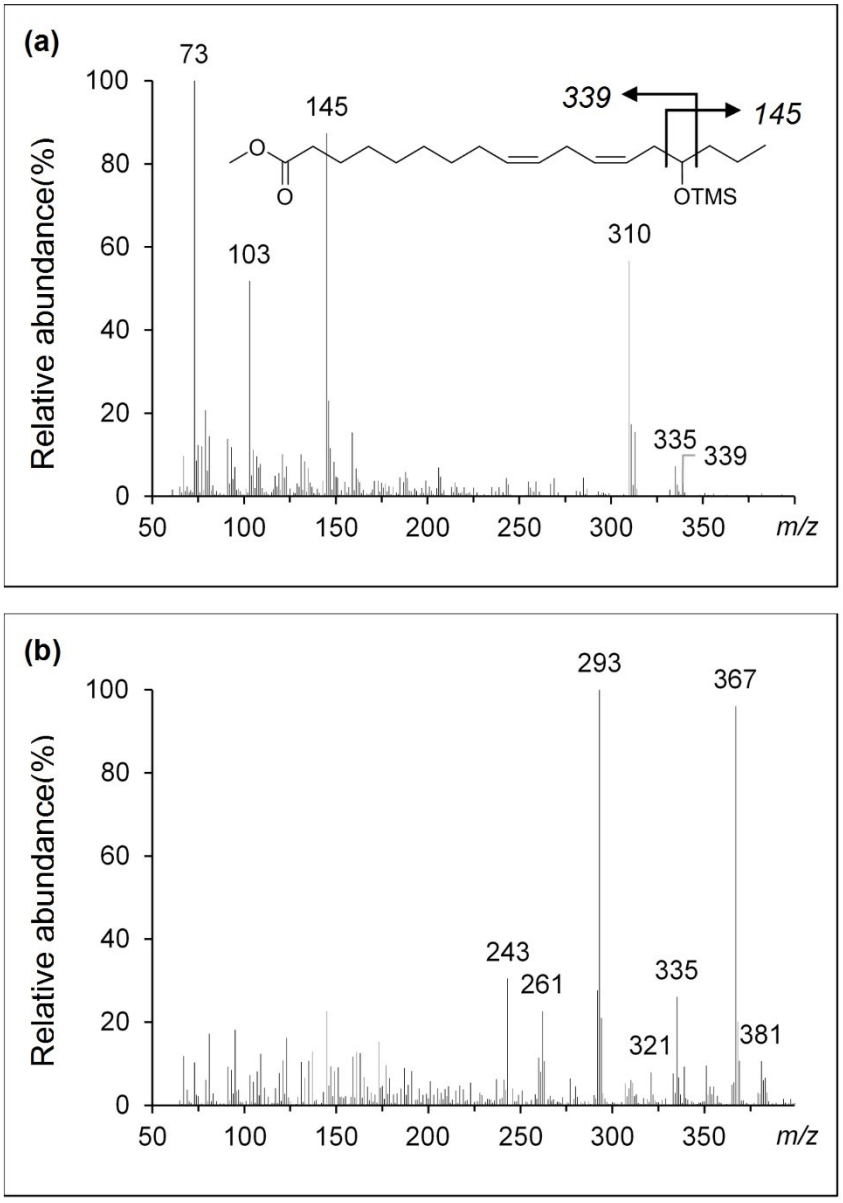


Figure 5.6 EI (a) and PCI (b) spectra of methyl 15-OTMS-octadeca-9,12-dienoate (molecular mass: 382). CI reagent gas: methane at 1.5 mL/min.

5.4.2.3.2 Conjugated dienes

Two conjugated OH-C18:2, 9-hydroxyoctadeca-10,12-dienoic acid and 13-hydroxyoctadeca-9,11-dienoic acid, were found in oxidized oils. The EI spectra of the conjugated structures are characteristic due to the base ions (Spiteller & Spiteller, 1997),

which resulted from the α-cleavage to the OTMS group occurring on the opposite side of the double bonds (Figure 5.7a). No cleavage was observable between the OTMS group and the conjugated double bonds. Moreover, the conjugated system, including the two double bonds and the OTMS group, was cleaved as a whole fragment (Figure 5.7a). This finding was consistent with the previous fragmentation patterns reported by Kleiman and Spencer (1973). Taking methyl 9-OTMS-octadeca-10,12-dienoate as an example, *m/z* 225 was the base ion in the EI spectrum, formed by cleavage between C8 and C9. The fragment of *m/z* 225 contained the OTMS group as well as the conjugated double bonds. Cleavage between C13 and C14 produced *m/z* 311, which also contained the conjugated system. In addition, the conjugated structures gave a clear molecular ion at *m/z* 382 and an obvious *m/z* 367 $[M-15]^+$, distinct from the EI spectra of other structures mentioned above.

The PCI spectra of methyl 9-OTMS-octadeca-10,12-dienoate and 13-OTMS-octadeca-9,11-dienoate were not distinguishable from each other and were quite similar to that of non-conjugated methyl 15-OTMS-octadeca-9,12-dienoate (Figure 5.6b). For instance, both conjugated structures had *m/z* 261, 243, and 321, with a base peak of *m/z* 293 $[M+H-90]^+$ (Figure 5.7b), which resembled the major peaks found in the PCI spectrum of methyl linolenate (Figure 5.7c). However, there were a number of ions that differentiated the conjugated and non-conjugated structures. Specifically, the PCI spectra of the conjugated diene structures showed a larger $[M+H]^+$ than $[M-H]^+$, while the opposite was observed for the other structures discussed above. Moreover, the conjugated diene structures produced a more intense *m/z* 321 than *m/z* 335 in the PCI spectra, while methyl 15-OTMS-octadeca-9,12-dienoate showed the opposite pattern. Last, as observed

for OH-C18:1, the non-conjugated/non-allylic structures gave a substantially stronger *m/z* 367 $[M-15]^+$ in PCI spectra than the conjugated/allylic structures.

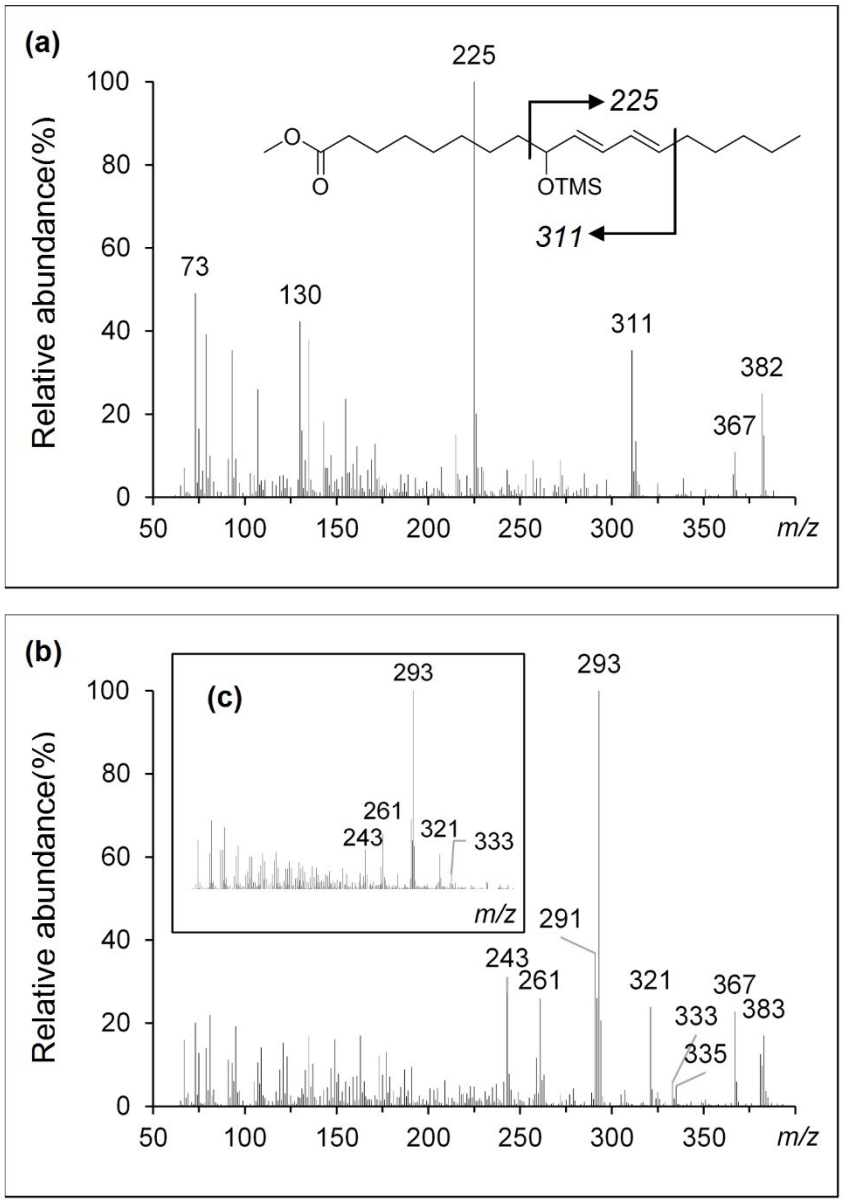


Figure 5.7 EI (a) and PCI (b) spectra of methyl 9-OTMS-octadeca-10,12-dienoate (molecular mass: 382). PCI (c) spectrum of methyl linolenate. CI reagent gas: methane at 1.5 mL/min.

5.4.2.4 DiOH-FAME

A dihydroxy fatty acid was identified as 9,10-dihydroxyoctadecanoic acid. In the EI spectrum of methyl 9,10-diOTMS-octadecanoate, neither the molecular ion nor the $[M-15]^+$ was visible, making it difficult to interpret the molecular weight (Figure 5.8a). However, the PCI spectrum of this compound indicated the molecular weight (MW: 474) by *m/z* 459 $[M-15]^+$ and a small but visible *m/z* 473 $[M-H]^+$ (Figure 5.8b). In the EI spectrum, the α-cleavage between C9 and C10 was shown by *m/z* 215 and 259 (Figure 5.8a). The ion *m/z* 361, resulting from the cleavage between C10 and C11, was not visible in the spectrum; however, its migration ion *m/z* 332, as an even numbered ion, was distinct from other ions.

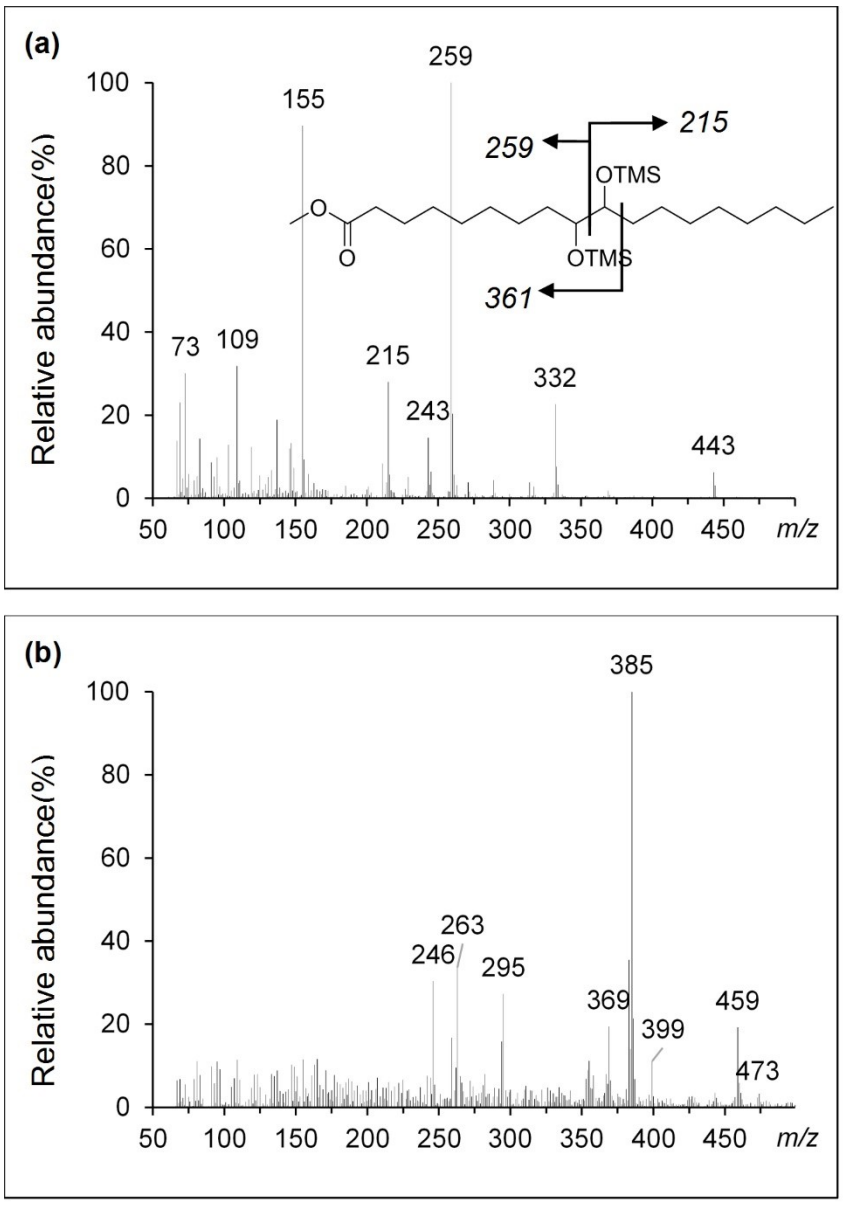


Figure 5.8 EI (a) and PCI (b) spectra of methyl 9,10-diOTMS-octadecanoate (molecular mass: 474). CI reagent gas: methane at 1.5 mL/min.

5.4.2.5 Epoxy-hydroxy FAME

Epoxy-hydroxy fatty acids found in Polar Fraction 2 have been reported to form from linoleic acid hydroperoxides (Gardner et al., 1974). In the oxidized oils (Figure 5.2), a group of peaks were identified as isomers of methyl 11-OTMS-9,10-epoxyoctadec-12-

enoate and 11-OTMS-12,13-epoxyoctadec-9-enoate. The base peaks in the EI spectra of methyl 11-OTMS-9,10-epoxyoctadec-12-enoate (Figure 5.9a) and 11-OTMS-12,13-epoxyoctadec-9-enoate (Figure 5.10a) were *m/z* 199 and 285, respectively, produced from α-cleavage occurring between the OTMS group and the epoxy group. Cleavage also occurred on the other side of the epoxy group, resulting in a significantly weaker ion in the high mass range: *m/z* 241 for 11-OTMS-9,10-epoxyoctadec-12-enoate and *m/z* 327 for 11-OTMS-12,13-epoxyoctadec-9-enoate. In both EI spectra, *m/z* 383 $[M-15]^+$ was visible but at extremely low intensity. The PCI spectra of these two structures were difficult to interpret (Figure 5.9b and 5.10b). Both showed *m/z* 383 $[M-15]^+$ to allow determination of molecular weight; however, much more fragmentation was found for epoxy-hydroxy structures in methane PCI spectra. The 11-hydroxy-12,13-epoxyoctadec-9-enoic acid and 11-hydroxy-12,13-epoxyoctadec-9-enoic acid observed in this study were possibly formed from 9-hydroperoxy-C18:2 and 13-hydroperoxy-C18:2 (Hamberg & Gotthammar, 1973).

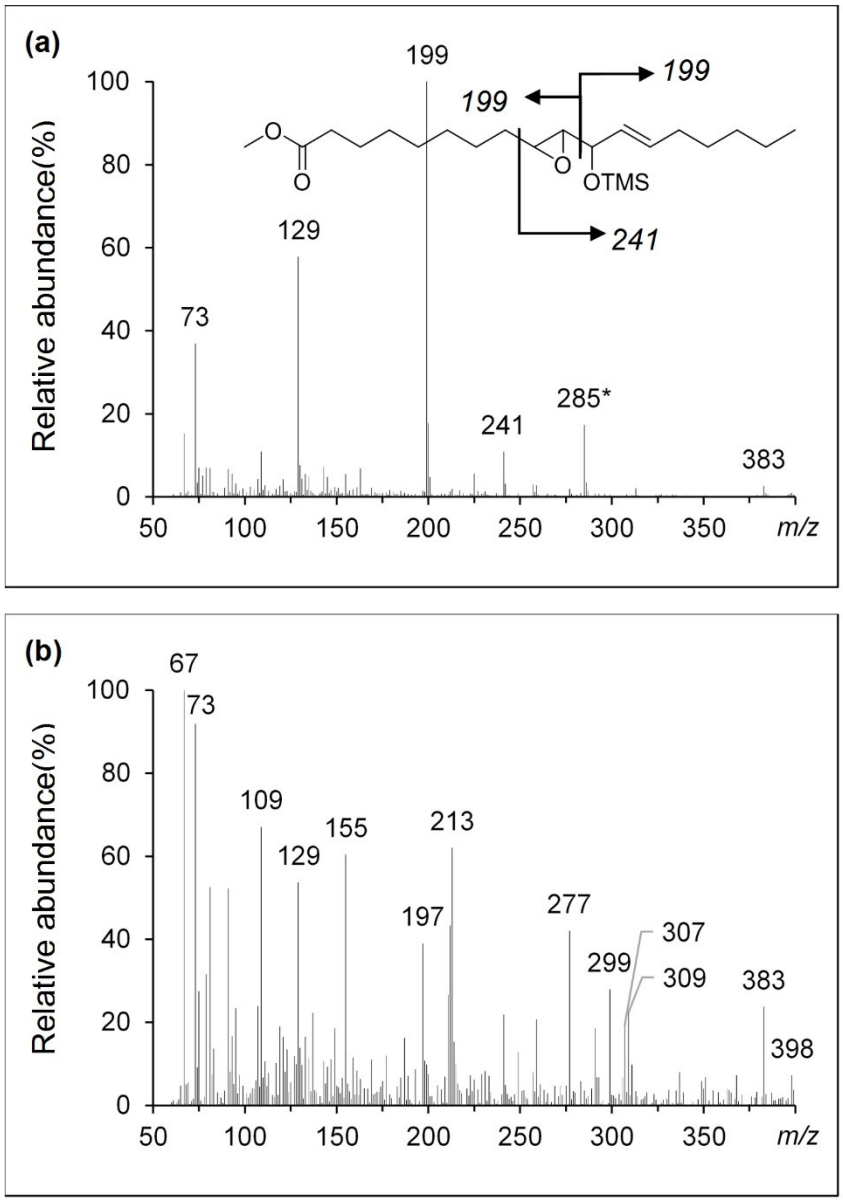


Figure 5.9 EI (a) and PCI (b) spectra of methyl 11-OTMS-9,10-epoxyoctadec-12-enoate (molecular mass: 398). CI reagent gas: methane at 1.5 mL/min. **m/z* 285 resulted from co-elution with an isomer of methyl 11-OTMS-12,13-epoxyoctadec-9-enoate.

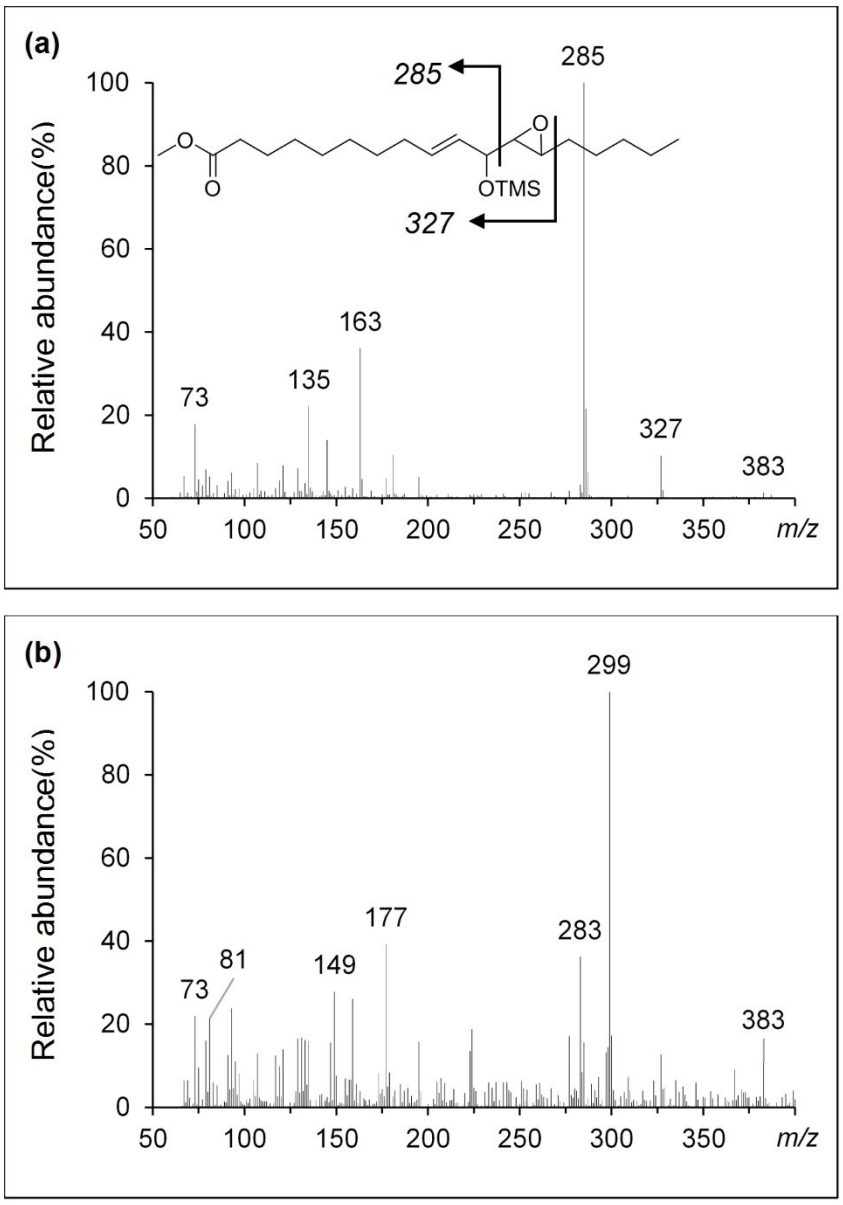


Figure 5.10 EI (a) and PCI (b) spectra of methyl 11-OTMS-12,13-epoxyoctadec-9-enoate (molecular mass: 398). CI reagent gas: methane at 1.5 mL/min.

5.5 Conclusion

This work identified both saturated and unsaturated hydroxy fatty acids as lipid oxidation products in edible oils using GC-MS analysis of their TMS derivatives and evaluation of the EI and PCI spectra. EI resulted in more characteristic ions that were useful in differentiating the positional isomers of hydroxy fatty acids but often failed to provide

information regarding the molecular weights. The fragmentation patterns of hydroxy fatty acids in their TMS ether forms in EI are summarized as follows:

1) In EI spectra, a saturated monohydroxy fatty acid is readily interpreted by the two ions produced from the α-cleavage on both sides of the OTMS group;

2) Interpretation of unsaturated hydroxy fatty acids can proceed by first distinguishing non-allylic and allylic structures: a non-allylic structure produces two fragments by α-cleavage on both sides of the OTMS group, with a migration ion generated from the fragment with the ester group. The migration ion is easily found as the only even numbered ion with a relatively high intensity in the spectrum. The non-allylic structures encountered in this study have only one methylene group between the hydroxy group and the double bond; therefore, the presence of the migration ion and the other fragment ion produced from the α-cleavage enables deduction of the structure of the hydroxy fatty acid;

3) Allylic structures, including conjugated dienes, always show base peaks resulting from the α-cleavage of OTMS group on the side opposite to the double bond(s). The allylic system is cleaved as a whole, without cleavage between the OTMS group and the double bond(s);

4) The number of epoxy-hydroxy structures encountered in this work is limited. Both compounds gave base peaks derived from the cleavage between the OTMS group and the epoxy group. As the OTMS group was allylic to the double bond, no cleavage was found between the allylic carbons.

In contrast, PCI, with methane as the reagent gas, provided little structural information from fragmentation patterns but did confirm the molecular weights of the compounds with $[M-15]^+$. The findings with PCI are summarized as follows:

1) $[M+H]^+$ and $[M-H]^+$ are both present for most of the TMS derivatives of hydroxy fatty acids found in this work; thus, $[M+H]^+$ and $[M-H]^+$, as well as $[M+H-90]^+$, are useful indicators of molecular weight;

2) PCI spectra of hydroxy fatty acids cannot differentiate positional isomers; however, the intensities of the major ions vary in allylic structures and non-allylic structures. Non-allylic structures tend to give an intense $[M-15]^+$ ion in the PCI spectrum, often larger than 90% of the base peak, in contrast to allylic/conjugated structures;

3) Apart from the $[M+H]^+$, $[M-H]^+$, and $[M-15]^+$ ions, the major ions shown in the PCI spectra of TMS ethers of hydroxy FAMEs resembled those ions in the PCI spectra of regular FAMEs with one more site of unsaturation. For example, the major ions in the PCI spectra of OH-C18:0 resembled the ions observed for methyl oleate.

By avoiding hydrogenation and applying these fragmentation patterns, we were able to characterize for the first time the structures of several unsaturated hydroxy fatty acids in oxidized canola and sunflower oils. The monounsaturated hydroxy fatty acids formed during lipid oxidation in the oils were 8-, 9-, 10-, and 11-substituted, with diunsaturated hydroxy fatty acids being 9- and 13-substituted. The identification of these unsaturated hydroxy fatty acids will facilitate quantitative studies of these compounds.

CHAPTER 6 SIMULTANEOUS QUANTIFICATION OF EPOXY AND HYDROXY FATTY ACIDS AS OXIDATION PRODUCTS OF TRIACYLGLYCEROLS IN EDIBLE OILS

6.1 Abstract

Unsaturated hydroxy fatty acids are considered oxidation products of TAGs but their formation has seldom been monitored due to the limitations of available methods. Epoxy fatty acids have also been found to be important intermediates during lipid oxidation; quantification of both structures may help evaluate the extent of competition among various lipid oxidation pathways. This article describes a method to simultaneously determine saturated and unsaturated, epoxy and hydroxy fatty acids derived from oxidation of vegetable oils. The experimental procedures employed transmethylation with sodium methoxide, separation of epoxy and hydroxy FAMEs using SPE, and TMS derivatization of hydroxy groups. GC-MS was used to identify the epoxy and hydroxy FAMEs in two different SPE fractions, while GC-FID was used to determine their quantities. An isomer of methyl 13-hydroxy-octadeca-9,11-enoate (13-HODE) TMS ether co-eluted with methyl 15-hydroxy-octadeca-9,12-enoate TMS ether, which was only present in canola oil; thus, GC-MS-SIM was used to determine the concentration of 13-HODE. Using GC-FID, the LOD of the method was 30 μg/g for epoxy FAMEs and 3-4 μg/g for hydroxy FAMEs, while the LOD was 40 μg/g for 13-HODE with GC-MS-SIM. In a stability study at 40 ºC, the proposed method has been successfully applied to monitor epoxy and hydroxy fatty acids in sunflower and canola oil. In future work, the LOD of the method can be lowered by increasing SPE sample load.

6.2 Introduction

Lipid oxidation is an important reaction that limits the shelf-life of fat-containing foods (Gordon, 2004). Oxidative deterioration of TAGs, the major lipid class present in foods, leads to a variety of oxidation products. The most commonly measured products are hydroperoxides, known as primary oxidation products, and conventionally determined by a titration method. In addition to hydroperoxides, conjugated dienes are considered primary products and are measured by a spectrophotometric method. Secondary oxidation products, including aldehydes and other volatiles derived from the decomposition of hydroperoxides, are also measured using a number of quantitative approaches (Barriuso et al., 2013).

Epoxy and hydroxy fatty acids have long been discussed as secondary lipid oxidation products (Frankel, 1987) but the formation of these oxidized fatty acids in oils and foods has gained more interest in recent years (Morales et al., 2014; Brühl et al., 2016). These oxidized fatty acids are normally present at low levels (e.g., mg/g levels in used frying oils and μg/g in fresh oils), so GC and HPLC have become the most suitable methods due to the sensitive detectors they employ. HPLC-UV methods enabled the quantification of hydroxydienes by measurement of absorption at 234 nm due to the conjugated double bonds, but for epoxy fatty acids, ELSD was necessary (Morales et al., 2012a). FID has been the major detector coupled with GC for the determination of epoxy fatty acids alone (Mubiru et al., 2013; Velasco et al., 2002) or together with hydroxy fatty acids (Marmesat et al., 2008). The major experimental setup in that approach was to prepare FAMEs from TAGs and then extract oxidized FAMEs from non-polar (non-oxidized) FAME for GC analysis. When simultaneously determining these two types of

compounds, hydrogenation was used to simplify the chromatograms for peak identification (Marmesat et al., 2008), which resulted in the loss of double bond structures.

Here, we improve on that earlier work by avoiding the use of hydrogenation and retaining the unsaturated structure. A three-step SPE separation was employed to eliminate co-elution of hydroxy compounds with epoxy FAMEs in chromatograms (Mubiru et al., 2013); thus, epoxy and hydroxy FAMEs were separated into two SPE fractions. The hydroxy groups were then converted into their TMS ethers enabling GC-MS identifications of both the epoxy and hydroxy FAMEs in the same analysis. With confirmation of the peak identities, the two SPE fractions were analyzed by GC-FID and GC-MS-SIM for quantification. This method was then applied to determine both saturated and unsaturated epoxy and hydroxy fatty acids in oxidized canola oil and sunflower oil.

6.3 Materials and Methods

6.3.1 Materials

6.3.1.1 Standards and oils

N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) (>99%, for GC derivatization), sodium methoxide solution (0.5M CH_3ONa in methanol), methyl 12-hydroxystearate (>99%), and methyl ricinoleate (>99%, methyl 12-hydroxyoctadec-9-enoate) were purchased from Sigma-Aldrich Inc (St. Louis, MO, US). 13(S)-HODE (13(S)-hydroxyoctadeca-9,11-dienoic acid) methyl ester (>98%) was obtained from Cayman Chemical Company (Ann Arbor, Michigan, US). Methyl nonadecanoate (C19:0) and

methyl octadec-9-enoate (C18:1) was purchased from Nu-chek Prep, Inc. (Elysian, MN, US). Si gel 40 (35-70 mesh) from Fluka Analytical (Switzerland) was used for SPE. Sunflower oil and canola oil were obtained from local markets. Their fatty acid compositions were determined after transmethylation (Table 6.1).

Table 6.1 Fatty acid composition (%, w/w total FAME) of the oils.

Oil	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	others
Canola oil	3.7	1.8	59.8	18.4	9.4	6.9
Sunflower oil	5.9	3.3	34.3	53.9	0.1	2.5

6.3.1.2 Synthesis of methyl 9,10-epoxyoctadecanoate

Methyl 9,10-epoxyoctadecanoate was chemically synthesized from methyl octadec-9-enoate using a hydrogen peroxide-formic acid method (Xia, Budge, & Lumsden, 2016) and was purified using the SPE conditions described in Section 5.3.4. The purity of methyl 9,10-epoxyoctadecanoate was determined to be >95% by GC-FID.

6.3.2 Transmethylation

Methylation with sodium methoxide was carried out following the procedures described by Berdeaux et al. (1999a). Aliquots of 50 mg oil were weighed into 10 mL test tubes, to which were then added 1mL of *tert*-butyl methyl ether and 0.5 mL of 0.2 M sodium methoxide in methanol. The test tubes were vortexed for 1 min and kept at room temperature for 2 min. Then, 0.1 mL of 0.5 M sulfuric acid solution was added, followed by addition of another 1.5 mL of water. After shaking and centrifugation, the organic

layer containing FAMEs was collected in another test tube and then diluted to 5 mL with *tert*-butyl methyl ether.

6.3.3 SPE conditions

Aliquots of 1 mL FAME solution, containing ~10 mg FAMEs, were dried under nitrogen and re-dissolved in 0.5 mL of 98:2 hexane: ethyl ether for SPE. The silica column was prepared by filling a Pasteur pipette with 1 g of activated Si gel. A three-step SPE was employed for extraction of epoxy and hydroxy FAMEs (Mubiru et al., 2013). First, the non-polar fraction, consisting of unaltered FAMEs (non-polar FAMEs) was eluted using 15 mL of 98:2 hexane: ethyl ether. Then, Polar Fraction 1, which contained epoxy FAMEs, was removed from the silica column using 15 mL of 90:10 hexane: ethyl ether. The third fraction, Polar Fraction 2, mainly containing hydroxy FAMEs, was eluted using 30 mL of ethyl ether.

6.3.4 TMS derivatization

Methyl nonadecanoate (C19:0) was added to Polar Fractions 1 (5 µg) and 2 (1 µg) as the internal standard. Both fractions were evaporated to dryness under nitrogen and derivatized with 50 µL BSTFA and 50 µL pyridine for 30 min at room temperature. After the reaction, the samples were again evaporated to dryness. Polar Fraction 1 was dissolved in 1 mL hexane and Polar Fraction 2 in 200 µL hexane for GC analysis. The final concentration of the internal standard was 5 µg/mL in both fractions.

6.3.5 GC analysis

6.3.5.1 GC-FID

After TMS derivatization, all the FAME samples were analyzed by GC-FID. Polar Fraction 1 was analyzed using an Rtx-2330 capillary column (90% biscyanopropyl/10% phenylcyanopropyl polysiloxane, 105m×0.25 mm i.d., 0.2 μm). The temperature program started at 50 ºC and was held for 2 min, increased to 174 ºC at 15 ºC/min and was held for 10 min, and finally reached 250 ºC at 20 ºC/min and was held for 31 min. The total analysis time was 55.07 min. The carrier gas was helium at 1.2 mL/min, with injector at 250 ºC in splitless mode. The FID detector temperature was set at 270 ºC, with argon, hydrogen, and air flow rates at 50, 30, 300 mL/min.

Polar Fraction 2 was analyzed using a DB-23 capillary column (50%-cyanopropyl-methylpolysiloxane, 30m×0.25 mm i.d., 0.25 μm). The temperature program started at 50 ºC and was held for 2 min, increased to 165 ºC at 15 ºC/min and was held for 15 min, then increased to 205 ºC at 10 ºC/min and was held for 10 min, increased to 230 ºC at 10 ºC/min and was held for 10 min with a total time of 51.17 min. The carrier gas was helium at 1.2 mL/min, with injector at 250 ºC in splitless mode. The FID detector parameters remained the same.

6.3.5.2 GC-MS

GC-MS was used to identify the epoxy and hydroxy FAMEs. The GC parameters remained the same for each type of column as applied in GC-FID. In the full scan mode, the ion source was kept at 200 ºC, with a mass scan range of *m/z* 60-600. In addition, GC-MS-SIM was employed to analyze Polar Fraction 2 derived from canola oil. In SIM

mode, *m/z* 312 was measured from 22 to 26 min for C19:0 (internal standard) and *m/z* 382 was measured from 26 min to the end of the run to determine 13-HODE.

6.3.6 Quantitative studies

6.3.6.1 Response factors

Methyl nonadecanoate (C19:0) was the internal standard for GC-FID quantifications. The response factors for hydroxy-C18:0, hydroxy-C18:1, and hydroxy-C18:2 FAMEs were obtained using methyl 12-hydroxystearate, methyl ricinoleate, and 13-HODE methyl ester, respectively. Synthetic 9,10-epoxyoctadecanoate was used to obtain the response factor for all epoxy FAMEs; as in other studies, the response factor of synthetic 9,10-epoxyoctadecanoate, named 9,10-epoxystearate (9,10-ES) in those studies, was applied to all epoxy FAMEs (Mubiru et al., 2013; Velasco et al., 2002; Velasco et al., 2004). Calibration curves were established by plotting the area ratio of analyte standard/internal standard versus the concentration ratio of analyte standard/internal standard. In addition to GC-FID, a calibration curve for 13-HODE methyl ester was obtained in GC-MS-SIM, taking methyl nonadecanoate as the internal standard. In both, GC-FID and GC-MS-SIM, the working ranges of the calibration curves were 0, 2, 4, 6, 8, and 10 µg/mL, with the internal standard at 5 µg/mL. For the calibration curves established by GC-FID, the instrumental LOD and LOQ were calculated using the standard deviation (σ) of the intercepts and the slope (s) of the calibration curves (LOD = 3σ/s and LOQ = 10σ/s) (Shrivastava & Gupta, 2011). The method LOQ, taking into account the sample analyzed here, was then converted from the instrumental LOQ using the sample mass carried through the experimental procedures.

6.3.6.2 Spiking and recovery

The SPE recovery tests of epoxy fatty acids in edible oils have been previously carried out using the three-step SPE approach (Mubiru et al., 2014). In order to test the recovery of hydroxy FAMEs here, saturated and unsaturated hydroxy FAME standards were used for the spiking and recovery test. Specifically, the test was conducted by adding known amounts of methyl 9, 10-epoxyoctadecanoate, methyl 12-hydroxystearate, methyl ricinoleate, and 13(S)-HODE methyl ester to the FAMEs derived from canola and sunflower oil. The amount of recovered standard was calculated by subtracting the amount of standard found in the unspiked matrix from the spiked matrix. The apparent recovery (%) was obtained by the mass ratio of recovered standard/spiked standard. The recoveries of epoxy and hydroxy FAMEs were tested in Polar Fraction 1 and Polar Fraction 2, respectively. The same data was used to construct standard addition curves. These were plotted for each spiked standard using the area ratio of analyte standard/internal standard versus the concentration ratio of analyte standard/internal standard. Matrix effects were evaluated for each standard material using analysis of covariance (ANCOVA) to examine the difference between the slopes of the calibration curve and the standard addition curve. A p-value > 0.05 indicated that there was no general matrix effect.

6.3.6.3 Thermo-oxidation of oils

For identification of epoxy and hydroxy FAMEs, 30 g of sunflower oil and canola oil in 100 mL beakers were kept in the dark at 70 ^{o}C for 7 days. For method application, a

stability study was conducted by applying the above method to monitor the levels of epoxy and hydroxy fatty acids in sunflower oil and canola oil during oxidation. Sunflower oil and canola oil (30 g) were weighed into 100 mL beakers and oxidized in the dark at 40 ºC for 40 days. The oils were analyzed every 10 days for concentrations of epoxy and hydroxy fatty acids as well as for PV (AOCS Method Cd 8-53) (Firestone, 2009c).

6.4 Results and Discussion

6.4.1 Identification of epoxy and hydroxy fatty acids in oils

The identities of the oxygenated fatty acids were confirmed by the retention times and their mass spectra. The chromatograms associated with the epoxy and hydroxy fatty acids quantified in oxidized oils are shown in Figure 6.1, with peak identities listed in Table 6.2.

The epoxy fatty acids in Polar Fraction 1 were the same as those reported in the model systems (Berdeaux et al., 1999a) and thermo-oxidized oils (Velasco et al., 2002; Velasco et al., 2004); specifically, two epoxy-C18:0 and four epoxy-C18:1 were found in oxidized oils. The formation of the epoxy group was expected to occur by replacement of an original double bond in the unsaturated fatty acids (Berdeaux et al., 1999a). Therefore, 9,10-epoxyoctadecanoate (9,10-epoxy-C18:0) formed from oxidation of oleic acid, with 12,13-epoxyoctadec-9-enoate (12,13-epoxy-C18:1) and 9,10-epoxyoctadec-12-enoate (9,10-epoxy-C18:1) derived from oxidation of linoleic acid. In addition, each positional isomer appeared as two isomers of *cis/trans* epoxides. The Rtx-2330 capillary column (105 m×0.25 mm i.d.) used here employed a biscyanopropyl cyanopropylphenyl

polysiloxane phase. Due to the stronger dipole moment provided by the cyano groups, the interaction between a *cis*-isomer and the cyano-dipole resulted in a longer retention time for *cis*-isomers than their *trans*-isomers (David, Sandra, & Vickers, 2005). Therefore, the Rtx-2330 capillary column tends to elute *trans* isomers before their *cis* isomers. Peak 1, 3, 4 in Figure 6.1 were *trans* epoxy isomers and Peak 2, 5, 6 were *cis* isomers. The assignments of the *cis/trans* epoxides were consistent with the findings reported by Mubiru et al. (2013), who employed a CP-Sil 88 (60 m×0.25 mm i.d.) column with a highly substituted cyanopropyl phase, for separation of epoxy FAME isomers.

The occurrence of unsaturated hydroxy fatty acids in oxidized oils has not been reported in the literature. In this study, TMS derivatives of saturated, monounsaturated, and diunsaturated hydroxy FAMEs were identified in Polar Fraction 2, which represented hydroxy-C18:0, hydroxy-C18:1, and hydroxy-C18:2 in oils. The only saturated hydroxy FAMEs were 9- and 10-hydroxyoctadecanoate (9- and 10-hydroxy-C18:0), appearing as a co-eluting peak in GC. Four positional isomers of allylic hydroxy-C18:1 eluted before hydroxy-C18:0, with hydroxy groups at the 8, 9, 10, and 11 positions. A pair of *cis/trans* isomers were associated with each positional isomer; therefore, each of 8-, 9-, 10-, and 11-hydroxy-C18:1 was associated with two GC peaks in Table 6.2. A non-allylic hydroxy-C18:1, ricinoleate (12-hydroxyoctadec-9-enoate), was also quantified in this study. The conjugated hydroxy dienes were identified as 13-hydroxyoctadeca-9,11-dienoate (13-HODE) and 9-hydroxyoctadeca-10,12-dienoate (9-HODE) and both occurred as two geometric isomers.

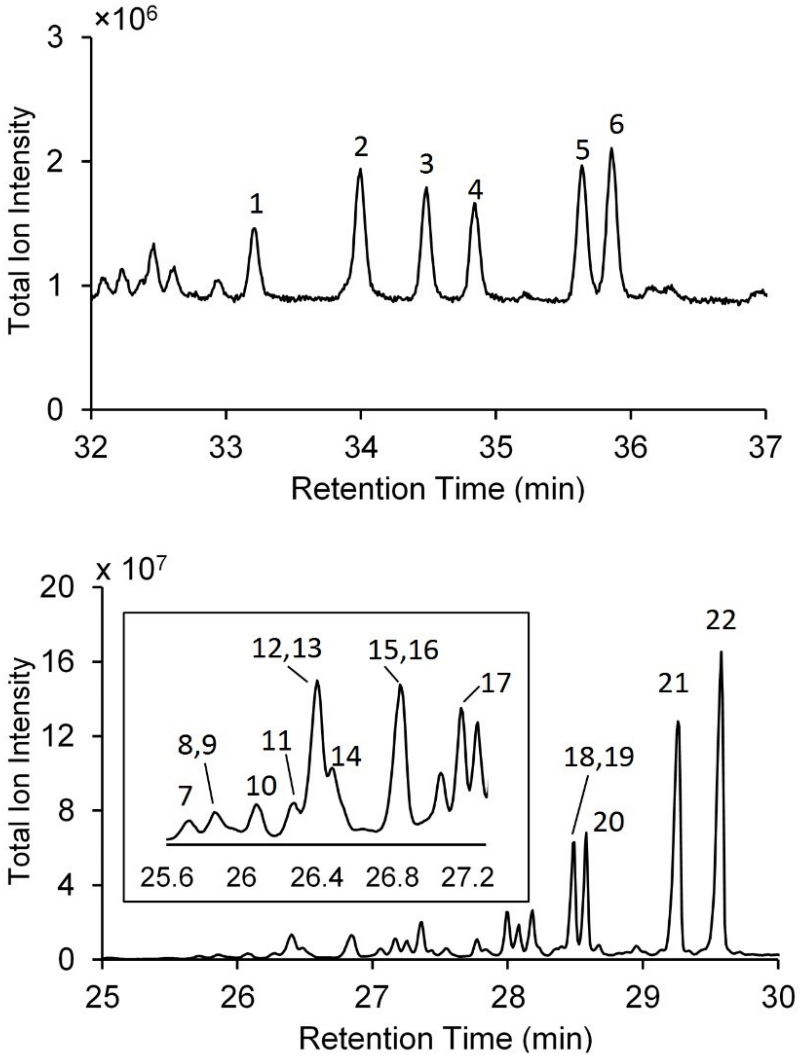


Figure 6.1 GC-MS total ion chromatograms of epoxy FAMEs in Polar Fraction 1 (top) and TMS ethers of hydroxy FAMEs in Polar Fraction 2 (bottom) derived from sunflower oil oxidized at 70 ^{o}C for 7 days, with peak identities listed in Table 6.2.

Table 6.2 Identification of epoxy and hydroxy FAMEs in Polar Fraction 1 and 2, corresponding to the peaks labelled in Figure 6.1.

Peak No. [a]	Compound	M^+	Base peak	High mass ions *m/z* >100 (Relative abundance %)
Epoxy FAME				
1,2	Methyl 9,10-epoxyoctadecanoate	312	67	199 (11), 171 (11), 167 (19), 155(55), 153(33), 139 (28), 127 (35), 121 (33), 109 (50)
3,5	Methyl 12,13-epoxyoctadec-9-enoate	310	79	217 (12), 189 (10), 164(14), 149 (22), 135 (30), 121 (44), 107 (24), 105 (21)
4,6	Methyl 9,10-epoxyoctadec-12-enoate	310	67	155 (20), 150 (26), 125 (23), 121 (24), 109 (36)
Hydroxy FAME	**(in TMS ether form)**			
7,11	Methyl 8-hydroxyoctadec-9-enoate	384	241	369 (2), 337 (10), 241 (100), 129 (52)
8,12	Methyl 9-hydroxyoctadec-10-enoate	384	227	369 (2), 337 (8), 227 (100), 129 (62)
9,13	Methyl 10-hydroxyoctadec-8-enoate	384	271	369 (2), 337 (12), 271 (100), 149 (80)
10,14	Methyl 11-hydroxyoctadec-9-enoate	384	285	369 (2), 337 (7), 285 (100), 163 (50), 135 (35)
15	Methyl 9-hydroxyoctadecanoate	386	259	371 (4), 339 (22), 259 (100), 229 (29), 155 (68)
16	Methyl 10-hydroxyoctadecanoate	386	273	371 (4), 339 (12), 273 (100), 215 (28), 169 (30)
17	Methyl 12-hydroxyoctadec-9-enoate (methyl ricinoleate)	384	187	369 (6), 337 (20), 299 (14), 270 (54), 187 (100), 159 (18), 146 (10), 103 (46)
18	Methyl 15-hydroxyoctadeca-9,12-dienoate	382	73	335 (10), 310 (67), 145 (70), 103 (49)
19,22	Methyl 13-hydroxyoctadeca-9,11-dienoate (13-HODE)	382	311	382 (40), 367 (4), 335 (7), 311(100), 269 (14), 259 (22), 225 (40), 207 (60),189 (40), 171 (84), 161 (50), 130 (67)
20,21	Methyl 9-hydroxyoctadeca-10,12-dienoate (9-HODE)	382	225	382 (42), 367 (2), 311(30), 272 (15), 225 (100), 215 (14), 155 (19), 135 (34), 130 (26), 107 (24)

[a] The compounds identified with two peak numbers were geometric isomers, giving identical mass spectra but different retention times.

In canola oil, a non-conjugated hydroxy-C18:2, 15-hydroxyoctadeca-9,12-dienoate, co-eluted with an isomer of 13-HODE in Polar Fraction 2 (Peak 18 and 19 in Figure 6.1). Such co-elution did not occur with sunflower oil since 15-hydroxyoctadeca-9,12-dienoate was not found in sunflower oil even by monitoring its diagnostic ion *m/z* 310. Due to the co-elution problem, GC-FID was not sufficient to determine the hydroxy diene concentrations in canola oil so GC-MS-SIM was employed to determine 13-HODE by monitoring *m/z* 382. The molecular ion (M^+, *m/z* 382) was not present in the mass spectrum of 15-hydroxyoctadeca-9,12-dienoate and thus no interference occurred in SIM mode.

6.4.2 Quantification of epoxy and hydroxy fatty acids in oils

6.4.2.1 Response factors

Calibration curves were plotted for epoxy and hydroxy FAME standards and their response factors are listed in Table 6.3. Using GC-FID, LOQ varied between 0.4-1.0 µg/mL, which was comparable to the LOQ reported previously for saturated epoxy (1.6 µg/mL) and hydroxy (2.1 µg/mL) FAMEs using GC-FID (Marmesat et al., 2008) and for hydroxydienes (0.6 µg/mL) using HPLC-UV (Morales, Dobarganes, Márquez-Ruiz, & Velasco, 2010). Using GC-MS-SIM, the calibration curve of 13-HODE methyl ester was $y=0.3183x-0.0722$, where x was the concentration ratio of 13-HODE/C19:0 and y was the area ratio of 13-HODE/C19:0. The intercept was a negative value, and was significantly different from 0 (one sample t-test p-value<0.001). Thus, the regression equation was only applicable for quantification of 13-HODE methyl ester within the linear range of 2-10 µg/mL in SIM mode.

Table 6.3 Determination of response factors with C19:0 ME as the internal standard.

Standard	Detection	Response factor	Correlation coefficient (R^2)	LOD (µg/mL)	LOQ (µg/mL)
methyl 9,10-epoxyoctadecanoate	FID	0.8170	0.9958	0.3	1.0
methyl 12-hydroxystearate[a]	FID	1.0650	0.9994	0.1	0.4
methyl ricinoleate[a]	FID	0.9763	0.9991	0.2	0.5
13-HODE methyl ester[a]	FID	0.9630	0.9991	0.1	0.5
13-HODE methyl ester[a]	SIM-MS	0.3183	0.9954	-[b]	2.0[c]

[a] The hydroxy FAME were analyzed in their TMS ether forms.
[b] LOD was not determined for 13-HODE in SIM because the intercept of the response curve was significantly different from 0 (one sample t-test p-value<0.001), which may indicate a non-linear response curve between 0 and 2 µg/mL.
[c] LOQ for 13-HODE in SIM-MS was determined as the lower limit of the working range, 2-10 µg/mL.

6.4.2.2 Recoveries

The SPE recoveries for epoxy and hydroxy standards were between 91-112% with RSD of 0.3-9% (Table 6.4). The matrix effect was then examined by comparing the slopes of the calibration curve and the standard addition curve for each type of standard (Table 6.5). P-values > 0.05 for all standards indicated that the two slopes were not different and that a matrix effect did not exist for sunflower and canola oil.

Table 6.4 SPE recoveries of epoxy and hydroxy FAME standards spiked in sunflower oil and canola oil.

Standard	Spiked mass (μg)	Sunflower oil		Canola oil	
		Apparent recovery mean±SD (%)	RSD (%)	Apparent recovery mean±SD (%)	RSD (%)
methyl 9,10-epoxyoctadecanoate	2	105±4	4	105±4	4
	4	91±5	5	101±4	4
	6	96±3	3	93±6	7
	8	108±7	7	105±2	2
	10	99±3	2	104±2	2
methyl 12-hydroxyoctadecanoate	0.4	103±1	1	110±0.35	0.3
	0.8	93±2	2	107±2	2
	1.2	94±2	2	99±4	4
	1.6	104±2	2	107±1	1
	2.0	95±1	1	101±2	2
methyl ricinoleate	0.4	100±6	6	106±4	4
	0.8	107±1	1	101±9	9
	1.2	95±5	5	94±6	7
	1.6	101±5	5	102±2	2
	2.0	106±3	3	95±5	5
13-HODE methyl ester	0.4	102±7	7	100±3	3
	0.8	97±3	4	97±5	6
	1.2	104±3	3	95±7	7
	1.6	101±6	6	112±7	6
	2.0	97±1	1	96±3	3

Table 6.5 The slopes and correlation coefficient (R^2) of the standard addition curves obtained for sunflower and canola oil, with matrix effects evaluated by the p-values of the ANCOVA tests.

Standard	Sunflower oil			Canola oil		
	slope	R^2	p-value	slope	R^2	p-value
methyl 9,10-epoxyoctadecanoate	0.8291	0.9877	0.651	0.8472	0.9930	0.181
methyl 12-hydroxyoctadecanoate	1.0387	0.9936	0.237	1.0426	0.9944	0.286
methyl ricinoleate	1.0099	0.9928	0.149	0.9324	0.9857	0.140
13-HODE methyl ester	0.9505	0.9946	0.517	0.9866	0.9737	0.569

6.4.2.3 Stability study

The LOD for epoxy fatty acids in oils was 30 µg/g, much higher than the LOD (1.45 µg/g) reported by Mubiru et al. (2013). The lower LOD of their method was likely due to the larger sample load (~200 mg) applied to the SPE column, compared to ~10 mg employed in our study. LOD obtained for saturated and unsaturated hydroxy fatty acids in our study ranged between 3-4 µg/g, better than those reported for hydroxy dienes using HPLC-UV and HPLC-ELSD without a concentration step like SPE (Morales et al., 2010; Morales et al., 2012). However, we are unable to compare our results for unsaturated hydroxy fatty acids using SPE procedures to literature values since such data do not exist. Although the LOQ for epoxy and hydroxy fatty acids can be interpreted as the lowest limit for quantification, the use of LOQ has been controversial and measurements below a LOQ may not be devoid of information (Thompson et al., 2002). Therefore, in this chapter, quantitative data above LOD but below LOQ is presented.

Upon artificial oxidation at 40 ºC, a number of epoxy and hydroxy fatty acids were apparent and measured in sunflower oil and canola oil (Table 6.6 and 6.7). Using GC-FID, RSDs for epoxy fatty acids in the oils were 0.6-13.7%, while RSDs obtained for hydroxy fatty acids were 1.2-15.1%. In MS-SIM, the RSDs for quantification of 13-HODE were 10.0-18.8%. The RSDs in this study were comparable to other studies which determined epoxy fatty acids in food samples (Mubiru et al., 2014), and epoxy and hydroxy fatty acids in oils (Marmesat et al., 2008).

Table 6.6 Quantification of epoxy and hydroxy fatty acids in sunflower oil oxidized at 40 ºC.

Day	PV (meq/kg)	Epoxy fatty acid concentration (µg/g)		
		cis-9,10-epoxy-C18:0[a]	*cis*-12,13-epoxy-C18:1[a]	*cis*-9,10-epoxy-C18:1[a]
0	13.7±0.1	111.1±7.3	38.8±2.5	194.1±16.8
10	31.8±0.3	122.7±8.7	44.8±6.1	205.4±11.0
20	57.9±0.6	117.5±4.4	50.2±4.3	195.9±7.6
30	88.9±0.3	150.2±7.0	74.1±3.4	238.1±12.6
40	122.8±0.3	146.1±10.8	59.1±0.3	214.8±9.2

Day	PV (meq/kg)	Hydroxy fatty acid concentration (µg/g)						
		9- and 10-hydroxy-C18:0	9- and 10-hydroxy-C18:1[b]	ricinoleate	13-HODE1[c]	9-HODE1[c]	9-HODE2[c]	13-HODE2[c]
0	13.7±0.1	32.8±2.4	35.4±3.0	134.6±8.0	63.2±5.7	76.7±6.7	58.5±4.4	52.4±4.1
10	31.8±0.3	33.6±0.6	35.7±1.7	123.5±5.8	78.3±11.0	103.0±15.9	67.1±4.3	58.4±4.5
20	57.9±0.6	31.5±0.4	33.5±0.6	128.6±1.9	111.2±2.6	132.1±3.0	63.7±1.2	55.9±1.3
30	88.9±0.3	34.7±2.6	37.1±3.0	140.9±11.0	140.4±6.7	171.7±7.9	74.3±2.7	65.2±1.9
40	122.8±0.3	33.0±2.6	35.7±2.5	134.2±12.5	176.8±7.7	208.6±9.5	78.9±3.4	67.6 ±2.6

[a] *cis*-9,10-epoxy-C18:0, *cis*-12,13-epoxy-C18:1, and *cis*-9,10-epoxy-C18:1 were abbreviated for *cis*-9,10-epoxyoctadecanoate, *cis*-12,13-epoxyoctadec-9-enoate, and *cis*-9,10-epoxyoctadec-12-enoate, respectively.

[b] 9- and 10-hydroxy-C18:1 were abbreviated for 9-hydroxyoctadec-10-enoate and 10-hydroxyoctadec-8-enoate, respectively.

[c] 13-HODE and 9-HODE were abbreviated for 13-hydroxyoctadeca-9,11-dienoate and 9-hydroxyoctadeca-10,12-dienoate, respectively. 13-HODE1, 9-HODE1, 9-HODE2, and 13-HODE2 were in correspondence with Peak 19, 20, 21, and 22 in Figure 6.1, following ascending order of their retention times.

Table 6.7 Quantification of epoxy and hydroxy fatty acids in canola oil oxidized at 40 °C.

Day	PV (meq/kg)	Epoxy fatty acid concentration (µg/g)		
		cis-9,10-epoxy-C18:0[a]	*cis*-12,13-epoxy-C18:1[a]	*cis*-9,10-epoxy-C18:1[a]
0	2.9±0.1	<30	<30	<30
10	9.7±0.1	43.6±5.7	<30	<30
20	37.3±0.6	32.5±3.2	<30	<30
30	71.2±1.0	89.2±2.7	<30	<30
40	99.7±0.9	94.0±9.4	<30	<30

Day	PV (meq/kg)	Hydroxy fatty acid concentration (µg/g)						
		9- and 10-hydroxy-C18:0	9- and 10-hydroxy-C18:1[b]	ricinoleate	13-HODE1[c,d]	9-HODE1[c]	9-HODE2[c]	13-HODE2[c]
0	2.9±0.1	239.7±3.2	<5	258.5±5.8	<40	12.2±1.3	<5	<5
10	9.7±0.1	247.3±6.7	<5	248.3±6.4	<40	13.1±1.7	<5	<5
20	37.3±0.6	231.7±22.3	<5	244.3±26.0	41.9±4.2	34.8±3.5	8.4±0.5	8.2±0.8
30	71.2±1.0	230.5±13.1	<5	246.5±13.6	55.1±10.3	56.6±3.4	14.6±1.6	13.9±1.7
40	99.7±0.9	246.3±13.0	8.9±0.5	261.7±14.5	95.6±9.5	94.0±9.0	34.2±5.1	31.3±4.2

[a] *cis*-9,10-epoxy-C18:0 was abbreviated for *cis*-9,10-epoxyoctadecanoate.
[b] 9- and 10-hydroxy-C18:1 were abbreviated for 9-hydroxyoctadec-10-enoate and 10-hydroxyoctadec-8-enoate, respectively.
[c] 13-HODE and 9-HODE were abbreviated for 13-hydroxyoctadeca-9,11-dienoate and 9-hydroxyoctadeca-10,12-dienoate, respectively. 13-HODE1, 9-HODE1, 9-HODE2, and 13-HODE2 were in correspondence with Peak 19, 20, 21, and 22 in Figure 6.1, following ascending order of their retention times.
[d] Quantification of 13-HODE1 in canola oil was carried out using GC-MS-SIM.

Among the epoxy fatty acids present in the fresh sunflower oil (Day 0), *cis*-9,10-epoxy-C18:1 was the highest in quantity. The levels of individual epoxy fatty acids in the sunflower oil were slightly lower than those found in five sunflower oils by Mubiru et al. (2013) (223-414 µg/g for *cis*-9,10-epoxy-C18:0, 55-110 µg/g for *cis*-12,13-epoxy-C18:1, and 748-1434 µg/g for *cis*-9,10-epoxy-C18:1), but within the range of those found in 13 sunflower oil samples tested by Brühl et al. (2016) (10-500 µg/g for *cis*-9,10-epoxy-C18:0, 20-1200 µg/g for *cis*-12,13-epoxy-C18:1, and 20-1140 µg/g for *cis*-9,10-epoxy-C18:1). During the stability study, the peaks associated with *cis*-12,13-epoxy-C18:1 and *cis*-9,10-epoxy-C18:1 were quantified in sunflower oil but they were present below 30 µg/g in canola oil throughout 40 days. No quantitative data of epoxy fatty acids has been reported for canola oil in literature to allow comparison. However, Mubiru et al. (2013) suggested a strong correlation between the fatty acid ratios of C18:1/C18:2 and epoxy-C18:0/epoxy-C18:1, so the lower content of epoxy-C18:1 in canola oil could be due to its lower concentration of linoleic acid (C18:2) compared to sunflower oil.

Peaks associated with *trans*-epoxy fatty acids were not observed in sunflower oil or canola oil over the period of 40 days, likely due to the mild oxidation temperature of 40 ºC. *Trans*-epoxy fatty acids are produced fast during frying of oils and thus are present in large amounts in used frying oils (Brühl et al., 2016; Velasco et al., 2004). In non-fried oils, the levels of *trans*-epoxy fatty acids are normally ~10-50 times lower than those of *cis*-epoxy fatty acids (Brühl et al., 2016). Therefore, in order to determine *trans*-epoxy fatty acids in fresh or mildly oxidized oils, the LOD needs to be pushed to a much lower level, e.g. <1 µg/g.

During the stability study at 40 ºC, the only observable allylic hydroxy-C18:1 were 9- and 10-hydroxy-C18:1 (Peak 12 and 13 in Figure 6.1), which were measurable in sunflower oil from Day 0 but only detectable in canola oil on Day 40. The other isomers of allylic hydroxy-C18:1 (Peak 7-11 and Peak 14 in Figure 6.1) were not seen in sunflower oil or canola oil at 40 ºC, although their signals were obvious in the oils oxidized at 70 ºC for 7 days. Therefore, neither 8- nor 11-hydroxy-C18:1 were quantified during the stability study at 40 ºC. Conjugated hydroxy-C18:2 were found during oxidation of sunflower oil and canola oil, with all four isomers presented as 13-HODE1, 9-HODE1, 9-HODE2, and 13-HODE2, based on the ascending order of their retention times (corresponding to Peak 19-22 in Figure 6.1, respectively). Again, the detection of 13-HODE1 in canola oil was limited to the linear range of the calibration curve (2-10 µg/mL) in SIM mode; therefore, the occurrence of 13-HODE1 was only quantified from Day 20. The concentration of 15-hydroxyoctadeca-9,12-dienoate (Peak 18 in Figure 6.1) was not determined in this study, although its concentration could have been calculated by subtracting the amount of 13-HODE1 from the mass of hydroxy-C18:2 determined by the area count of the co-eluting peak; however, this approach could have introduced large errors in the measurements so was not pursued.

The exclusion of hydrogenation suggests new findings about the formation of unsaturated hydroxy fatty acids in edible oils. First, the differences in the concentrations of hydroxy-C18:0, hydroxy-C18:1, and hydroxy-C18:2 would not be observed with hydrogenation applied, as they would all have been measured as saturated structures. The method introduced here allowed determinations of allylic hydroxy-C18:1 and conjugated hydroxy-C18:2, which would be considered the main products of hydrogen abstraction by

oleic and linoleic alkoxy radicals. Interestingly, although linoleic acid only comprised <20% of total fatty acids in canola oil, the total concentration of conjugated hydroxy-C18:2 was always higher than that of allylic hydroxy-C18:1 in canola oil over 40 days. Second, the concentration of an individual conjugated HODE isomer was quite consistent relative to the total content of conjugated HODE isomers. Specifically, in sunflower oil, 9-HODE1, 9-HODE2, 13-HODE1, and 13-HODE2 comprised 36±4%, 19±4%, 29±4%, and 16±3% of the total HODE in each sample over 40 days. In canola oil, since most conjugated HODEs were only detectable from Day 20, the percentages of 9-HODE1, 9-HODE2, 13-HODE1, and 13-HODE2 in total HODE from Day 20 to Day 40 were: 38±2%, 11±2%, 41±4%, and 10±2%. Third, significant changes were observed in the concentrations of allylic hydroxy-C18:1 and conjugated hydroxy-C18:2 during the stability study at 40 ºC, while hydroxy-C18:0 and ricinoleate remained constant over the 40-day period, which suggested that the saturated and methylene-interrupted structures may not arise from autoxidation. Last, this method allowed comparison of the epoxy and hydroxy products formed from the same fatty acid, which can be a potential approach to compare the lipid oxidation pathways. For example, epoxy-C18:0 and hydroxy-C18:1 are both considered oxidation products of oleic acid, and epoxy-C18:1 and hydroxy-C18:2 are derived from linoleic acid. In both oils examined in this study, the concentration of epoxy-C18:0 was always higher than hydroxy-C18:1 but total epoxy-C18:1 were less than total hydroxy-C18:2. Further investigations of these findings can be carried out using the proposed method for the study of lipid oxidation mechanisms.

In summary, the method presented here allowed simultaneous determinations of both saturated and unsaturated, epoxy and hydroxy fatty acids in vegetable oils. To date,

this is the first report of the occurrences of both saturated and unsaturated, epoxy and hydroxy fatty acids during oxidation. The focus of this paper was given to method development including the identification of the oxidized FAMEs, validation of SPE conditions, and application to quantitative studies of these oxygenated products derived from lipid oxidation. Modifications can be made to increase the method LOD by, for example, increasing the SPE sample load, since this would result in more analyte collected for GC analysis. With appropriate validation of the SPE recoveries, the experimental procedures employed in this study will serve as a foundation for future studies on oxygenated fatty acids for applications to food lipids and oils.

CHAPTER 7 CONCLUSIONS

7.1 General Conclusions

Epoxides and alcohols have been recognized as lipid oxidation products for decades but only a few studies have determined their concentrations in edible oils. The greatest challenge of quantification was that those structures are normally present at low levels in oils, along with a large amount of non-altered fatty acids; therefore the methods for quantification required high selectivity and sensitivity of the analytical approach. In this thesis, a ^{1}H NMR method and a GC method have been developed to determine epoxides and alcohols in edible oils.

As a basis for quantitative work, the ^{1}H NMR chemical shifts of epoxides were first examined by chemical synthesis of epoxides from edible oils. Initially, epoxides derived from vegetable and fish oils were both examined for their chemical shifts; the chemical shifts of epoxides, between 2.9-3.2 ppm, are distinct from other chemical groups in oils. For quantification, the signals of glycerol protons were taken as the internal standard, eliminating the use of an additional standard. The method did not employ any chemical derivatization or extraction and thus minimized the potential for artificial alteration of the oils during experimental procedures. The ^{1}H NMR method has been successfully applied to monitor epoxides derived from soybean oil oxidized at 100 ^{o}C with reasonable reproducibility. However, the use of the ^{1}H NMR method did not detect any changes between 2.9-3.2 ppm in the spectra of fresh fish oil and highly oxidized fish oil. Additional research will be required to determine whether this apparent

absence of epoxides results from limitations of method sensitivity or from low production, i.e. oxidation by other reactions dominate in the highly unsaturated fish oil.

In contrast, the GC method was 100-fold more sensitive than the ^{1}H NMR method and thus was not as constrained by its detection limit. The major improvement of the GC method from previous work is that, without hydrogenation, information regarding double bond positions in hydroxy fatty acids becomes known. This method has been successfully applied to monitor the concentrations of epoxy and hydroxy fatty acids in vegetable oils oxidized at 40 ºC. The sensitivity of the GC method can even be pushed to a lower level if using a modified SPE procedure, e.g. a larger sample load. Therefore, the GC method is anticipated to reach a lower limit of detection with further modifications of the SPE procedures. However, the GC method is much more time-consuming than ^{1}H NMR, with the SPE being the most tedious preparation step. The SPE columns employed are Pasture pipettes. If commercial SPE columns and vacuum manifolds are employed to reproduce the procedures, the sample preparation time can be substantially reduced, resulting in greater time efficiency. It should also be pointed out that the epoxides determined by ^{1}H NMR include all the internal epoxy groups present in the oils but the GC method measures only specific epoxy fatty acids so there is a discrepancy in the levels of analytes measured between the two methods presented in this work.

Overall, two new methods have been developed to monitor epoxides and alcohols as minor oxidation products derived from oils. The ^{1}H NMR method showed its reliability and convenience for sample analysis and therefore is expected to be useful if rapid analyses are the goals. The sensitivity of the GC method is superior to the ^{1}H NMR technique and is anticipated to have a wide range of applications to oils and foods.

7.2 Future Work

The starting point of this project was to use ^{1}H NMR to quantify epoxides derived from edible oils (Chapter 3 and 4). Although being a convenient and reliable technique, the ^{1}H NMR method does have an important drawback in being insufficiently sensitive to track changes in fresh oils or mildly oxidized oils. The sensitivity of ^{1}H NMR is limited due to the small number of protons associated with the analyte, which are, in this case, the epoxy protons. The analytes are present at extremely low levels compared to the large amount of other protons in TAGs, such as the methylene and glycerol protons. In order to increase the signal-to-noise ratio for the analytes, the receiver gain has to be increased. The receiver gain is normally automatically calculated to maximize the signal-to-noise ratio; therefore, a high receiver gain is optimum for small peaks. However, a low receiver gain is preferred for strong signals to avoid saturation of the receiver, since an over limit receiver gain produces distorted spectra. Therefore, the receiver gain used in our work is limited by the large signal of methylene protons and it prevents the detection of small numbers of epoxy protons.

In order to increase the sensitivity of the ^{1}H NMR method, two approaches can be attempted in further work. First, the ^{1}H NMR method can be improved by employing a pre-treatment step, such as SPE, to increase the sensitivity. For example, if the polar fractions obtained in the GC methods are subject to ^{1}H NMR analysis, the SPE step will have removed the un-altered (non-polar) structures and the protons unassociated with the analytes will be significantly reduced. The signals associated with epoxides will therefore become relatively stronger. Nevertheless, this SPE step will extend the preparation time

and offset the advantages of ^{1}H NMR. The second approach is to selectively suppress the signals of methylene protons in ^{1}H NMR spectra to achieve better sensitivity for minor components including epoxy protons because the suppression of strong signals will increase the signal-to-noise ratio for minor peaks by increasing the receiver gain. Such approaches will be particularly useful in determining epoxides in fish oils and mildly oxidized vegetable oils as these oils require a higher sensitivity for determination of epoxides. This is particularly important since GC-MS identifications of epoxy compounds derived from fish oils are difficult due to the less characteristic mass spectra of epoxy FAMEs. Since ^{1}H NMR gave distinct signals for epoxides in fish oils, it would facilitate determination of epoxides in fish oils.

In order to further validate the GC method (Chapter 5 and 6), the stability of lipid hydroperoxides formed during lipid oxidation should be examined by carrying fatty acid hydroperoxide standards through the experimental procedures. Such investigation has not been discussed in the literature and is particularly important to confirm that the epoxy and hydroxy fatty acids determined by the GC approach are not decomposition products of hydroperoxides formed during sample preparation.

Future work can be conducted to modify the SPE procedures employed to lower the detection limit of the GC method. As more sample is loaded onto the SPE column, more analytes can be detected by the instruments (GC-FID and GC-MS). However, if the SPE column is overloaded, full separation between the polar fractions will not be achieved. Therefore, in order to ensure the recoveries of the analytes, further validation must be conducted if sample size is to be increased. If necessary, a larger mass of Si gel can be packed in the column.

The application of this new GC method will allow further studies of lipid oxidation mechanisms. For example, oxidation of oleic acid and linoleic acid in canola and sunflower oil at 40 ºC showed different preferences for formation of epoxy and hydroxy fatty acids. Such differences may result from competition between the oxidation pathways. In addition, allylic and conjugated hydroxy fatty acids were present at extremely low levels in fresh oils but they were produced in greater amounts during autoxidation; in contrast, the concentrations of saturated and methylene-interrupted hydroxy fatty acids remained constant during the stability study at 40 ºC, suggesting that: 1) these saturated and methylene-interrupted hydroxy fatty acids may not be formed from lipid autoxidation; or 2) the saturated and methylene-interrupted hydroxy fatty acids may have been formed and decomposed at a steady rate over time. Further investigations should be conducted to verify these findings using lipid standards and under different oxidation conditions. The concentrations of these oxygenated products are indicators of competition among different lipid oxidation pathways.

Future work can apply the developed methods to look at the effects of various antioxidants on the oxidation of oils. For instance, hydroxy compounds are involved in important reactions associated with the antioxidant properties of tocopherols. The antioxidant effect of tocopherols is related to their hydrogen donation to peroxyl radicals (Frankel, 2005). Similarly, their hydrogen donation to alkoxyl radicals produces hydroxy fatty acids, which has been considered one of the main reactions inhibiting the decomposition of hydroperoxides into short-chain volatiles. In the literature, volatiles are more often measured in stability studies and information about tocopherol activity as a contributor to the formation of hydroxy oxidation products is unknown. On the other

hand, hydrogen donation of tocopherols to alkoxyl radicals is thought to stabilize the alkoxyl radicals formed from homolytic decomposition of hydroperoxides by producing 'stable' hydroxy compounds, and this should be enhanced by tocopherols and other antioxidants (Frankel, 2005). The competition between hydrogen abstraction and cyclization of alkoxyl radicals is also rarely studied. Epoxy compounds are the reaction products from cyclization. Thus, the GC method presented in this work is a feasible way to examine the effects of tocopherols on the formation of these secondary oxidation products – both epoxy and hydroxy. This information will help us understand the participation of antioxidants in pathways other than hydroperoxide formation, which may lead to a more comprehensive understanding of antioxidant properties and the achievement of long-term stability against lipid oxidation.

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APPENDIX A SUPPLEMENTARY FIGURES

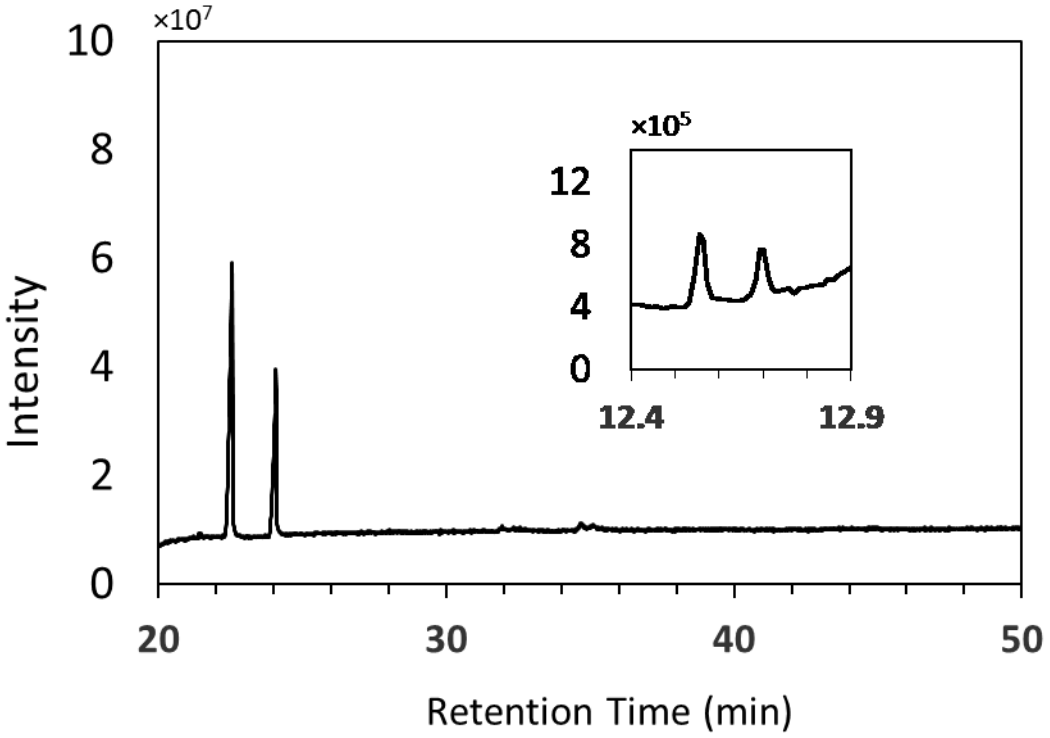

Figure A-1 EI total ion chromatogram of methyl esters derived from fully-epoxidized trilinolein. The diepoxide stereoisomers eluted at 22.6 and 24.1 min, while the impurities eluted at 12.6 min and 12.7 min (shown in inset).

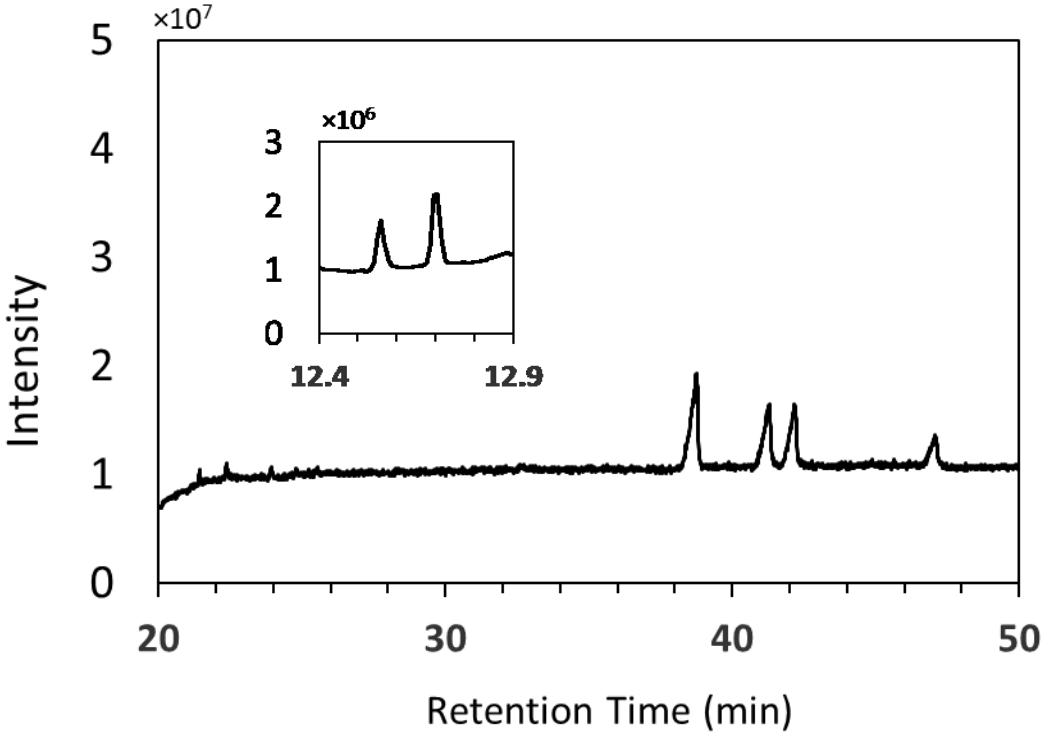

Figure A-2 EI total ion chromatogram of methyl esters derived from fully-epoxidized trilinolenin. The triepoxide stereoisomers eluted at 38.8, 41.3, 42.2, and 47.1 min, while the impurities eluted at 12.6 min and 12.7 min (shown in inset).

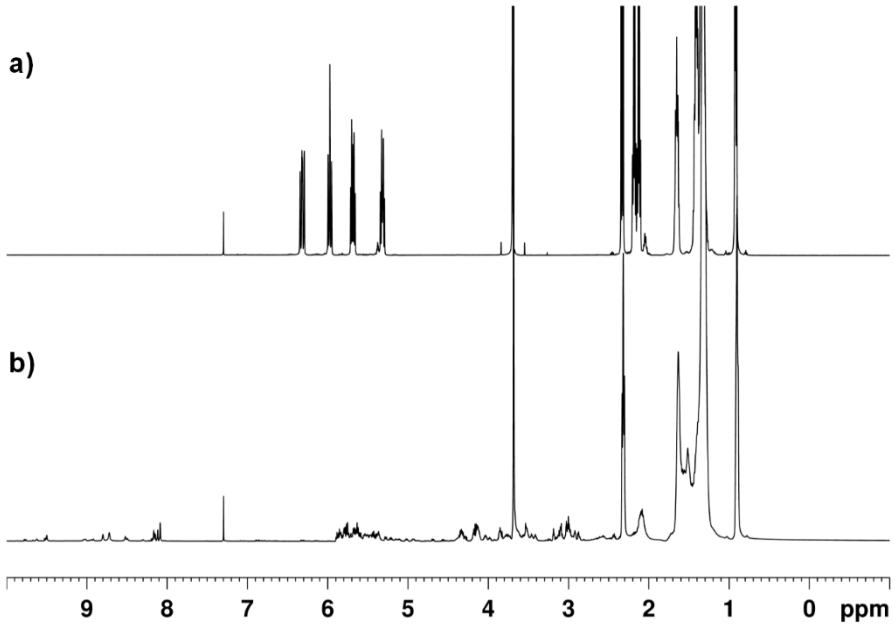

Figure A-3 ^{1}H NMR spectrum of a) conjugated methyl linoleate standard (C18:2, *cis*-9, *trans*-11), and b) conjugated methyl linoleate treated with 50% hydrogen peroxide and formic acid and heated for 6 hours.

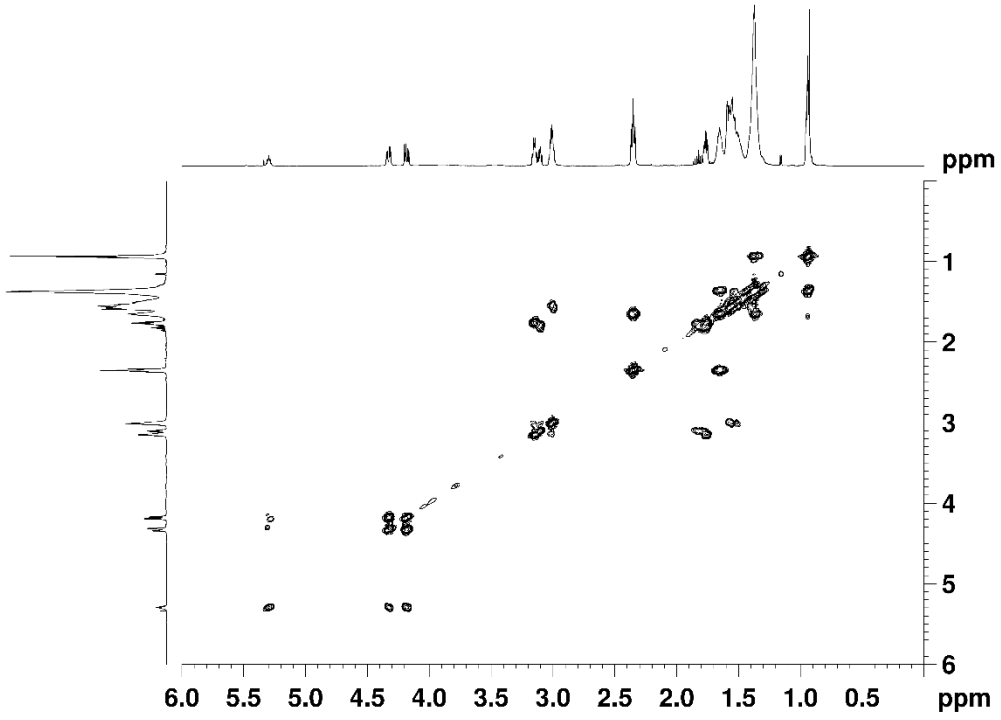

Figure A-4 2D COSY spectrum of fully-epoxidized trilinolein, corresponding to Figure 2.1c.

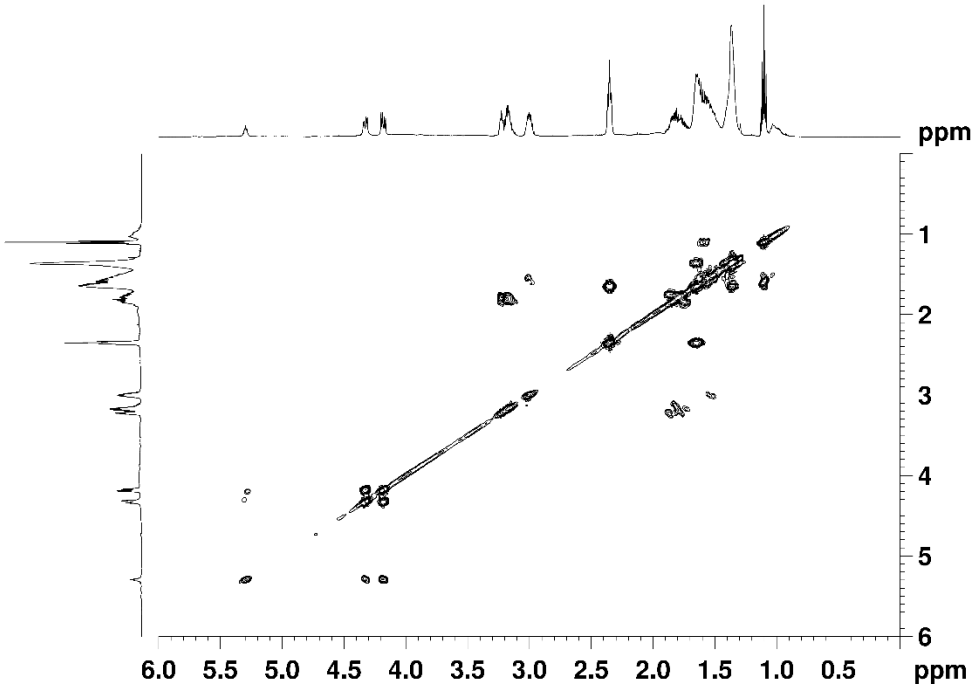

Figure A-5 2D COSY spectrum of fully-epoxidized trilinolenin, corresponding to Figure 2.2c.

APPENDIX B SUPPLEMENTARY TABLES

Table B-1 The integrals of the relevant signals in Figure 4.1 by taking the *sn*-1,3 glycerol protons as the internal standard (4.00), where overlapped peaks are deconvolved with Lorentzian line shapes.

chemical shift (δ, ppm)	protons	spectrum[a]				
		a	b	c	d	e
1.45-1.60	$C\underline{\mathbf{H}}_2$-CH(O)CH	nd[b]	1.74	6.32	10.23	11.21
1.70-1.85	CH(O)CH-$C\underline{\mathbf{H}}_2$-CH(O)CH	nd	0.09	0.61	1.77	3.24
2.00-2.10	CH_2-$C\underline{\mathbf{H}}_2$-CH=CH	9.60	8.21	5.13	2.44	0.65
2.80-2.85	CH=CH-$C\underline{\mathbf{H}}_2$-CH=CH	3.86	2.94	1.22	0.29	nd
2.90-2.95[c]	$C\underline{\mathbf{H}}$(O)$C\underline{\mathbf{H}}$ / $C\underline{\mathbf{H}}$(O)$C\underline{\mathbf{H}}$-CH_2-CH=CH	nd	1.07	2.43	2.86	1.99
3.00	$C\underline{\mathbf{H}}$(O)CH-CH_2-CH(O)$C\underline{\mathbf{H}}$	nd	0.02	0.61	1.46	2.68
3.06-3.24	CH(O)$C\underline{\mathbf{H}}$-CH_2-$C\underline{\mathbf{H}}$(O)CH	nd	0.04	0.48	1.49	2.92
5.35-5.45	$C\underline{\mathbf{H}}$=$C\underline{\mathbf{H}}$	7.83	6.33	1.94	0.42	0.02
5.40-5.50	CH(O)CH-CH_2-CH=$C\underline{\mathbf{H}}$	nd	nd	1.55	0.92	0.30
5.50-5.60	CH(O)CH-CH_2-$C\underline{\mathbf{H}}$=CH	nd	0.46	1.09	0.82	0.27
5.60-5.65	CH(O)CH-CH_2-$C\underline{\mathbf{H}}$=$C\underline{\mathbf{H}}$-CH_2-CH(O)CH	nd	nd	0.06	0.10	0.10

[a] The sequence of the spectra corresponds to Figure 4.1.
[b] nd = 'not detected'.
[c] The signals due to the epoxy protons in saturated monoepoxides completely coincide with those of the epoxy groups β to the double bond.

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Author: Wei Xia, Suzanne M. Budge, Michael D. Lumsden

Publication: Journal of Agricultural and Food Chemistry

Publisher: American Chemical Society

Date: Jun 1, 2015

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