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Techniques for the Analysis of Minor Lipid Oxidation Products Derived from Triacylglycerols: Epoxides, Alcohols, and Ketones

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Abstract: Lipid oxidation can lead to flavor 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 oxygenated 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 gas chromatography (GC), high-performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR). For characterization purposes, GC-mass spectrometry (GC-MS) provides valuable information regarding the structures of individual oxygenated fatty acids, typically as methyl esters, isolated from oxygenated triacylglycerols (TAGs), while the use of liquid chromatography–MS (LC-MS) techniques allows analysis of intact oxygenated TAGs and offers information about the position of the oxygenated acyl chain on the glycerol backbone. 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.

Keywords: GC, HPLC, lipid oxidation, NMR

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 and Decker 2008). Lipid molecules with double bonds, such as unsaturated fatty acids, are particularly susceptible to oxidative degradation. Triacylglycerols (TAGs), containing unsaturated fatty acids and present as oils and fats in foods, will decompose during lipid oxidation and produce off-flavors 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 flavors 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 and others 2012; Roman and others 2012), detection of changes in concentrations of lipid reactants (Tyagi and Vasishtha 1996; Kim and others 2013), and determination of oxidation products, including primary and secondary products (Barriuso and others 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 peroxide values (PVs). Hydroperoxides are formed from peroxy 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 polyunsaturated fatty acids, 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 and others 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 and others 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

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of lipid oxidation, where recombination of radicals occurs (Neff and others 1988).

In the absence of antioxidants, oxidative degradation of lipids occurs quickly. 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 and Min 2009). The presence of antioxidants at low concentrations in food inhibits oxidation, thereby increasing the shelf-life of foods (Gülçin 2012).

Normally, the antioxidant activities of these compounds are investigated by measuring the progress of lipid oxidation in the lipid samples and comparing them to control samples without antioxidant added. As an indication of the extent of the oxidation process, the above tests are normally conducted when assessing the efficacy of antioxidants (The American Oil Chemists' Society Method Cg 7-05) (Firestone 2009); however, 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. The other tests, such as PAV, TBARS, and volatile analysis, monitor the short-chain carbonyl compounds produced in the propagation stage of lipid oxidation. Thus, the above 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 peroxy radicals directly, for example, epoxides, or from hydroperoxides through pathways other than scission, such as ketones, alcohols, and epoxides.

Reactions including the generation of oxygenated fatty acids from hydrogen abstraction and rearrangement are rarely discussed in the literature. Fatty acids containing oxygenated groups were found to contribute to the formation of acrylamide, which may be carcinogenic to humans (Zamora and Hidalgo 2008). A lack of information concerning the concentrations of these oxygenated compounds also precluded the evaluation of antioxidant effects on their formation. A previous study found that high concentrations of α-tocopherol accelerated the formation of keto- and hydroxy-compounds during the induction period of oxidation (Kamal-Eldin and others 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 and others 2016). These effects would not be observed in common studies of antioxidants using chemical tests such as PVs or TBARS.

The concentrations of these oxygenated fatty acids are rarely reported when studying lipid oxidation in foods because there is 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 fatty acids.

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 3 stages: initiation, propagation, and termination. At the initiation stage, radicals are formed, usually with an initiator present, such as metals, light, or heat (Schaich 2005). The initiators can remove an electron from lipids and oxygen to form lipid and oxygen radicals (Min and Boff 2002). In the propagation stage, the free radicals take part in further reactions, which allows the unpaired electron to transfer along the carbon chain or initiate the formation of more radicals by abstracting hydrogen from other lipid molecules. The lipid radicals may also interact with atmospheric oxygen to yield peroxy radicals. These processes will repeat themselves until the radicals are consumed to form nonradical products, which is considered the termination stage.

Secondary products of lipid oxidation produced from scission of alkoxy 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 oxygenated fatty acids found in oxidized lipids (Berdeaux and others 2002), formation of oxygenated fatty acids with the original chain length have also been identified as lipid oxidation products (Velasco and others 2002; Morales and others 2010). Therefore, alternative pathways to the classic mechanism of formation of secondary products need to be investigated. Their competition with chain scissions should not be neglected.

Epoxides are formed through the replacement of a double bond with an epoxide (Figure 1a and 2a), where a peroxy radical directly attacks a double bond, generating an epoxide and an alkoxy radical (Frankel 1984; Giuffrida and others 2004a). Also, they can be produced from the rearrangement of alkoxy radicals (Frankel 1984). In this case, they are thought to be secondary products and only form from the alkoxy radicals produced from decomposition of hydroperoxides (Figure 1b and 2b). It should be noted that the peroxide addition forms an epoxy group at the position of the original double bond (Figure 1a); however, the rearrangement of alkoxy radicals can form positional isomers due to the rearrangement of radicals (Figure 1b). Neff and Byrdwell (1998) observed 2 classes of epoxides during oxidation of model TAGs. 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. The results suggested that 2 processes produce epoxides, although the specific pathways were not discussed (Neff and Byrdwell 1998). Therefore, epoxides can form either from rearrangement of alkoxy radicals as secondary products or independently from hydroperoxide formation (Schaich 2012). Moreover, the alkoxy radicals produced in the process (Figure 1a) can react further to generate epoxides, alcohols, ketones, and other lipid oxidation products.

In the propagation stage, alcohols are produced from hydrogen abstraction by alkoxy radicals (Figure 1c and 2c). Hydrogen abstraction from lipid molecules by peroxy radicals forms hydroperoxides, the major class of primary oxidation products. However, hydrogen abstraction by alkoxy radicals is even faster. Alkoxy radicals can abstract hydrogens directly from hydroperoxides; therefore, the process is a competitive reaction to the formation of hydroperoxides and has an important effect during the propagation stage (Schaich 2005). Yet, lipid alcohols from hydrogen abstraction are considered minor products (Schaich 2012), in contrast to hydroperoxides. Combination of hydroxy radicals with lipid radicals can also form hydroxy compounds (Figure 1d and 2d) (Frankel 1984). These hydroxy radicals can be produced from the decomposed lipid hydroperoxides in the presence of transition metals (Repetto and others 2012). They are highly reactive and thus not stable. Finally, in the termination stage, recombination of a secondary alkoxy radical with a primary alkoxy radical produces a ketone and an alcohol (Figure 1e and 2e) (Schaich 2005). Ketones can also form from recombination of a secondary alkoxy radical with an alkyl radical (Figure 1f and 2f) (Schaich 2005) or by elimination of OH^- from hydroperoxides (Figure 1g and 2g) (Terao and others 1975).

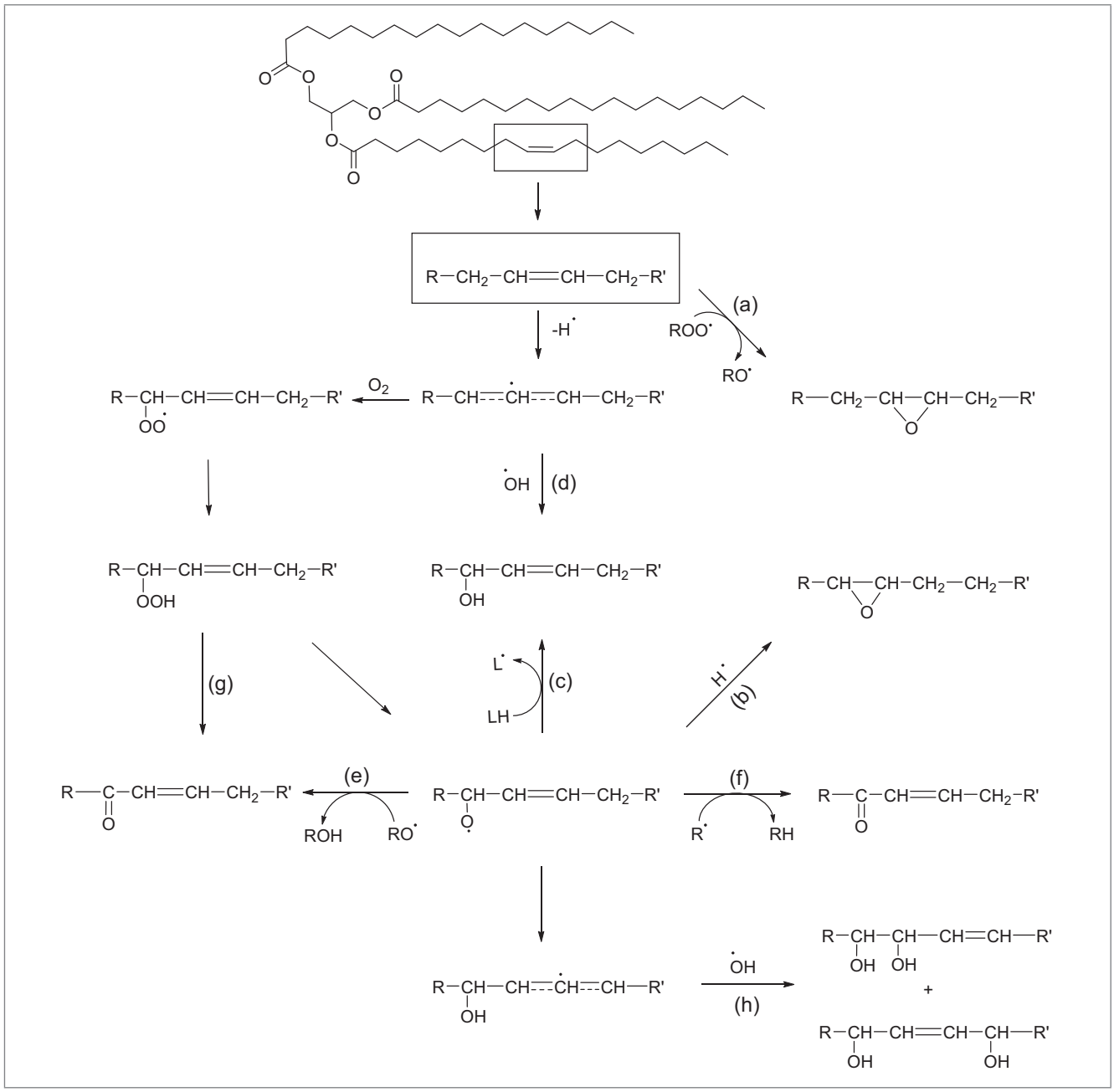

Figure 1–Formation of lipid oxidation products from an unsaturated site in TAG.

Fatty acids with more than one of these oxygenated functional groups have been reported to form from both mono- and polyunsaturated fatty acids during lipid oxidation. Epoxy hydroxy monoenes result from cyclization of alkoxy radicals and addition of hydroxy radicals with and without double bond migration (Figure 2h and 2i) (Frankel 1984). Gardner and others (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 2j). In addition, dihydroxy fatty acids are formed by the reaction between allylic enols and hydroxy radicals (Figure 1h) (Frankel 1987).

In summary, the locations of the oxygenated groups are highly related to the position of original double bonds. Allylic and conjugated structures are likely to occur during lipid oxidation due to double bond migration, leading to a variety of different structures, and causing difficulties in separation and identification of the oxidation products. In addition, previous studies on the characterization of oxygenated fatty acid structures have mainly focused on model systems such as pure TAGs and fatty acid methyl esters (FAMEs), and pure hydroperoxide standards. The extremely low levels of those oxygenated compounds have caused even more difficulties in detection in nonmodel systems, even in vegetable oils which primarily consist of TAGs. Thus, this review will evaluate the available methods that make use of gas chromatography (GC), high-performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR) techniques in terms of their selectivity to identify the compounds and their sensitivity to

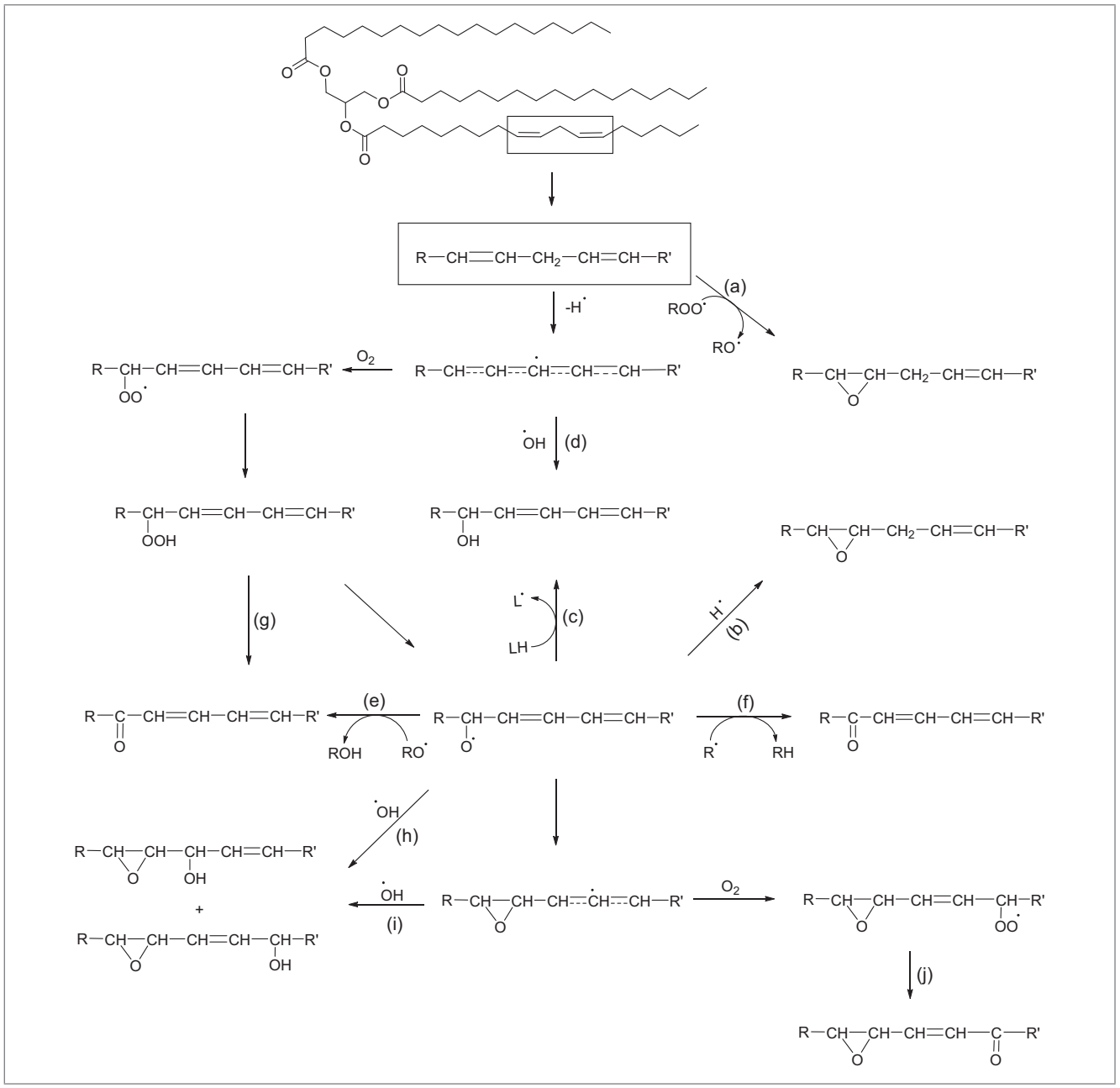

Figure 2–Formation of lipid oxidation products from a pentadiene system in TAG.

determine these products at low levels in food lipids and edible oils.

Occurrence of Epoxides, Alcohols, and Ketones

Epoxides were first monitored in oxidized methyl esters and TAGs (Berdeaux and others 1999b). Methyl oleate, methyl linoleate, triolein, and trilinolein were heated at 180 °C for 15 h and were tested every 5 h for epoxy fatty acid content. After heating methyl oleate for 15 h, 2 methyl monoepoxystearates (*trans*-9,10 epoxy and *cis*-9,10 epoxy) were present at 20.3 and 14.9 mg/g, respectively. Under the same conditions, 4 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, and 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 and others 1999b). In addition, less unsaturated substrates (methyl oleate and triolein) produced larger amounts of epoxides than more unsaturated substrates (methyl linoleate and trilinolein) (Berdeaux and others 1999b). The 6 monoepoxy fatty acids reported in this study, including the 2 methyl monoepoxystearates and 4 methyl monoepoxyoleates, have become the 6 most commonly measured epoxy fatty acids in the literature.

Velasco and others (2004) determined monoepoxy fatty acids in oils heated using the same temperature and heating time as Berdeaux and others (1999b). The total amounts of the 6 common monoepoxy fatty acids were 14.24 and 9.44 mg/g in olive oil and sunflower oils, respectively (Velasco and others 2004). The

authors determined methyl epoxysterates and methyl epoxyoleates in 10 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 and others (1999b), which may be attributed to a lower tendency of epoxystearates to form polymers and to participate in further oxidative reactions (Velasco and others 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 6 commonly determined (Mubiru and others 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 to 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 and others 2013).

Mubiru and others (2014) measured the 6 common monoepoxy fatty acids in food matrices including biscuits, meat, butter, and nuts. A range of 3 to 171 μg/g of total epoxy fatty acids was 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 epoxysterate (53.66 μg/g) was reported for the butter sample, while the other food matrices had lower concentrations of *trans*-9,10 epoxysterate, ranging from 0.75 to 17.33 μg/g (Mubiru and others 2014).

Considering the locations of the epoxy groups in the 6 most measured monoepoxy fatty acids, in which the epoxy groups take the place of the original double bond in position 9 or 12 in methyl oleate and linoleate, the 6 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 alkoxy radicals (Figure 1b and 2b), are not reported.

The above results reported for epoxides were determined by GC; therefore, individual epoxy fatty acids were identified and quantified. In contrast, NMR methods determine total epoxide content. For instance, Goicoechea and Guillén (2010) determined monoepoxides and diepoxides in sunflower oils heated at 70 °C and 100 °C with aeration. At 100 °C, both monoepoxides and diepoxides reached a maximum value at 161 and 107 mmol/L oil (approximately 56 and 39 mg/g) and showed a declining trend afterwards. At 70 °C, the concentrations of mono- and diepoxides reached 41 and 123 mmol/L oil (approximately 14 and 45 mg/g) (Goicoechea and Guillén 2010).

Available data on the occurrence of alcohols and ketones reported are scarcer than for epoxides. Hydroxy fatty acids were first quantified in hydrogenated ground nut oil and individual C18 hydroxy fatty acids were present between 2 and 494 μg/g with a total amount of 1422 μg/g (Wilson and others 1997). In fried sunflower oil (180 °C for 10 h), total C18 hydroxy FAME ranged between 1.9 and 5.5 mg/g oil; C18 keto FAME and C18 epoxy FAME were also measured, determined at 0.5 to 2.5 mg/g oil and 1.3 to 4.4 mg/g oil, respectively (Marmesat and others 2008). In extra virgin olive oil heated at 190 °C for 7.5 to 32 h, primary alcohols were determined by NMR at 2 to 5 mmol/mol TAG (approximately 0.7 to 1.7 mg/g TAG), while secondary alcohols were between 4 and 20 mmol/mol TAG (approximately 1.4 to 7.0 mg/g TAG) (Martínez-Yusta and Guillén 2014a). In the experiment, (E)- and (Z)-epoxides reached concentrations of 20 and 12 mmol/mol TAG (approximately 7.0 and 4.2 mg/g), respectively. Three other food types, Spanish doughnut, 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 and Guillén 2014a). However, in a later study, the same authors (Martínez-Yusta and Guillén (2014b) compared the formation of primary alcohols in fried soybean oils and the same 3 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 3 media. The authors also reported that the primary alcohols began to be detectable after 7.5 h of frying in soybean oil and Spanish doughnut dough, and after 10 h of frying in pork adipose tissue and salmon fillet.

Hydroxy dienes and keto dienes were monitored during oxidation of sunflower oil-derived FAME at 40 °C (Morales and others 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 h of heating, 3.1 mg/g of ketodienes and 1.3 mg/g of hydroxy dienes were found in the FAMEs 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 and others 2010). For high linoleic sunflower oil oxidized under similar conditions, the same group found concentrations of ketodienes and hydroxydienes up to 1.6 and 0.7 mg/g, respectively (Morales and others 2014).

Steenhorst-Slikkerveer and others (2000) determined the concentrations of epoxy-, oxo-, and hydroxy-TAGs in oxidized rapeseed oil and linseed/safflower oil. The oils were analyzed when received, without any artificial oxidation. Epoxy- and oxo-TAGs coeluted 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 and 3549 ppm (approximately 8.8 and 3.5 mg/g) in rapeseed oil and linseed/safflower oil, respectively (Steenhorst-Slikkerveer and others 2000).

Quantitative results on the occurrence of epoxides, alcohols, and ketones are summarized in Table 1. It should be noted that possible discrepancies exist among the results due to 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 unoxidized oils, these oxygenated compounds were quantifiable using chromatographic techniques but present at much lower levels at μg/g.

GC

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 in the chain, where the analytes can be subjected to GC analysis directly, normally using low temperatures. In contrast, TAGs are large, nonvolatile 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 FAMEs, 3-hydroxymethylpyridinyl (3-pyridylcarbinol)

Table 1–Occurrence of epoxides, alcohols, and ketones in FAMEs, TAGs, oils, and foods with/without artificial oxidation.

Sample	Oxidation condition	Quantitative technique	Analyte	Range of quantity	Reference
FAMEs					
Methyl oleate	Heated at 180 °C for 5 to 15 h	GC-FID	Monoepoxy fatty acids	14.2 to 35.2 mg/g	Berdeaux and others (1999b)
Methyl linoleate	Heated at 180 °C for 5 to 15 h	GC-FID	Monoepoxy fatty acids	9.2 to 19.1 mg/g	Berdeaux and others (1999b)
FAME derived from high linoleic sunflower oil	Heated at 40 °C for 0 to 91 h	HPLC-UV	Ketodienes	Up to 3.094 mg/g	Morales and others (2010)
		HPLC-UV	Hydroxydienes	Up to 1.338 mg/g	Morales and others (2010)
FAME derived from high oleic sunflower oil	Heated at 40 °C for 0 to 192 h	HPLC-UV	Ketodienes	Up to 1.726 mg/g	Morales and others (2010)
		HPLC-UV	Hydroxydienes	Up to 0.590 mg/g	Morales and others (2010)
FAME derived from high linoleic sunflower oil	Heated at 80 °C for 0 to 6 h	HPLC-UV	Ketodienes	Up to 0.38 mg/g	Morales and others (2012a)
		HPLC-UV	Hydroxydienes	Up to 0.46 mg/g	Morales and others (2012a)
FAME derived from high oleic sunflower oil	Heated at 80 °C for 0 to 17 h	HPLC-UV	Ketodienes	Up to 0.78 mg/g	Morales and others (2012a)
		HPLC-UV	Hydroxydienes	Up to 0.31 mg/g	Morales and others (2012a)
		HPLC-ELS	Methyl *trans*-epoxystearate	Up to 1.33 mg/g	Morales and others (2012a)
		HPLC-ELS	Methyl *cis*-epoxystearate	Up to 1.52 mg/g	Morales and others (2012a)
TAGs					
Triolein	Heated at 180 °C for 5 to 15 h	GC-FID	Monoepoxy fatty acids	13.3 to 35.7 mg/g after CH_3ONa catalyzed transmethylation	Berdeaux and others (1999b)
Trilinolein	Heated at 180 °C for 5 to 15 h	GC-FID	Monoepoxy fatty acids	6.9 to 18.3 mg/g after CH_3ONa catalyzed transmethylation	Berdeaux and others (1999b)
Oils					
Hydrogenated ground nut oil	-	GC-MS-SIM	C18 hydroxy fatty acid isomers (C6-C17 substitution)	2 to 494 μg/g	Wilson and others (1997)
Olive oil	Heated at 180 °C for 15 h	GC-FID	Monoepoxy fatty acids	13.52 mg/g with on-column injection, 13.72 mg/g with split injection	Velasco and others (2002)
Sunflower oil	Heated at 180 °C for 15 h	GC-FID	Monoepoxy fatty acids	10.88 mg/g with on-column injection, 10.87 mg/g with split injection	Velasco and others (2002)
Olive oil	Heated at 180 °C for 5 to 15 h	GC-FID	Monoepoxy fatty acids	4.29 to 14.24 mg/g	Velasco and others (2004)
Sunflower oil	Heated at 180 °C for 5 to 15 h	GC-FID	Monoepoxy fatty acids	5.10 to 9.44 mg/g	Velasco and others (2004)
Used frying oils	-	GC-FID	Monoepoxy fatty acids	3.37 to 14.42 mg/g	Velasco and others (2004)
Edible oils	-	GC-FID	*cis*-epoxy-oleic acid	110 to 2300 μg/g	Fankhauser-Noti and others (2006)
		GC-FID	*cis*-epoxy-linoleic acid	150 to 1550 μg/g	Fankhauser-Noti and others (2006)
		GC-FID	Diepoxy linoleic acid	0.2 to 1.5 μg/g	Fankhauser-Noti and others (2006)
Frying oils	-	GC-FID	*cis*-epoxy-oleic acid	1900 to 7300 μg/g	Fankhauser-Noti and others (2006)
		GC-FID	*trans*-epoxy-oleic acid	2800 to 16400 μg/g	Fankhauser-Noti and others (2006)
		GC-FID	*cis*-epoxy-linoleic acid	1400 to 2000 μg/g	Fankhauser-Noti and others (2006)
		GC-FID	*trans*-epoxy-linoleic acid	1800 to 3300 μg/g	Fankhauser-Noti and others (2006)

(*Continued*)

Table 1–Continued.

Sample	Oxidation condition	Quantitative technique	Analyte	Range of quantity	Reference
		GC-FID	Diepoxy linoleic acid	4.9 to 16 μg/g	Fankhauser-Noti and others (2006)
Sunflower oils	Heated at 180 °C for 10 h	GC-FID	Epoxy fatty acids	1.3 to 4.4 mg/g	Marmesat and others (2008)
		GC-FID	Keto fatty acids	0.5 to 2.5 mg/g	Marmesat and others (2008)
		GC-FID	Hydroxy fatty acids	1.9 to 5.5 mg/g	Marmesat and others (2008)
Fresh oils	-	GC-FID	Epoxy fatty acids	0.03 to 2 mg/g	Mubiru and others (2013)
Used frying fat/oil	-	GC-FID	Epoxy fatty acids	0.05 to 16.57 mg/g	Brühl and others (2016)
Pumpkin seed oil	-	GC-FID	Epoxy fatty acids	1.12 to 5.10 mg/g	Brühl and others (2016)
Sweet almond oil	-	GC-FID	Epoxy fatty acids	0.72 to 2.65 mg/g	Brühl and others (2016)
Sunflower oil	-	GC-FID	Epoxy fatty acids	0.06 to 1.90 mg/g	Brühl and others (2016)
Groundnut oil	-	GC-FID	Epoxy fatty acids	0.10 to 7.25 mg/g	Brühl and others (2016)
Olive oil	-	GC-FID	Epoxy fatty acids	0.10 to 0.32 mg/g	Brühl and others (2016)
Rapeseed oil	-	HPLC-MS	Epoxy- and oxo-TAG	2734 ppm (2734 μg/g)[a]	Steenhorst-Slikkerveer and others (2000)
		HPLC-MS	Oxo-2$^1/_2$-glycerides	506 ppm (506 μg/g)[a]	Steenhorst-Slikkerveer and others (2000)
		HPLC-MS	Hydroxy-TAG	8759 ppm (8759 μg/g)[a]	Steenhorst-Slikkerveer and others (2000)
		HPLC-MS	Dihydroxy-TAG	1049 ppm (1049 μg/g)[a]	Steenhorst-Slikkerveer and others (2000)
Linseed/safflower oil	-	HPLC-MS	Epoxy- and oxo-TAG	28701 ppm (28701 μg/g)[a]	Steenhorst-Slikkerveer and others (2000)
		HPLC-MS	Oxo-2$^1/_2$-glycerides	2146 ppm (2146 μg/g)[a]	Steenhorst-Slikkerveer and others (2000)
		HPLC-MS	Hydroxy-TAG	3549 ppm (3549 μg/g)[a]	Steenhorst-Slikkerveer and others (2000)
		HPLC-MS	Dihydroxy-TAG	1823 ppm (1823 μg/g)[a]	Steenhorst-Slikkerveer and others (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 8 h/d for 5 d	^{1}H NMR	(*E*)-9,10-epoxystearic acyl groups	22.8 mmol/L (7.9 mg/g)[b]	Guillén and Uriarte (2012c)
		^{1}H NMR	(*Z*)-9,10-epoxystearic acyl groups	14.5 mmol/L (5.0 mg/g)[b]	Guillén and Uriarte (2012c)
Soybean oil	Heated at 100 °C for 4 to 20 d	^{1}H NMR	Epoxides	8.4 to 90.7 mmol/kg (2.6 to 28.3 mg/g)[b]	Xia and others (2015)
High oleic sunflower oil	Heated at 40 °C for 0 to 86 d	HPLC-UV	Ketodienes	0.13 to 1.34 mg/g	Morales and others (2012c)
		HPLC-UV	Hydroxydienes	0.13 to 0.72 mg/g	Morales and others (2012c)
High linoleic sunflower oil	Heated at 40 °C for 0 to 41 d	HPLC-UV	Ketodienes	0.12 to 0.79 mg/g	Morales and others (2012c)
		HPLC-UV	Hydroxydienes	0.36 to 1.12 mg/g	Morales and others (2012c)
Soybean oil	Heated at 40 °C for 0 to 53 d	HPLC-UV	Ketodienes	0.07 to 2.97 mg/g	Morales and others (2014)
		HPLC-UV	Hydroxydienes	1.02 to 3.53 mg/g	Morales and others (2014)
Rapeseed oil	Heated at 40 °C for 0 to 46 d	HPLC-UV	Ketodienes	Up to 2.1 mg/g	Morales and others (2014)

(*Continued*)

Table 1–Continued.

Sample	Oxidation condition	Quantitative technique	Analyte	Range of quantity	Reference
		HPLC-UV	Hydroxydienes	Up to 3.71 mg/g	Morales and others (2014)
Foods					
Infant foods	-	GC-FID	Epoxidized soybean oil	9 to 86 μg/g	Fankhauser-Noti and others (2005)
Oily sauces	-	GC-FID	Epoxidized soybean oil	47 to 580 μg/g	Fankhauser-Noti and others (2005)
Products in oil	-	GC-FID	Epoxidized soybean oil	85 to 350 μg/g	Fankhauser-Noti and others (2005)
Bakery foods	-	GC-FID	*cis*-epoxy oleic acid	150 to 4240 μg/g	Fankhauser-Noti and others (2006)
		GC-FID	*cis*-epoxy linoleic acid	60 to 3460 μg/g	Fankhauser-Noti and others (2006)
		GC-FID	Diepoxy linoleic acids	0.5 to 5.3 μg/g	Fankhauser-Noti and others (2006)
Products in oil	-	GC-FID/GC-MS	Epoxidized soybean oil	36 to 374 μg/g (GC-FID) and 42 to 363 μg/g (GC-MS)	Biedermann-Brem and others (2007)
Biscuits	-	GC-FID	Epoxy fatty acids	46.95 to 74.49 μg/g	Mubiru and others (2014)
Mayonnaise	-	GC-FID	Epoxy fatty acids	32.6 to 49.16 μg/g	Mubiru and others (2014)
Nuts	-	GC-FID	Epoxy fatty acids	48.88 to 170.78 μg/g	Mubiru and others (2014)
Butter	-	GC-FID	Epoxy fatty acids	115.34 μg/g	Mubiru and others (2014)
Meat	-	GC-FID	Epoxy fatty acids	3.24 to 5.58 μg/g	Mubiru and others (2014)
Chocolate, cocoa butter	-	GC-FID	Epoxy fatty acids	0.57 to 3.38 mg/g	Brühl and others (2016)
Extra virgin olive oil fried with doughnut/pork/salmon fillets	Heated at 190 °C for 7.5 to 32 h	^{1}H NMR	(*E*)-9,10-epoxystearic acyl groups	4 to 20 mmol/mol TAG (1.4 to 7.0 mg/g) [c]	Martínez-Yusta and Guillén (2014a)
			(*Z*)-9,10-epoxystearic acyl groups	4 to 12 mmol/mol TAG (1.4 to 4.2 mg/g) [c]	Martínez-Yusta and Guillén (2014a)
			Primary alcohols	2 to 5 mmol/mol TAG (0.7 to 1.7 mg/g) [c]	Martínez-Yusta and Guillén (2014a)
			Secondary alcohols	4 to 20 mmol/mol TAG (1.4 to 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 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 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 900 g/mol for calculation.

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 and others 1997). The common methylation reagents, BF_3 and HCl, could convert allylic hydroxy fatty acids into conjugated dienoic acids (Yurawecz and others 1994). In addition, when applying BF_3 or HCl with methanol, a great loss of the vernolic acid (*cis*-12-epoxy-*cis*-9-octadecenoic acid) peak was observed in GC using a thermal conductivity detector (TCD) (Kleiman and others 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 and others 1969; Kleiman and 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 and others 1999a), 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-mass spectrometry (MS) has been reviewed by Christie and others (1986), Christie (1998), and Harvey (1998). Although direct preparation of 3-pyridylcarbinol esters from TAGs was initially considered impossible (Christie 1998), a

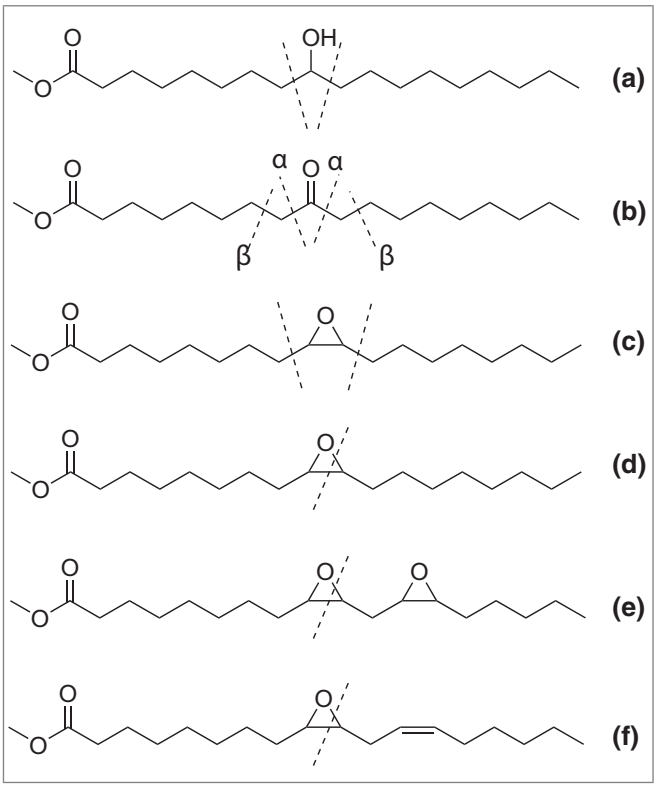

Figure 3–Major fragmentations of oxygenated FAMEs in EI-MS.

one-step transesterification method was later applied to prepare 3-pyridylcarbinol esters from TAGs (Destaillats and Angers 2002; Dubois and others 2006). However, transesterification of oxygenated TAGs using this method needs verification.

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 (Table 2).

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 and Spencer 1973; Weihrauch and others 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 3a and b) (Weihrauch and others 1974; Wilson and others 1997). For keto-FAMEs, both α- and β-cleavages to the carbonyl group take place (Figure 3b) (Weihrauch and others 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 7-keto-11-octadecenoic acid methyl ester (Daulatabad and Jamkhandi 1997). The least characteristic spectra are found for epoxy FAMEs, where only a single diagnostic ion is formed. For methyl 9,10-epoxy-octadecanoate, for example, the diagnostic ion is m/z 155 (Kleiman and Spencer 1973); the same diagnostic ion was found for methyl 9,10:12,13-diepoxy-octadecanoate (Blee and Schuber 1990) and methyl 9,10-epoxy-12-octadecenoate (Figure 3f) (Piazza and others 2003). Two mechanisms of formation of m/z 155 have been proposed in previous work (Kleiman and Spencer 1973; Piazza and others 2003): (1) by cleavages on each side of the epoxy group and loss of m/z 157 [$(CH_2)_7COOCH_3$] (Figure 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 $[HO{=}CH(CH_2)_7COOCH_3]^+$ (Figure 3d–f) (Piazza and others 2003). Mubiru and others (2013) presented spectra for monoepoxy FAMEs with 0, 1, and 2 sites of unsaturation, where characteristic ions were identified for each epoxy FAMEs. Although the spectra were distinguishable from each other, the relative intensities of the characteristic ions were low. In the spectra of unsaturated epoxy FAMEs, intense ions with m/z <100 were present, which caused difficulty 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. A significant advantage of CI is that retention times of the target peaks are retained from EI when employing the same GC conditions, and therefore CI provides supplementary information for structural analysis, including molecular weights of oxygenated FAMEs (Plattner and others 1983; Fankhauser-Noti and others 2006).

The functional groups in FAMEs can also be derivatized for different purposes. Use of such derivatives increases sensitivity and selectivity for a specific class of compounds and, in some cases, produces 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 hydroxyl groups using various methods, including alkylsilyl (Poole 2013), acyl (Orata 2012), and methoxy derivatives (Wilson and others 1997).

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 cleavages on each side of the carbon attached to the trimethylsilyloxy (OTMS) groups (Figure 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, cleavage occurred on both sides of the OTMS group (Figure 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 4c) (Kleiman and 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 2 of the major ions in the spectra, as for methyl 9-hydroxy-10-octadecenoate (Figure 4d) and 9-hydroxy-10, 12-octadecadienoate (Figure 4e), where cleavages occurred on both sides of the allylic or conjugated dienol system (Kleiman and 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 and others 2013). Moreover, the rule that allylic OTMS cleaved as a whole in EI spectra has been extensively used to characterize

Table 2–Selected GC-MS characterization of oxygenated fatty acids in literature sources

Ionization	Major ions (fragments)	Systematic name	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-epoxy-12-octadecenoic 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 and others (2003)
EI	164, 207	12,13-epoxy-9-octadecenoic acid ME	Mubiru and others (2013)
EI	207, 111	12,13-epoxy-9,15-octadecadienoic acid ME	Mubiru and others (2013)
EI	236, 247	15,16-epoxy-9,12-octadecadienoic acid ME	Mubiru and others (2013)
EI	185, 155, 108	9,10-epoxy-12,15-octadecadienoic acid ME	Mubiru and others (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 and others (1983)
PCI (methane)	327 (M+H), 309, 291, 277	9,10:12,13-diepoxyoctadecanoic acid ME	Biedermann-Brem and others (2007)
PCI (ammonia)	344 ($M+NH_4$), 309 (M+H-18)	9,10:12,13-diepoxyoctadecanoic acid ME	Biedermann-Brem and others (2007)
NCI (ammonia)	325 (M-H)	9,10:12,13-diepoxyoctadecanoic acid ME	Biedermann-Brem and others (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 and others (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 and others (1997)
EI	371 (M-15)[a], 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-hydroxy-9-octadecenoic acid ME TMS ether	Kleiman and Spencer (1973); Mubiru and others (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-hydroxy-12-octadecenoic 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-hydroxy-9-octadecenoic 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-hydroxy-11,17-eicosadienoic 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-hydroxy-10-octadecenoic 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-hydroxy-10,12-octadecadienoic acid ME TMS ether	Kleiman and Spencer (1973)
EI	313 ($CH_3O_2C(CH_2)_9$-CH=CH-$CHOSi(CH_3)_3^+$), 199 ($CH_3(CH_2)_4$-$CHOSi(CH_3)_3$CH=CH^+)	13-hydroxy-11-octadecenoic 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 ($CH_3(CH_2)_4$-$CHOSi(CH_3)_3$CH=CH-CH=CH^+)	13-hydroxy-9,11-octadecadienoic acid ME TMS ether	Kleiman and Spencer (1973)
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-dihydroxy eicosanoic acid ME TMS ether	Borgeat and others (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-dihydroxy eicosatetraenoic acid ME TMS ether	Borgeat and others (1982)
EI	229, 259	9-hydroxyoctadecanoic acid ME TMS ether	Mubiru and others (2013)
EI	215, 273	10-hydroxyoctadecanoic acid TMS ether	Mubiru and others (2013)
EI	213, 273	10-hydroxy-12-octadecenoic acid TMS ether	Mubiru and others (2013)
EI	227, 259	9-hydroxy-12-octadecenoic acid TMS ether	Mubiru and others (2013)
EI	173, 313	13-hydroxy-9-octadecenoicd acid TMS ether	Mubiru and others (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 and others (1996)

(*Continued*)

Table 2–Continued.

Ionization	Major ions (fragments)	Systematic name	Reference
EI	299 ($CH_3O_2C(CH_2)_7CH{=}CH\text{-}CH_2\text{-}CHOSi(CH_3)_3^+$), 275 ($(CH_3(CH_2)_4CHOSi(CH_3)_3\text{-}CHOSi(CH_3)_3^+$), 173 ($CH_3(CH_2)_4CHOSi(CH_3)_3^+$)	12,13-dihydroxy-9-octadecenoic acid ME TMS ether	Street and others (1996)
EI	361 ($CH_3O_2C(CH_2)_7CHOSi(CH_3)_3\text{-}CHOSi(CH_3)_3^+$), 271 (361-trimethylsilanol), 259 ($CH_3O_2C(CH_2)_7\text{-}CHOSi(CH_3)_3^+$), 213 ($(CH_3(CH_2)_4CH{=}CH\text{-}CH_2CHOSi(CH_3)_3^+$)	9,10-dihydroxy-12-octadecenoic acid ME TMS ether	Street and others (1996)
EI	275 ($CH_3(CH_2)_4$ $CH(OSi(CH_3)_3)\text{-}CHOSi(CH_3)_3^+$), 173 ($CH_3(CH_2)_4CHOSi(CH_3)_3^+$)	12,13-dihydroxy-6,9-octadecadienoic acid ME TMS ether	Street and others (1996)
EI	339 ($CH_3O_2C(CH_2)_7\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2$ $CHOSi(CH_3)_3^+$), 233(CH_3CH_2CH $(OSi(CH_3)_3)\text{-}CHOSi(CH_3)_3^+$), 131 ($CH_3CH_2CHOSi(CH_3)_3^+$)	15,16-dihydroxy-9,12-octadecadienoic acid ME TMS ether	Street and others (1996)
EI	259, 171	9, 13-dihydroxy-11, 15-octadecadienoic acid ME bisTMS ether	Miakar and Spiteller (1994)
EI	299, 273, 131	12, 16-dihydroxy-9,13-octadecadienoic 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 and others (1983)
PCI (isobutane)	387 (M+H), 385 (M-H), 297 (M+H-90), 301, 187	12-hydroxyoctadecanoic acid ME TMS ether	Plattner and others (1983)
Keto-			
EI	422 (M),390 (M-32), 312 ($CH_3O_2C(CH_2)_{15}\text{-}C(O)\text{-}CH_3$), 297 ($CH_3O_2C(CH_2)_{15}\text{-}C^+(O)$)	17-oxo-20-hexacosaenoic acid ME	Kleiman and Spencer (1973)
EI	310 (M), 279 (M-31), 239 ($CH_3O_2C(CH_2)_7\text{-}CH{=}CH\text{-}(CH_2)_2\text{-}C^+(O)$), 99 ($CH_3(CH_2)_4\text{-}C^+(O)$)	13-oxo-9-octadecenoic acid ME	Weihrauch and others (1974)
EI	310 (M), 279 (M-31), 185 ($CH_3O_2C(CH_2)_7\text{-}C^+(O)$), 153 ($CH_3(CH_2)_4\text{-}CH{=}CH\text{-}(CH_2)_2\text{-}C^+(O)$)	9-oxo-12-octadecenoic acid ME	Weihrauch and others (1974)
EI	310 (M), 239 ($CH_3O_2C(CH_2)_5\text{-}C(O)$ $(CH_2)_3CH{=}CH\text{-}CH_2^+$), 181 ($CH_3(CH_2)_5\text{-}CH{=}CH(CH_2)_3C^+(O)$), 157 ($CH_3O_2C(CH_2)_5\text{-}C^+(O)$, 125 ($CH_3\text{-}(CH_2)_5CH{=}CH\text{-}CH_2^+$)	7-oxo-11-octadecenoic 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-oxo-9-octadecenoate	Brechany and Christie (1994)
EI	304, 276, 248, 234	3-pyridylcarbinol 10-oxo-14-octadecenoate	Brechany and Christie (1994)
PCI (isobutane)	313 (M+1), 281 (M+1-32)	methyl 13-ketooctadecanoate	Plattner and others (1983)
Multi-oxygenated			
EI	241 ($CH_3(CH_2)_4CH{=}CH\text{-}CH(OTMS)\text{-}CHOCH^+$), 199 ($CH_3O_2C(CH_2)_7CHOCH^+$), or 199 ($CH_3(CH_2)_4CH{=}CH\text{-}CH(OTMS)^+$)	9,10-epoxy-11-hydroxy-12-octadecenoic acid ME TMS ether	Gardner and others (1974)
EI	327 ($CH_3O_2C(CH_2)_7CH{=}CH\text{-}CH(OTMS)\text{-}CHOCH^+$), 298 ($CH_3O_2C(CH_2)_7CH{=}CH\text{-}CH(OTMS)\text{-}CH^+$), 285 ($CH_3O_2C(CH_2)_7CH{=}CH\text{-}CH(OTMS)^+$), 241 ($CH_3(CH_2)_4\text{-}CHOCH\text{-}CH(OTMS)\text{-}CH=CH^+$)	12,13-epoxy-11-hydroxy-9-octadecenoic acid ME TMS ether	Gardner and others (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 and others (1983)

methyl esters and pentafluorobenzyl (PFB) esters of monohydroxy fatty acids containing more than 2 double bonds (Wheelan and others 1995). However, that rule did not apply to TMS-dihydroxy fatty acid PFB esters with 3 conjugated double bonds (Wheelan and others 1995), which means that it remains challenging to interpret spectra derived from PUFAs with more than 3 double bonds, as in fish oils, without reference standards of hydroxy fatty acids.

Common acylation methods include acylation of a hydroxyl group using carboxylic acid anhydride and fluorinated reagents. Fluorinated reagents, such as heptafluorobutyrate anhydride (HFBA), have been used in combination with 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 and others 2000). However, precise determination of the position of the double bond in unsaturated hydroxy FAMEs was not possible (Pons and others 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 and others 1997). Hydrogenation simplified the interpretation of spectra with fragments formed by cleavage of the fatty acid chain on both sides of the methoxy group, giving 2 intense ions in the spectrum (Figure 4f) (Wilson and others 1997).

Few derivatization methods have been developed for epoxides as reactions result in ring opening. Methanolysis of epoxy FAMEs was discussed by Kleiman and Spencer (1973), where the use

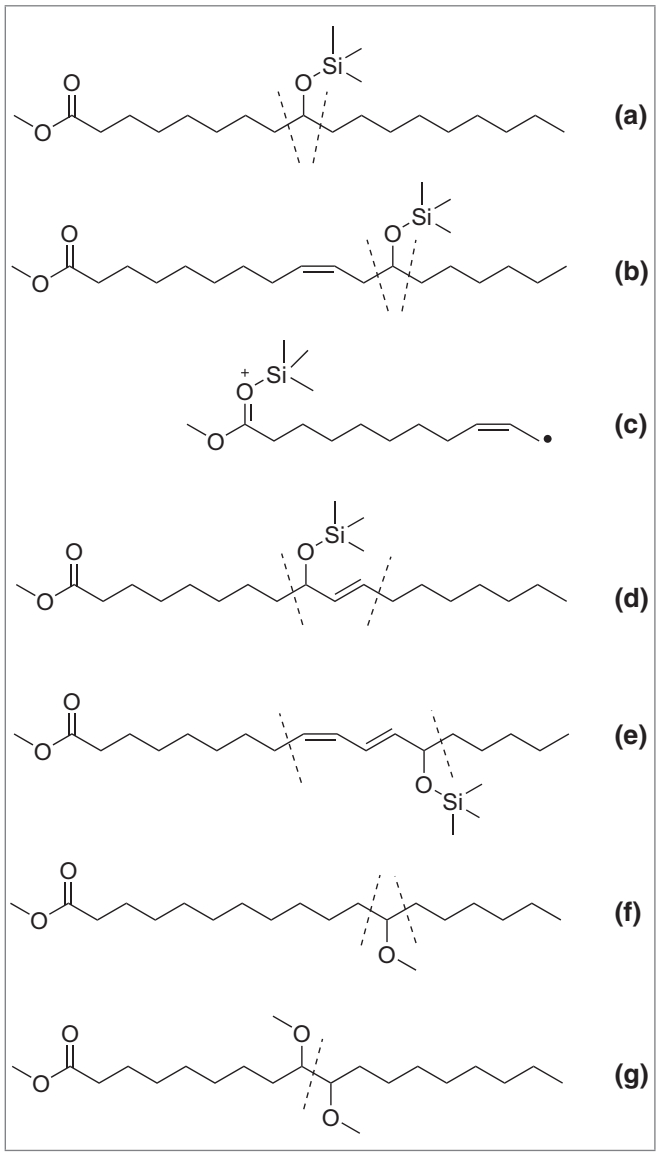

Figure 4–Major fragmentations of derivatives of hydroxy FAMEs in EI-MS.

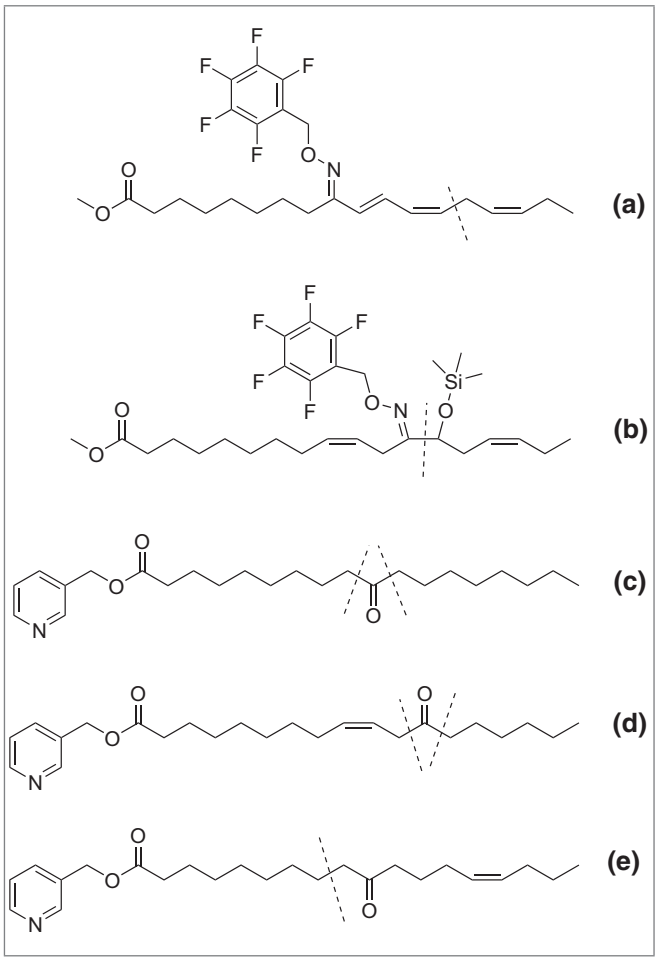

Figure 5–Major fragmentations of derivatives of keto fatty acids in EI-MS.

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 and Lyall 2002). Two major fragments were formed from the cleavage between the 2 vicinal methoxy groups (Figure 4g), characteristic in MS spectra (Wilson and Lyall 2002).

Ketones do not contain active hydrogens as alcohols do. Derivatization can be made using PFB hydroxylamine (PFBHA) to convert keto-fatty acids into their O-2,3,4,5,6-PFB oximes (Figure 5a). Generally, in EI, *m/z* 181 characterized PFB oximes, as 181 is the mass of the $[C_6F_5CH_2]$ moiety (Hachey and others 1991). When present, m/z $[M\text{-}181]^{\cdot+}$ resulted from the loss of the $C_6F_5CH_2$, and *m/z* $[M\text{-}197]^{\cdot+}$ 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 $[C_6F_5CH_2O]^{\cdot-}$ were characteristic ions for the PFB oxime in NCI (Hachey and others 1991; Schulze and others 2006), with methane as the reagent gas. The combination of TMS and PFBHA derivatizing reagents was also employed for the identification of ketols (Schulze and others 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 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 and others 2006). An exception was 9,12-dioxo-10-dodecenoic acid which went through enolization in the presence of MSTFA to form methyl 12-O-2,3,4,5,6-PFB oximino-9-trimethylsilyloxy-8,10-dodecadienoate (Gallasch and 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.

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 5c) except for 3-pyridylcarbinyl 5-oxo-stearate (Brechany and 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 and 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 5d); otherwise, the cleavage β to the keto group resulted in intense ions in the spectra (Figure 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.

Quantification of oxygenated fatty acids by GC

While GC-MS may help to identify the structures of the oxygenated fatty acids, standards of commercial fatty acids are useful as references to determine retention times and response factors in GC-flame ionization detector (GC-FID). The major challenge to measure oxygenated fatty acids in oils is that they are present at low concentrations. In order to make those products detectable, it is necessary to extract and concentrate them from large amounts of the nonaltered fatty acids. Therefore, one useful approach to such separation employs solid-phase extraction (SPE) with a silica column as the stationary phase. When this technique was applied to oxidized fats and oils directly, the relatively polar compounds, including diacylglycerols (DAGs), monoacylglycerols, and free fatty acids, were separated from TAGs (Dobarganes and others 2000). For SPE separation between nonoxygenated and oxygenated FAMEs, the 1st elution solvent, comprised of a less polar solvent mixture such as hexane:ethyl acetate 98:2 (v/v) (Jenske and Vetter 2008), hexane: diethyl ether 95:5 (v/v) (Velasco and others 2002), and hexane:diethyl ether 98:2 (v/v) (Marmesat and others 2008), removes unaltered FAMEs, so oxygenated FAMEs were retained. The 2nd elution solvent, normally ethyl acetate or diethyl ether, is sufficiently polar to elute the oxygenated FAMEs from the silica column. By removing the unaltered FAMEs, SPE has been considered a concentration step and has been useful to quantify trace levels of oxygenated fatty acids (Velasco and others 2002; Jenske and Vetter 2008; Marmesat and others 2008). The SPE steps can also be modified for specific purposes. For example, Wilson and others (1997) used 3 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 and others (2013) also used 3 elutions on a silica column to elute epoxy fatty acids with n-hexane: diethyl ether (90:10, v/v), and they successfully removed coeluting hydroxy fatty acid interferences from monoepoxy fatty acids. The pretreatment methods and detection conditions are summarized in Table 3.

With appropriate pretreatment, including transmethylation and SPE, GC-FID has been reported to be a powerful tool to simultaneously determine epoxy, hydroxy, and keto fatty acids (Marmesat and others 2008), and also monoepoxy fatty acids alone (Berdeaux and others 1999b; Velasco and others 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 coeluted in their unsaturated forms. An obvious disadvantage is that hydrogenation resulted in the 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 their structures in nonmodel systems has prevented easy quantification by GC. Measurements of unsaturated monoepoxy fatty acids in fresh oils have been conducted by Mubiru and others (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 and others 2016).

Methoxy derivatives of C18 hydroxy FAME isomers were employed as a marker of lipid peroxidation (Wilson and others 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 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 and others 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 and Lyall 2002); however, no applications have been reported for food.

Fluorinated reagents, such as fluorinated anhydride and pentafluorobenzoyl 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 and Scheutwinkel-Reich 1980; Jenske and Vetter 2008). A recent study reported the detection limits to be 300 fg to 2 pg for 2-hydroxy FAMEs and 50 to 500 fg for 3-hydroxy FAMEs in bovine milk fat (Jenske and 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 negative CI (NCI) mode, PFB derivatives have been mainly applied for quantification, with a detection limit of 0.1 pg (Hachey and others 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 oxygenated fatty acids as minor products of lipid oxidation but there remain unresolved problems in coelution and identification of unknown compounds. Coelution results in even less characteristic spectra and further difficulties in the 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 and others 2004). This technique has potential in obtaining improved separations of complex oxygenated oxidation products in GC.

HPLC

HPLC characterization of oxygenated TAGs

HPLC has long been used to separate epoxy fatty acids (Gérard and others 1992), hydroxy fatty acids (Bussell and Miller 1979; Hennion and others 1983; Bandi and Reynolds 1985; Bandi and Ansari 1986), and keto fatty acids (MacMillan and Murphycor 1995; Byrdwell and 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.

Table 3–GC quantitative methods and corresponding pretreatment and detection conditions for analysis of oxygenated fatty acids in foods

Analytes	Samples	Pretreatment	Extraction condition	Detection	Column	LOD/LOQ	References
Monohydroxy (plus hydroperoxy) fatty acids	Nut oil	Hydrogenation, transmethylation, SPE, and methylation of OH group	SPE: 1st elution: 95:5 hexane: ethyl acetate; 2nd elution: 80:20 hexane: ethyl acetate	EI-MS-SIM	CP-Sil 19 fused silica (25 m × 0.25 mm i.d.)	LOD = 0.2 ng (injection volume = 1 μL)	Wilson and others (1997)
Mono epoxy fatty acids	Thermoxidized olive and sunflower oils	Transmethylation and SPE	SPE: 1st elution: 95:5 hexane: diethyl ether; 2nd elution: diethyl ether	FID	DB-Wax fused silica (30 m × 0.25 mm i.d.)	Not reported	Velasco and others (2002)
Mono epoxy fatty acids	Thermoxidized olive and sunflower oils/used frying oils	Transmethylation and SPE	SPE: 1st elution: 95:5 hexane: diethyl ether; 2nd elution: diethyl ether	FID	HP-Innowax fused silica (30 m × 0.25 mm i.d.)	Not reported	Velasco and others (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 = 2 mg/kg extract and LOQ = 6 mg/kg	Fankhauser-Noti and others (2005); Fankhauser-Noti and others (2006)
Monoepoxy fatty acids	Oils/snacks	Transmethylation	N/A	FID	SP-2560 (100 m × 0.25 mm i.d.)	Not reported	Fankhauser-Noti and others (2006); Biedermann-Brem and others (2007)
Methyl mono-/di-epoxy linoleate	Oily foods	Transmethylation	N/A	EI/CI-MS	Rtx 2330 (20 m × 0.25 mm i.d.)	LOD < 1 mg/kg (CI) LOQ = 3 mg/kg (EI)	Biedermann-Brem and others (2007)
Epoxy, keto and hydroxy fatty acids	Thermoxidized sunflower oils	Transmethylation, SPE, and hydrogenation	SPE: 1st elution: 98:2 hexane: diethyl ether; 2nd elution: diethyl ether	FID	DB-Wax fused silica (60 m × 0.25 mm i.d.)	LOQ = 1.6 to 2.1 μg/mL	Marmesat and others (2008)
Keto and hydroxy fatty acids	Milk fat	Transmethylation and SPE	SPE: 1st elution: 98:2 hexane: diethyl ether; 2nd elution: diethyl ether	FID	DB-Wax fused silica (30 m × 0.32 mm i.d.)	Not reported	Márquez-Ruiz and others (2011)
Mono epoxy fatty acids	Arachid/colza/corn/ frying/ olive/soya/ sunflower/ salad/mixed/ soya oils	Transmethylation and SPE	SPE: 1st elution: 98:2 hexane: diethyl ether; 2nd elution: 90:10 hexane: diethyl ether; 3rd 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 and others (2013)
Mono epoxy fatty acids	Oil/crisps/pork/milk powder	Modified Bligh and Dyer method, transmethylation and SPE	SPE: 1st elution: 98:2 hexane: diethyl ether; 2nd elution: 90:10 hexane: diethyl ether	FID	CP-Sil 88 (50 m × 0.25 mm i.d.)	LOD = 1.7 to 10.2 μg/g and LOQ = 3.3 to 20.5 μg/g	Mubiru and others (2014)

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. Generally, NP-HPLC, employing a polar stationary phase and nonpolar mobile phase, is more often used for analysis of fatty acids in their free or methyl ester forms, while RP-HPLC, using a nonpolar stationary phase and a polar mobile phase, has been mainly used for analysis of TAG. 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, 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 and others 2000).

Although HPLC allows analysis of intact oxygenated TAGs, it is a challenging task because a large number of individual TAGs species are encountered as a result of the many possible combinations of esterified fatty acids (Buchgraber and others 2004). In addition, TAGs are normally homologs or regio- and geometric isomers overlapping with each other in chromatograms (Buchgraber and others 2004). Therefore, HPLC coupled with MS has been useful in identifying their structures in vegetable oil, lard, and plasma samples (Marai and others 1983). Among the MS techniques that have been applied with HPLC, electrospray ionization (ESI) and atmospheric pressure CI (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 fragments that are generated from cleavage in the hydrocarbon chain, which is normally observed with GC-MS.

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 and Byrdwell 1995b) and hydroxy groups (Byrdwell and Neff 1998), as well as mono- and di-epoxides (Neff and Byrdwell 1998) and a ketone (Byrdwell and Neff 1999) derived from the oxidation of triacylglycerol 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 and Byrdwell 1995a). 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 and Emken 1995); protonated molecular ions were not observable in the mass spectra of saturated TAGs (Byrdwell and 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 (Byrdwell and Neff 1998; Neff and Byrdwell 1998).

Two different mechanisms have been reported for nonmethylene-interrupted and methylene-interrupted situations for a double bond and an epoxy group (Neff and Byrdwell 1995b). When the epoxy group was adjacent to a double bond (nonmethylene-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-epoxy-*cis*-9-octadecenoic acid), $[M-H_2O+H]^+$ was observed due to the loss of H_2O.

A ketone was also characterized in APCI (Byrdwell and 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, 4 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 (Byrdwell and Neff 1998). Using APCI, it was concluded that hydroxy fatty acids are more likely to occur at *sn*-1 and *sn*-3 acyl chains than *sn*-2, which would not be possible to discover when using GC due to the necessary transmethylation (Byrdwell and Neff 1998).

ESI was introduced more recently than APCI in lipid oxidation studies. ESI analysis of nonoxygenated TAGs produced molecular adduct ions derived from the mobile phase instead of protonated molecules (Holčapek and others 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 and De Giulio 2011). However, when DAG fragments were absent, a MS/MS technique was applied to characterize the ammonia adduct of the molecular ion (Duffin and others 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 and others 1991).

Giuffrida and others (2004b) studied the MS/MS spectra of ammonium adduct 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. The comparison of the spectra confirmed the mono-oxygenated TAGs, 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 and others 2004b).

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 identifications of TAGs (McAnoy and others 2005). To date, ESI-MS has been applied to characterize TAGs containing naturally occurring hydroxy fatty acids, such as ricinoleic acid (12-hydroxy-9-cis-octadecenoic acid) and lesquerolic acid ((11Z, 14R)-14-hydroxyicos-11-enoic acid), in plant oils with subsequent fragmentation using MS/MS, MS^3, and MS^4 (Lin and Arcinas 2008; Lin and others 2008; Lin 2009; Lin and Chen 2010; Lin and 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 the solvent system and increased the ionization efficiency in ESI, which facilitated identification of the molecular adducts of TAGs. Thus, MS/MS was not required for identification. The prominent ions observed with APCI were still applicable. For example, an epoxy TAG gave $[M+H\text{-}H_2O]^+$ 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 $[M+H\text{-}H_2O]^+$. 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 and 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 and Murkovic 2011), corn oil (Zeb and Murkovic 2013), and camellia oil (Zeb 2012).

In addition to the interpretation of their mass spectra, identification of oxidation products of TAGs using ESI was also conducted by studying the elution orders of synthetic oxygenated TAGs, such as epoxy- and hydroxyl-TAGs, with RP-HPLC, using a linear gradient of 20% to 80% isopropanol in methanol as eluting solvent (Sjövall and others 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 nonoxygenated and oxygenated TAG standards compared with 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 0. With reference to a series of the saturated TAGs standards, the incremental elution factors of these oxygenated TAGs were calculated for the distribution of functional groups and double bonds in TAGs. Therefore, when analyzing oxidized TAGs, by referring to the homologous series of saturated TAGs, the retention times of unknown oxygenated TAGs could be estimated, which allowed identification of oxidation products of TAGs (Sjovall and others 2001). Based on this research, keto TAGs were also synthesized and identified using elution factors (Suomela and others 2004b). This method has been mainly applied to the study of TAG oxidation in biological samples rather than foods (Suomela and others 2004a, 2005).

ESI and APCI have also been used for a dual parallel MS approach to determine the oxidation products produced in canola oil (Byrdwell and Neff 2002). While APCI-MS was more sensitive to saturated TAGs, ESI-MS showed the opposite and was generally more sensitive overall to TAGs. 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 $[M+1]^+$ using APCI. Using the dual parallel MS approach, APCI-MS and ESI-MS gave complementary information about the structure of the 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 and others 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 and others 2008).

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. HPLC-APCI applied to oxygenated fatty acids has been limited to hydroxy fatty acids in the literature (Ikeda and Kusaka 1992; Kusaka and Ikeda 1993), where RP-HPLC was employed. 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 18:0 and ricinoleic acid, showed a base peak of $[M\text{-}H_2O+H]^+$. 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 $[M+H\text{-}H_2O]^+$, while $[M+H]^+$ was small. However, the same derivatives of 2-hydroxy 18:0 and 12-hydroxy 18:0 gave similar spectra (Kusaka and Ikeda 1993); therefore, there was 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 and others 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 $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, and $[M+H-H_2O]^+$ (Yamane and others 1994). With the electron beam on, cleavage occurred at the epoxy ring and formed ions indicating the location of the epoxy group (Yamane and others 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-PUFA, stepwise elimination of water molecules from the hydroxy groups generated many fragment ions and decreased sensitivity. Polyhydroxy-PUFA 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 and 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 and Abe 1992; Yamane and others 1992). Sensitivity was much improved with acetylation as a result of less fragmentation and higher intensity of the base ion (Yamane and Abe 1992). TSP-MS in the discharge mode facilitated the observation of molecular ions of analytes at low levels. 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 2 water molecules from $[M+H]^+$.

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 TAGs by taking advantage of simpler procedures. In addition, HPLC allows the determination of the distribution of oxygenated fatty acids in acyl chains attached to glycerol backbones, which is not possible with GC since derivatization is normally necessary.

Quantification by HPLC

Quantification of oxygenated products with HPLC techniques has been mainly reported for their FAME forms (Table 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 and others (2012c). The procedure employing KOH/methanol resulted in approximately 90% wt. loss of hydroperoxy dienes, while the loss of hydroperoxy dienes caused by sodium methoxide was less than 10% wt. Therefore, transmethylation with sodium methoxide was shown to be a more appropriate method and thereby employed with HPLC-UV and HPLC-ELSD to analyze epoxy, hydroxy, and keto fatty acids (Morales and others 2012c, 2014).

Conjugated diene structures have enabled the detection of a number of oxygenated compounds by UV, such as keto dienes and hydroxy dienes. Keto dienes exhibit maximum absorbance at 268 nm, while hydroxy dienes and hydroperoxy dienes are monitored at 233 nm (Hopia and others 1996). A significant improvement in this method was made by Morales and others (2010) where conjugated dienes with C13-substituted hydroperoxy, hydroxy, and keto groups were chemically synthesized 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 and others (1996). The HPLC-UV method was then extensively applied to sunflower oils with different levels of oleic and linoleic acids, with quantification limits of 0.3 μg/mL for hydroperoxy and keto dienes and 0.6 μg/mL for hydroxy dienes (Morales and others 2012c).

Esterified glycerols and methyl esters do not absorb at wavelengths higher than 220 nm, except for those with conjugated structures (Holčapek and others 1999), causing difficulties in the detection of nonconjugated oxygenated fatty acids. Therefore, ELSD has been coupled with HPLC, in addition to UV detection (Morales and others 2012a). By connecting HPLC with UV and ELSD in series, it was possible to quantify hydroperoxides, monoepoxy fatty acids, ketodienes, and hydroxydienes derived from oxidation of sunflower oils (Morales and others 2014). Response curves were established for methyl 13-hydroperoxy-, 13-hydroxy-, and 13-keto-octadecadienoate, as well as *cis*- and *trans*-9,10-epoxy methyl stearates, with limits of quantification 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 *cis*- and *trans*-9,10-epoxy methyl stearates, although the peaks were not completely resolved from the nonoxidized FAMEs converted from sunflower oils (Morales and others 2012a). However, due to the relatively low sensitivity of ELSD compared to UV, hydroperoxides and keto-dienes were only detectable in the sunflower oils with high oxidation levels, while hydroxy dienes were not detectable after oxidation for 17 h at 80 °C. The LOQ for keto-dienes was 0.4 mg/g of FAMEs (Morales and others 2012a). Thus, the relatively low sensitivity of ELSD limited its applications in quantifying oxygenated fatty acids in minimally oxidized oils.

Applications of HPLC for the quantification of intact TAGs have been mainly developed for the analysis of molecular species of naturally occurring oxygenated acylglycerols, such as castor bean oil, using RP-HPLC (Lin and others 2003). In terms of oxygenated TAG products, the large number of structures resulted in highly complex RP-HPLC chromatograms, which caused difficulties in interpretation. Thus, Steenhorst-Slikkerveer and others (2000) synthesized reference standards for each class of oxidation products and employed NP-HPLC to resolve the oxygenated TAGs based on their classes. A similar response was obtained for different oxygenated classes by ESI. With identification by ESI, the LOD was 5 ng in the TIC chromatograms and 0.1 to 0.5 ng for the extracted ion chromatograms (Steenhorst-Slikkerveer and others 2000). This method was applied to determine epoxy- and oxo- (coeluting), and hydroxyl-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-HPLC (UHPLC-ESI-MS). Lithium was used as an ionization enhancer to increase the sensitivity (Tarvainen and others 2012). Relative contents of TAGs were estimated by the intensities of the molecular ion represented by $[M+Li]^+$ (Lin and 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 and others 2012). This method has been applied to analyze oxidized TAGs in Atlantic salmon fillets but absolute contents were not reported (Tarvainen and others 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. ESI-MS has been the most popular choice for the analysis of intact TAGs. However, direct analysis of intact oxygenated 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.

NMR Spectroscopy

NMR has been widely used in the study of edible oils, including analysis of fatty acid profiles (Knothe and Kenar 2004), acyl positional distribution of TAGs (Mannina and others 1999), determination of ω-3 fatty acids in fish oil (Sacchi and others 2006), analysis of adulteration (Mannina and others 2009), and quality control and authentication (Dais and Spyros 2007; Dais and Hatzakis 2013), where ^{1}H, ^{13}C, and ^{31}P NMR were used. However, previous NMR studies on lipid oxidation products mainly focused on ^{1}H NMR as a result of its relatively higher sensitivity among the commonly studied nuclei (Table 5). Martínez-Yusta and others (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

Table 4–HPLC quantitative methods and corresponding pretreatment and detection conditions for analysis of oxygenated fatty acids in foods

Analytes	Samples	Pretreatment	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 and others (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 and others (1996)
Epoxy-, hydroxy-, and oxo TAG	Rapeseed oil and linseed/safflower oil	N/A	Si (25 cm × 2.1 mm, 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 to 0.5 ng for extracted ion chromatogram	Steenhorst-Slikkerveer and others (2000)
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 and others (2010); Morales and others (2012b) Morales and others (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 to 5.2 μg/mL and LOQ = 2.5 to 11.6 μg/mL	Morales and others (2012a); Morales and others (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 and others (2003)
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 (1 mM lithium formate as ionization enhancer)	LOD = 40.3 pg and LOQ = 134.3 pg for 12-hydroxystearic acid (injection volume: 3 μL)	Tarvainen and others (2012)

Table 5–NMR quantitative studies of epoxides and alcohols as nonvolatiles 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 h at 190 °C (8 h/d for 5 d) 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 (2012c)
Alcohols	Frying soybean oil with dough, pork adipose tissue, and farmed salmon fillets	Up to 32 h at 190 °C (8 h/d for 4 d) 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 h at 190 °C (8 h/d for 4 d) 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 d in amber bottles at 100 °C	Natural air	Between 2.90 and 3.24 ppm (total epoxides)	6.3 mmol/kg oil	Xia and others (2015)

review, chemical shifts of alcohol-, epoxide-, and ketone-related structures were summarized mainly based on standard compounds.

In terms of epoxides, 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 and Guillén 2010). Alcohol protons (-O**H**) have a large range of chemical shifts, from 1 to 5 ppm; the exact chemical shift depends on a number of conditions, such as temperature, solvent, and concentration. In conjugated hydroxy diene structures, alcohol protons appeared as a broad peak around 7 ppm (Gardner and Weisleder 1972). Therefore, in contrast to epoxides, previous studies have relied 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 protons directly. In $CDCl_3$, the CH groups (-C**H**OH) in unsaturated hydroxy FAMEs gave signals between 4.0 and 4.4 ppm (Porter and others 1994); when using CCl_4 as solvent, the CH groups (-C**H**OH) exhibited signals between 3.38 and 3.93 ppm (Gunstone and others 2007). A ketone group, unlike an aldehyde, does not contain any 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 and 2.38, can be monitored, but these coincide with the acyl C2 protons in esterified glycerol (Kuo and others 1999). It is only when a double bond is conjugated to a carbonyl group that 2 usable signals at 6.8 and 6.1 ppm become apparent (Porter and Wujek 1987). Thus, unless the carbonyl group is comprised of a conjugated structure, the ketone structure is difficult to observe in a ^{1}H NMR spectrum.

A number of advantages have been reported with the use of NMR (Martínez-Yusta and others 2014). Unlike other techniques, such as GC or HPLC, derivatization and pretreatment of a lipid sample is not necessary for NMR analysis, and it has been proven to be a reliable quantitative method. 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 and others 1994; Wanasundara and others 1995; Skiera and others 2012a, b). 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. Moreover, by simply dissolving samples in a deuterated solvent, NMR allows simultaneous determination of several kinds of oxidation products in a single run. For example, Goicoechea and Guillén (2010) monitored mono- and di-epoxides 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, 4-oxoalkanals (Guillén and Uriarte 2009, 2012a, b), which resulted from scissions in the chain. Conjugated hydroxy dienes, showing 2 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 and Guillén 2014a, b). Ketones have been seldom monitored on their own.

It is worth noting that NMR has one major drawback in its low sensitivity compared to the chromatographic methods (Table 5). In a very recent study, the LOQ of epoxides using ^{1}H NMR was 6.3 mmol per kg of oil (Xia and others 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 TAGs was the most used unit when reporting concentrations of the oxidation products (Martínez-Yusta and Guillén 2014a, b). 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 oxygenated lipid oxidation products can improve the selectivity and sensitivity through pretreatment before NMR analysis in order to meet the requirements for analysis of those trace compounds; however, pretreatments are time-consuming and thereby offset any advantage gained by using rapid NMR methods.

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 and others 2009; Badu and Awudza 2017), high-resolution matrix-assisted laser desorption ionization Fourier transform mass spectrometry (MALDI-FTMS) (van den Brink and others 2001), and Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS) (Proschogo and others 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 and others 2001; Picariello and others 2009; Proschogo and others 2012). Quantitative determinations are still to be verified.

Another technique, which has been studied for lipid oxidation, is infrared (IR) spectroscopy. In IR, ester carbonyl groups (C=O) exhibited 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 and Cabo 2000; Vlachos and others 2006). Therefore, this region was often monitored in IR. Specifically, maximum absorbances were found for ketones and aldehydes at 1715 and 1725 cm^{-1}, respectively (Vlachos and others 2006). Formation of aldehydes and ketones resulted in the broadening of the peak at 1746 cm^{-1} (Vlachos and others 2006). However, those studies monitored C=O bonds mainly to assess the extent of formation of secondary oxidation products (1728 cm^{-1}). Characteristic bands for alcohols appeared at 3535 cm^{-1} (Ledreau and others 2009), which showed a widening band in the Fourier transform infrared spectroscopy (FTIR) spectra of sunflower oils stored at room temperature and oxidized at 70 °C with aeration (Guillén and Goicoechea 2007). However, FTIR was said to be even less sensitive than ^{1}H NMR to detect low levels of oxidation (Guillén and 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 and others 2001; Guillén and 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, these techniques require validation for quantitative applications.

Conclusions and Future Prospects

Recent progress in the characterization and quantification of oxygenated 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 oxygenated TAGs and offer information regarding the location of the functional group in acyl chains, which would not be observed in GC, because routine GC analysis of fatty acids requires methylation. 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 may be used for quantitative purposes. 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 whole class of compounds in a single run by simply monitoring the corresponding chemical shifts. It also analyzes 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 in mildly oxidized lipids.

Since SPE has enabled concentration of oxygenated FAMEs with removal of nonaltered 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 analysis.

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 the characterization of oxygenated fatty acids. 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 the analysis of intact TAGs and, therefore, ESI and subsequent fragmentation can be applied for both qualitative and quantitative purposes. Rather than characterization of the oxygenated TAGs, validation of a quantitative method should be conducted for HPLC-MS methods.

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