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Occurrence of epoxy fatty acids in oils and their formation during photo-oxidation in o/w emulsion

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ABSTRACT

This research work was carried out to study the occurrence of C18 epoxy fatty acids in fresh market oil samples and their formation during riboflavin mediated photo-oxidation in casein stabilized o/w emulsion system. The analysis of epoxy fatty acids involved the transmethylation of oils using sodium methoxide followed by SPE fractionation to obtain polar fatty acid methyl esters (FAMEs) that were analyzed in GC-FID. The quantification was carried out using methyl *cis*-10,11- epoxyheptadecanoate as an internal standard. Epoxy fatty acids were present in the range 6.6 to 2057.1 μg/g in the fresh market oil samples, despite having low peroxide value in the range of 0.1 to 10.0 meq/kg of oil. Hence, epoxy fatty acids could be the marker of history of lipid oxidation even before the refining steps. Furthermore, methyl *cis*-9,10 epoxy stearate was spiked in the casein stabilized o/w emulsion systems using buffer at different pH levels (5.0 to 12.0) and the spiked epoxy compound was monitored during storage at 25^{o}C in dark. It was observed that epoxy compound could be stable for two weeks in the casein stabilized o/w emulsion system using phosphate buffer (pH 7.4). Hence, such o/w emulsion system was used for the study on the formation of epoxy compounds from sunflower oil during photo-oxidation, keeping the control samples in dark condition. Photo-oxidized samples showed the formation of higher quantity of epoxy fatty acids, mainly the *trans* isomers, compared to the control samples kept in dark.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
EO	epoxy oleate
EOL	epoxy linoleate
ES	epoxy stearate
FAMEs	fatty acid methyl esters
GC-FID	gas chromatography with flame ionization detector
MUFA	monounsaturated fatty acid
p-AnV	*p*-anisidine value
PUFAs	polyunsaturated fatty acids
PV	peroxide value
SPE	solid phase extraction
tBME	*tert*-butyl methyl ether
FFAs	free fatty acids
w/o emulsion	water in oil emulsion
o/w	oil in water emulsion
ppm	parts per million

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1. INTRODUCTION AND AIMS OF THE STUDY

Lipid oxidation is one of the most important mechanisms of chemical deterioration of food. It causes rancidity, off-flavours, loss of nutritional quality and formation of toxic secondary oxidation products such as hydroxylated alkenals, alpha beta unsaturated aldehydes, malondialdehyde, oxygenated fatty acids such as epoxy-, oxo- and hydroxy- fatty acids etc (Damodaran, Parkin, & Fennema, 2007; Frankel, 2012a; Mubiru, Shrestha, Papastergiadis, & De Meulenaer, 2013). Epoxy fatty acids are reported to be protoxins, i.e. it becomes toxic after it is altered during metabolism (Greene, Newman, Williamson, & Hammock, 2000). The absorption of such epoxy fatty acids in healthy women was observed during the previous study making it a relevant food safety issue (Wilson *et al.*, 2002).

Epoxy group can be formed in the unsaturated fatty acyl chain of the triglycerides during lipid oxidation (Schaich, 2005). Such epoxy fatty acids were reported to be formed in high amounts during thermoxidation. Most of the research in epoxy fatty acid analysis was focused on the heat treated or fried products such as French fries (Marmesat, Velasco, & Dobarganes, 2008; J Velasco, Berdeaux, Márquez-Ruiz, & Dobarganes, 2002; Joaquin Velasco, Marmesat, Bordeaux, Marquez-Ruiz, & Dobarganes, 2004). Recently, Mubiru *et al.* (2013) developed a method for the analysis of epoxy fatty acids which involves the transmethylation of fatty acids using sodium methoxide followed by SPE fractionation of polar fraction of fatty acid methyl esters (FAMEs) that was analyzed in GC-FID. The developed method was also validated in different food matrices such as oil, crisps, pork and milk powder (Mubiru, Shrestha, Papastergiadis, & De Meulenaer, 2014). Mubiru *et al.* (2013) showed for the first time that epoxy fatty acids were present in significant quantity (0.03 to 2 mg/g of oil) even in the fresh market oil samples having low peroxide value. Therefore, epoxy fatty acids content could be an indicator of history of lipid oxidation, probably even before the oil refining steps. Moreover, epoxy fatty acids were also present in crisps, biscuits, speculoos, mayonnaise, peanuts butter, cooked ham, minced beef, walnuts etc collected from the Belgian market in the range of 18 to 615 μg/g of fat (Mubiru *et al.*, 2014). Based on the amounts detected in those samples, it can be understood that safety issue of epoxy fatty acids is still being underestimated. Such results can have significant consequences in future and hence it is necessary to reconfirm such observations. Moreover, previous studies mainly focused on the formation of epoxy fatty acids during thermoxidation. Since, epoxy fatty acids

were present in significant quantity even in fresh market oil samples, formation of such compounds via autoxidation and photo-oxidation needs to be evaluated in details.

Therefore, the first objective of the research was to reconfirm the observations of Mubiru *et al.* (2013) which was about the presence of epoxy fatty acids in significant amount even in fresh market oil samples with low peroxide. The second objective of the study was to evaluate the stability of methyl ester of epoxy fatty acid in casein stabilized o/w emulsion systems using buffers at different pH levels (5.0 to 12.0). This experiment was performed to select a model system in which epoxy fatty acids could remain stable for the experimental period so that further on its formation during lipid oxidation could be carried out. Finally, the third objective of the research work was to study the formation of the epoxy fatty acids during riboflavin mediated photo-oxidation of sunflower oil in the casein stabilized o/w emulsion model system.

2. LITERATURE REVIEW

2.1. Lipids

Lipids represent a broad group of chemically diverse compounds which are soluble in organic solvents. Generally, fats and oils are considered as food lipids. Fat refers to the solid state and oil refers to the liquid state at ambient temperature. The major components of food lipids are- acylglycerols, fatty acids, phospholipids, sphingolipids, sterols, waxes and some miscellaneous lipids like fat soluble vitamins and carotenoids. Food lipids can be further subdivided into non polar (e.g. triacylglycerol, sterols, cholesterol) and polar lipids (e.g. phospholipids). Polar lipids may also act as surfactants because of having hydrophilic "head" group and lipophilic "tail" group (Damodaran *et al.*, 2007).

Fatty acids consist of hydrocarbon chains with carboxylic acid and are aliphatic (Gunstone, Frank D., Harwood, John L. & Dijkstra, 2007). The fatty acids can be classified into groups on the basis of chain length, number, position, configuration of their double bonds, and the occurrence of additional functional groups along the chains (Belitz, Grosch, & Schieberle, 2004). The chain length in the fatty acid varies commonly from 4 to 26 carbon (Belitz *et al.*, 2004). Generally, fatty acids can be saturated and unsaturated (containing double bond fatty acids). Unsaturated fatty acids are known as monounsaturated fatty acids (MUFA) - if containing only one double bond and polyunsaturated fatty acids (PUFA)- if containing two or more double bonds (Damodaran *et al.*, 2007).

2.2 Lipid oxidation

Generally, the term "lipid oxidation" refers to a complex sequence of chemical reactions resulting from the interaction of lipids with oxygen. The fatty acids can decompose to form small, volatile molecules producing the odours during the lipid oxidation. This is known as oxidative rancidity. These volatile compounds are desirable in small amounts for some products like fried foods, dried cereals and cheese, but in general, these compounds are detrimental to the food quality (Damodaran *et al.*, 2007). Lipid oxidation can occur either in bulk oils or in emulsion such as butter, margarine (w/o emulsions); and milk, ice-cream, mayonnaise (o/w emulsion) etc. Food deterioration via lipid oxidation can occur differently according to the types of food system (Ruxton, Calder, Reed, & Simpson, 2005).

Lipid oxidation is affected by many factors, such as temperature, antioxidants, pro-oxidants, fatty acid composition, presence of oxygen etc. (Choe & Min, 2006; Frankel, 2012a). The

presence of metals, light, water activity also affects the lipid oxidation. The presence of photosensitizers initiates photo-oxidation in the presence of light (Frankel, 2012a).

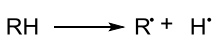
2.2.1 Mechanism of lipid oxidation

A number of mechanisms have been proposed for lipid oxidation (Steele, 2004). These include mainly photo-oxidation (Johnson & Decker, 2015), autoxidation (Steele, 2004) and enzymatic oxidation (Belitz *et al.*, 2004). Due to the limitation of this research, only the mechanisms of autoxidation and photo-oxidation are mentioned.

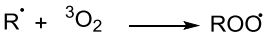
2.2.1.1 Autoxidation

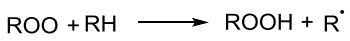
Autoxidation refers to the direct reaction of molecular oxygen with organic compounds under mild conditions. It is a classical theory of lipid oxidation and proceeds via free radical chain mechanism. In general, there are three steps during autoxidation of lipids - initiation, propagation and termination as shown in **Figure 1** (Frankel, 2012b).

Initiation

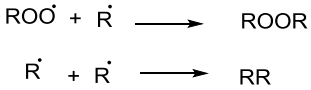


Propagation





Termination



(R: lipid alkyl)

Figure 1. Mechanism of autoxidation (Adapted from Belitz *et al.* (2004); Gunstone, Frank D., Harwood, John L. & Dijkstra (2007))

The initiation step proceeds with the abstraction of hydrogen atom from a fatty acids giving an alkyl radical ($R^{\cdot}$) (Damodaran *et al.*, 2007). Lipid radicals can be produced either by thermal or photochemical homolytic cleavage of a RH bond or by the abstraction of a hydrogen atom from a RH bond by free radical (Porter, Caldwell, & Mills, 1995; Steele, 2004).

During the propagation step, oxygen is added to the alkyl radical forming a peroxyl radical ($ROO^{\cdot}$) which is highly energetic. Subsequently, it abstracts a hydrogen atom from another unsaturated fatty acid. This results in the formation of a fatty acid hydroperoxide (ROOH) and another fatty acid free radical ($R^{\cdot}$) (Damodaran *et al.*, 2007; Velisek, 2014). These hydroperoxides are known as primary oxidation products (Shahidi & Zhong, 2005). Due to the bis-allylic methylene group between the two double bonds on carbon-11 in linoleate, abstraction of hydrogen atom is 40 times more rapid than from oleate (Koppenol, 1990; Marquez-Ruiz, Holagdo, & Velasco, 2013; Porter *et al.*, 1995; Steele, 2004). Linoleic acid is 10-40 times more susceptible to oxidation than oleic. Similarly, linolenic acid oxidizes twice as fast as linoleic acid (Damodaran *et al.*, 2007).

Termination step consists of combination of two radicals to form non radical species. The peroxyl radical becomes the predominant free radical in the presence of oxygen and thus termination step ends by combining peroxyl and alkoxyl radicals. However, in a low oxygen environment, fatty acids dimers are formed by termination reactions between alkyl radicals (Damodaran *et al.*, 2007).

The alkoxyl radicals which are formed initially by hydroperoxide decomposition may decompose to volatile compounds such as hydrocarbons, alcohols, aldehydes, organic acids etc. This also results in the formation of non-volatile compounds such as hydroxy-fatty acids, oxo-fatty acids, ketones, epoxy fatty acids (Schaich, 2005; Shahidi & Zhong, 2005). All these compounds are known as secondary oxidation products (Gunstone, Frank D., Harwood, John L. & Dijkstra, 2007). Such secondary oxidation products, along with the free radicals, form the bases for the measurement of oxidative deterioration of food lipids (Shahidi & Zhong, 2005).

2.2.1.2 Photo-oxidation

The mechanism of photo-sensitization can occur by two ways - either reacting directly with the substrate (type -I sensitizer) or activating oxygen to the singlet state (type II sensitizer) (DeMan, 2013).

In type I photo-sensitization (**Figure 2**), photosensitizers absorb the light energy and convert into excited singlet state ($^1Sen^*$ represents excited singlet state in **Figure 2**). The excited singlet state photosensitizer can abstract the hydrogen or electron from unsaturated oil to form free radicals. Since these free radicals are produced photochemically, chain breaking antioxidants cannot stop this mechanism (Frankel, 2012a; Scrimgeour, 2005).

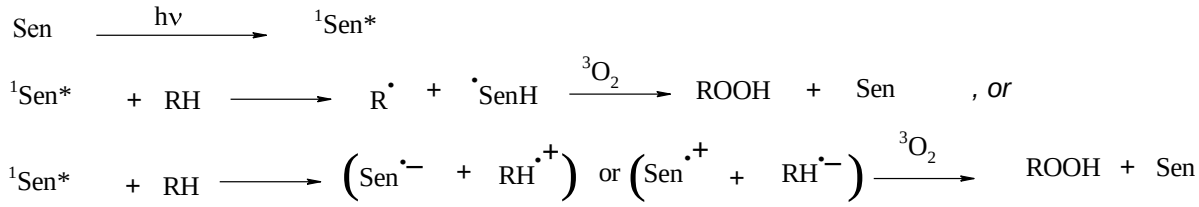


Figure 2. Scheme showing type I photo-oxidation reaction (Shahidi & Zhong, 2010)

In the type II photosensitization (**Figure 3**), the excited singlet state photosensitizer reacts with the triplet state oxygen to form non radical singlet oxygen ($^1O_2$) in the presence of light. These photosensitizers can be riboflavin, chlorophyll, porphyrins, myoglobin and synthetic colorants (Scrimgeour, 2005). Singlet oxygen is a highly reactive molecule. It cannot be quenched by free radical antioxidants (Frankel, 2012a). Only singlet oxygen quenchers like carotenoids can inhibit it. The higher energy of singlet oxygen ($^1O_2$) makes it more reactive towards the double bond in unsaturated lipid. This reaction generates hydroperoxides by the shifting of the double bond in the molecules (Shahidi & Zhong, 2010). The reaction of single oxygen with oleic acid generates hydroperoxides in 9 and 10 positions in the ratio of 48:52, while the autoxidation mechanism (reaction with triplet oxygen) results in the formation hydroperoxides in 8, 9, 10 and 11 positions in the ratio of 27:23:23:27 (Belitz *et al.*, 2004). Some naturally occurring pigments like chlorophyll initiate photo-oxidation by both mechanisms.

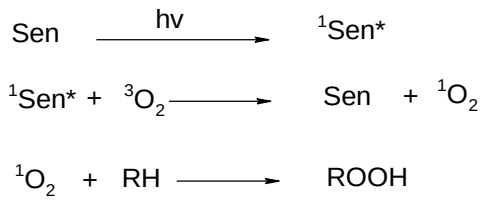


Figure 3. Scheme showing type II photo-oxidation reaction (Shahidi & Zhong, 2010)

2.1.2.3.1 Riboflavin mediated photo-oxidation

The structure of riboflavin **(Figure 4**) shows that it consists of many conjugated double bonds and nitrogen in its ring structure. Although it is heat stable, it is highly sensitive to light (Choe, Huang, & Min, 2005). However, the absence of oxygen makes it more stable under light (Gutiérrez, Criado, Bertolotti, & García, 2001). Allen & Parks (1979) have reported that the degradation of riboflavin follows first order kinetics. Mestdagh *et al.* (2011) observed that

riboflavin is mostly degraded (more than 95%) after two weeks of continuous light exposure in an o/w emulsion. The type of the oil used in the formation of o/w emulsion did not affect the degradation of riboflavin in the illuminated emulsion (Mestdagh *et al.*, 2011). The riboflavin bound to protein is less labile to light than the free riboflavin in solution (Maniere & Dimick, 1976).

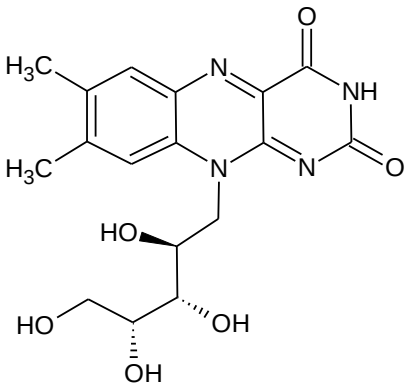


Figure 4. Structure of riboflavin (Adapted from Choe *et al.*, (2005))

Riboflavin photosensitization promotes lipid oxidation in o/w emulsions (Bradley & Min, 1992; Lee & Min, 2010; Min & Boff, 2002). The dairy products which are rich in riboflavin, are prone to riboflavin induced photo-oxidation in the presence of light (Borle, Sieber, & Bosset, 2001). The lipid oxidation was mediated by singlet oxygen and was accelerated by transition metals in riboflavin photosensitized o/w emulsions. Therefore, the antioxidant system should include the combination of inhibition of both the reactive oxygen species (such as singlet oxygen) and pro-oxidant metals (Lee & Decker, 2011).

2.2.2 Lipid oxidation in oil in water (o/w) emulsion

In o/w emulsions or in foods, oil droplets are dispersed into an aqueous matrix. Hence, for lipid oxidation, oxygen needs to diffuse through the aqueous phase and pass through the oil water interface in order to gain access to lipids. Chaiyasit, McClements, & Decker (2005); McClements & Decker (2000); Schwarz, Huang, German, & Tiersch (2000) have proposed the interaction between lipid hydroperoxides and transition metals at the droplet surface as one of the major pathways of lipid oxidation in o/w emulsion.

Various factors influence the rate of oxidation, such as type and concentration of emulsifier, size of oil droplets, emulsion droplet charge, interfacial area, thickness, viscosity of the aqueous phase, composition and porosity of the aqueous phase, pH etc (Chaiyasit *et al.*, 2005; Fennema, 1996; McClements & Decker, 2000; Schwarz *et al.*, 2000; Waraho, McClements,

& Decker, 2011). The diffusion of oxygen through the aqueous phase is the rate determining step for lipid oxidation. The rate of lipid oxidation decreased when the holding temperature of the sample was increased. This was due to the low solubility of the oxygen in water at higher temperature (Coupland & McClements, 1996).

The lipid oxidation products have been reported to interact with other components in the food system. Cucu *et al.* (2011) observed protein aggregation during the autoxidation of dairy protein based emulsion. Similar results were observed in whey protein based emulsion during photo-oxidation (Mestdagh *et al.*, 2011). A decrease in nutritional quality of the dairy product was reported due to interaction of these oxidized lipids with amino acids (Obando, Papastergiadis, Li, & De Meulenaer, 2015). In addition, Vandemoortele & De Meulenaer (2015) also stated that the secondary oxidation product, such as malondialdehyde reacts with protein. Mestdagh *et al.* (2011) & Obando *et al.* (2015) observed that emulsion based on oils consisting high PUFA levels are more susceptible to lipid oxidation and showed a higher impact on protein digestibility. Due to chemical interaction of lipids and proteins during the oxidation process, the study of photo-oxidation in o/w emulsion is very complicated (Yamaki, Kato, & Kikugawa, 1992).

2.2.3 Measurement of lipid oxidation

Lipid oxidation results in the formation of primary and secondary oxidation products. The hydroperoxides formed during the initial stage of lipid oxidation are known as primary oxidation products whereas their degradation products such as hydroxy fatty acids, oxo-fatty acids, epoxy fatty acids, aldehydes, ketones, alcohols, polar fractions etc. are known as secondary oxidation products.

Primary oxidation products can be determined by peroxide value and conjugated dienes and trienes. It is an indicator of the initial stages of oxidative changes and represents the hydroperoxide content in the oil (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002; Ruiz, Canada, & Lendl, 2001). The most regularly used methods for the determination of peroxide value are the iodometric titration, and spectrophotometric method (Shantha & Decker, 1994; Yildiz, Wehling, & Cuppett, 2003).

Secondary oxidation products vary in their chemical structure. Measurement of secondary oxidation products includes *p*-anisidine value, malondialdehyde, hexenals, epoxy fatty acids, hydroxy fatty acids, alcohols, ketones, volatile compounds etc.

Among the different markers of the lipid oxidation, epoxy fatty acids content is emerging as an important indicator of the lipid oxidation. Mubiru *et al.* (2013) showed for the first time that epoxy fatty acids content could be present in significant amounts even in the fresh market oils samples, having low peroxide value. Epoxy fatty acids could have been formed due to lipid oxidation before the refining step. The refining step might have decreased the peroxide value, while epoxy fatty acids were still in significant amounts, probably indicating the history of lipid oxidation. Therefore, it is important to further investigate the chemistry of the epoxy fatty acids.

2.2.4 Epoxy fatty acids as a marker of lipid oxidation

Epoxy fatty acids are the secondary oxidation products of lipid oxidation. They are formed from the decomposition of hydroperoxides. They occur in high amounts in thermally oxidized oils (Marmesat *et al.*, 2008; J Velasco *et al.*, 2002; Joaquin Velasco *et al.*, 2004). They have also been found in the fresh market oil samples, crisps, biscuits, speculoos, mayonnaise, peanuts butter, cooked ham, minced beef, walnuts etc. collected from the Belgian market (Mubiru *et al.*, 2013, 2014).

Two different mechanisms have been mainly proposed for the formation of epoxy fatty acids (**Figure 5**). The first one is the direct addition of a peroxyl radical ($LOO^{\cdot}$) to an isolated or nonconjugated double bond, that undergoes 1,3-cyclization to form an epoxide with elimination of alkoxy radical ($LO^{\cdot}$). In the second one, epoxyallylic radical is formed by the rearrangement or cyclization, involving a 1,2- addition to an adjacent double bond (**Figure 5**) (Dobarganes, 2009; Mubiru *et al.*, 2014; Schaich, 2005).

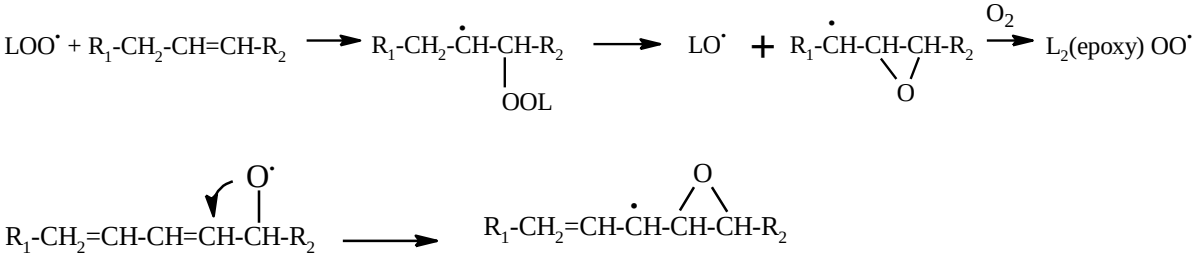


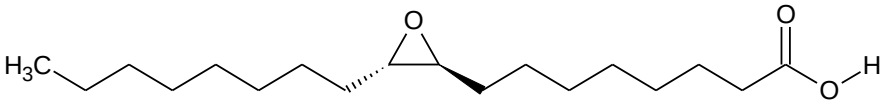
Figure 5. Formation of epoxy fatty acids during lipid oxidation (Adapted from Schaich (2005)).

Mubiru *et al.* (2013, 2014) studied different C18 mono epoxy fatty acids from oleic acid, linoleic acid and linolenic acid. The lists of these mono epoxy fatty acids have been given in **Table 1** and their structures have been shown in **Figure 6**.

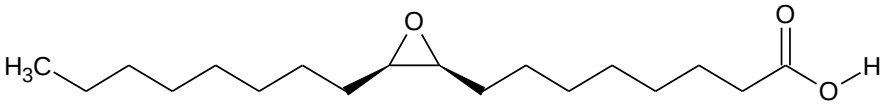
Table 1. Mono-epoxy fatty acids from C18 fatty acids (adapted from Dobarganes (2009); Mubiru *et al.* (2013))

Originating fatty acids	Epoxy fatty acids	Abbreviation
Oleic acid (C18:1)	*trans*-9,10-epoxyoctadecanoate	*trans*-9,10-ES
	cis-9,10-epoxyoctadecanoate	*cis*-9,10-ES
Linoleic acid (C18:2)	*trans*-12,13-epoxy-octadec-9-enoate	*trans*-12,13-EO
	cis-12,13-epoxy-octadec-9-enoate	*cis*-12,13-EO
	trans-9,10-epoxy-octadec-12-enoate	*trans*-9,10-EO
	cis-9,10-epoxy-octadec-12-enoate	*cis*-9,10-EO
Linolenic acid (C18:3)	*trans*-12,13-epoxy-9,15-octadecadienoate	*trans*-12,13-EOL
	cis-12,13-epoxy-9,15-octadecadienoate	*cis*-12,13-EOL
	trans-15,16-epoxy-9,12-octadecadienoate	*trans*-15,16-EOL
	cis-15,16-epoxy-9,12-octadecadienoate	*cis*-15,16-EOL
	trans-9,10-epoxy-12,15-octadecadienoate	*trans*-9,10-EOL
	cis-9,10-epoxy-12,15-octadecadienoate	*cis*-9,10-EOL

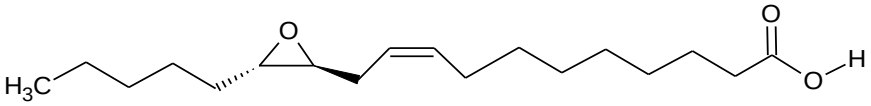
Analysis of epoxy fatty acids involves the transmethylation of oil followed by SPE fractionation of polar FAMEs which can be analyzed in GC-FID (Mubiru *et al.*, 2013, 2014). Base catalyzed transmethylation using sodium methoxide at room temperature is commonly used for the analysis of epoxy fatty acids (Mubiru *et al.*, 2013, 2014). This procedure can methylate the fatty acids in esterified form, but it is not suitable for the methylation of free fatty acids. Therefore, to prevent the hydrolysis of triglycerides during the transmethylation, the reaction medium is made water free. This is achieved by using tBME (*tert*- butyl methyl ether) as mediator solvent. This helps to precede reaction faster and is achieved in one minute. It is then stopped by addition of an acid to neutralize the reaction medium. It also helps to avoid potential further saponification (Suter, Grob, & Pacciarelli, 1997).



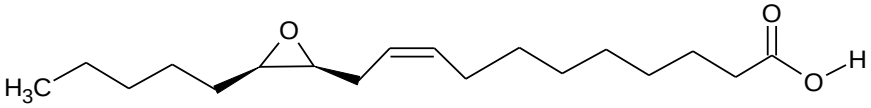
trans-9,10-epoxyoctadecanoate (*trans*-9,10-ES)



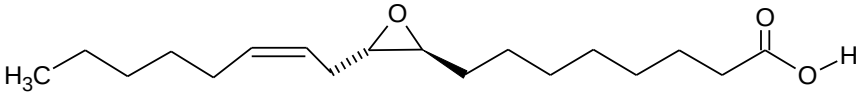
cis-9,10-epoxyoctadecanoate (*cis*-9,10-ES)



trans-12,13- epoxy-octadec-9-enoate (*trans*-12,13-EO)



cis-12,13-epoxy-octadec-9-enoate (*cis*-12,13-EO)



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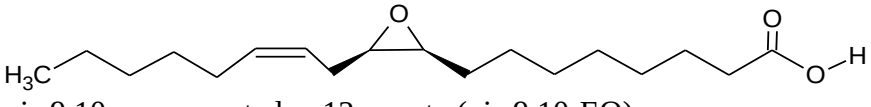

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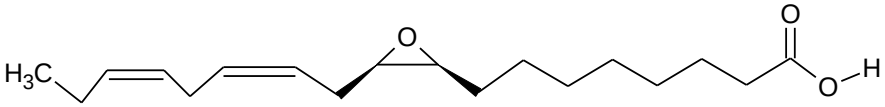
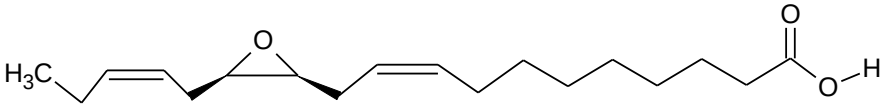
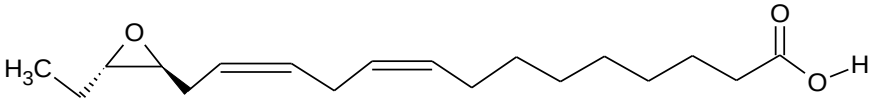


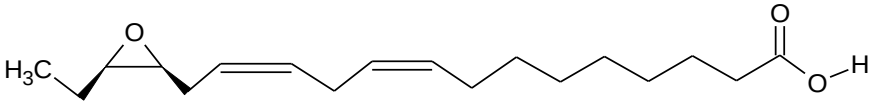
trans-12,13-epoxy-9,15-octadecadienoate (*trans*-12,13-EOL)



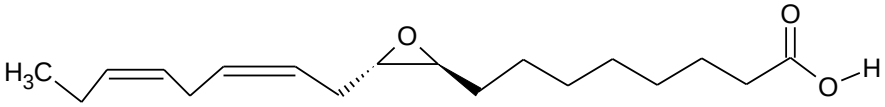
cis-12,13-epoxy-9,15-octadecadienoate (*cis*-12,13-EOL)



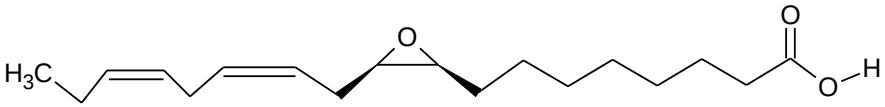
trans-15,16-epoxy-9,12-octadecadienoate (*trans*-15,16-EOL)



cis-15,16-epoxy-9,12-octadecadienoate (*cis*-15,16-EOL)



trans-9,10-epoxy-12,15-octadecadienoate (*trans*-9,10-EOL)



cis-9,10-epoxy-12,15-octadecadienoate (*cis*-9,10-EOL)

Figure 6. Structures of different mono-epoxy fatty acids

2.2.4.1 Effect of epoxy fatty acids on health

The most important epoxy fatty acid related to the food safety is C18 epoxy fatty acids and is found to have toxic effects (Greene *et al.*, 2000). When healthy women consumed milkshake containing uniformly labelled [U-^{13}C] monoepoxy or diepoxy triglyceride, absorption of dietary epoxy fatty acids were found. Better absorption was reported for the mono-epoxy triglyceride. Moreover, the higher levels of [U-^{13}C] mono-epoxy and di-epoxy triglyceride were reported in plasma. As the dietary epoxides react with amines, SH groups of proteins and DNA leading to toxic effects, the estimation of dietary epoxides is of great interest (Wilson *et al.*, 2002).

Cardio-depressant and thrombotic effects have been observed with the intravenous injection of epoxy linoleic acid (Siegfried, Aoki, Lefer, Elisseou, & Zipkin, 1990). Epoxy fatty acids such as 9,10-epoxy-12-octadecenoic acid and 12,13-epoxy-9-octadecenoic acid are found to be related with acute respiratory distress syndrome (Ozawa *et al.*, 1991). Demling (1995) reported that exposure to 9,10-epoxy-12-octadecanoic acid results in histological changes including edema, hemorrhage and emphysema.

3. MATERIALS AND METHODS

3.1. Chemicals and materials

tert-Butyl methyl ether (tBME) and *p*-anisidine reagent (99%) were purchased from Acros Organics (Geel, Belgium). Hexane (95+%), isooctane (99.5%), sodium chloride (99.8%), sodium sulfate, sea sand (acid washed and calcinated), diethyl ether, barium chloride, potassium dihydrogen orthophosphate (KH_2PO_4, 99.9+%), potassium phosphate dibasic (K_2HPO_4, 99.9+%), sodium hydroxide (99.8%), hydrochloric acid (37%), ammonium thiocyanate, citric acid monohydrate (99.5+ %) and dichloromethane (99%) were purchased from Chem-Lab NV (Zedelgem, Belgium). Aluminium oxide (Brockmann I activated), sodium methoxide (25%,w/v), tri-sodium citrate dihydrate, sodium azide and ferrous sulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Silica gel 60 (0.063-0.1 mm) was bought from Merck Chemicals (Overijse, Belgium). Petroleum ether, methanol, sulfuric acid (95%), glacial acetic acid (99.7%) were purchased from Fischer scientific (Tournai, Belgium). The glycerol triheptanoate (C7 triglyceride) was purchased from Stearinerie DUBOIS (France). Sodium caseinate (Microdan 30) was obtained from Acatris Food Belgium (LonderZeel, Belgium). Methyl *cis*-10,11- epoxyheptadecanoate was used as internal standard (IS). The internal standard (IS) and epoxy FAMEs mixture from C18:1, C18:2 and C18:3 were provided in the laboratory. They were synthesized and identified using GC-MS by Mubiru *et al.* (2013, 2014).

3.2 Samples

A total of twenty fresh market oil samples were purchased from five different supermarkets in Belgium. They were brought to the laboratory and analyzed immediately for the C18 epoxy fatty acids and peroxide value.

3.3 Experiments

Three sets of experiments were carried out as per the three objectives of the research work. At first, the epoxy fatty acids were analyzed in the market oil samples. This was done to confirm the previous study about epoxy fatty acids in market oil samples that was carried out in the lab. The second experiment was related to the stability of epoxy fatty acids in the presence of proteins. The third experiment was carried out to evaluate the formation of epoxy fatty acids at low temperature during riboflavin supported photo-oxidation, with the control samples in dark.

3.3.1 Experiment 1: comparison of the two lipid oxidation markers (epoxy fatty acids and peroxide value) in the fresh market oil samples

The fresh market oil samples were analyzed for epoxy fatty acids and peroxide value as the lipid oxidation markers. The obtained results were compared with the previous work of Mubiru *et al.* (2013).

3.3.2 Experiment 2: stability of epoxy fatty acids in protein stabilized emulsion

In order to study the stability of epoxy fatty acids in protein stabilized emulsions, casein stabilized emulsions spiked with one particular epoxy fatty acid methyl ester in a saturated oil was prepared at different pH levels and stability of the spiked epoxy compounds was monitored during storage.

First of all, aqueous solution of the sodium caseinate (6 mg/mL) was prepared by dispersing it in 0.1 M phosphate buffers (pH 7.4 and pH 12.0) and 0.1 M citrate buffer (pH 5.0 and pH 7.0) at room temperature. It was subsequently stirred with a magnetic stirrer overnight to ensure complete hydration and dispersion of casein. Sodium azide (0.02%) was added in the solution to prevent microbial growth. To these solutions, 3% of C7 triglyceride oil spiked with methyl *cis*-9,10 epoxy stearate (9.0 μg/mL emulsion) was added and coarse emulsion was prepared using an Ultra turrax (Janke and Kunkel, IKA-Werk, Staufeb, Germany) for 2 minutes at 12000 rpm. Subsequently, the coarse emulsion was homogenized at 250 bar/50 bar through a high-pressure valve, two-stage APV lab 1000 homogenizer (APV Benelux NV/SA, Erpe Mere, Belgium) at 40°C and then cooled to room temperature. In addition, same procedure was repeated without C7 triglyceride oil for the sodium casienate solutions in phosphate butter (pH 7.4 and pH 12.0).

About 30 mL of epoxy spiked o/w emulsions (pH 5.0, 7.0, 7.4 and 12.0) and casein solutions (pH 7.4 and 12.0) were transferred in the sterilized Duran bottles (100 mL) and were incubated at 25°C in dark for one month. Every week, incubated samples in triplicate were taken for the analysis of epoxy fatty acids.

3.3.3 Experiment 3: formation of C18 epoxy fatty acids in oil in water emulsion during riboflavin supported photo-oxidation

Another set of experiment was carried out to study the formation of epoxy fatty acids during riboflavin supported photo-oxidation. Casein (6 mg/mL) and riboflavin (2 μg/mL) was dissolved in 0.1 M phosphate buffer (pH 7.4). Sunflower oil (3%) was added to the solution

and coarse emulsion was prepared using an Ultra turrax (Janke and Kunkel, IKA-Werk, Staufeb, Germany) for 2 minutes at 12000 rpm. Subsequently, the coarse emulsion was homogenized at 250 bar/50 bar through a high-pressure valve, two-stage APV lab 1000 homogenizer (APV Benelux NV/SA, Erpe Mere, Belgium) at 40°C and then cooled to room temperature. After homogenization the emulsion was pasteurized at 70°C for three minutes so as to minimize the microbial load during incubation. Afterwards, the emulsion (30 mL) was transferred to sterilized Duran bottles (100 mL) and cooled to room temperature. Half of the sample bottles were incubated at 4°C on an orbital shaker (Edmund Buhler, Hechingen, Germany) under illumination (1875 lux at the bottle neck, 1140 lux at the bottom of bottle and 1729 at the side of the bottom) obtained in the light cabinet using Philips TL-D 36 W/840 fluorescent tubes, fixed at 0.5 m above the bottles. Light intensity was measured using a Lux meter (PAR-cell 532, 400-700 nm, Skye Instruments, Llandrindod Wells, UK). The remaining control samples were incubated in dark at 4°C. The incubated samples in triplicates were analyzed weekly for the C18 epoxy fatty acid content.

3.4 Analytical methods

3.4.1 Determination of epoxy fatty acids content

The analytical method described by Mubiru *et al.* (2013, 2014) was used for the extraction of oil from the emulsion, transmethylation, SPE fractionation of polar FAMEs and GC-FID analysis and has been described below.

3.4.1.1 Extraction of oil from emulsion

First of all, 500 μL of methyl *cis*-10,11- epoxyheptadecanoate (IS) solution in hexane (100 μg/mL) was accurately pipetted into a 100 mL glass centrifuge tube and was dried under nitrogen using a nitrogen generator (Domnick Hunter, Parker, Cleveland, USA). Afterwards, 10 mL of emulsion was accurately pipetted into the glass centrifuge tubes containing the previously dried internal standard. Water was adjusted so as to reach a final water content of 14.5 g as determined by the initial water content of the sample. Thereafter, 26 mL of methanol and 11 mL of dichloromethane were added and homogenized using an Ultra-Turrax blender (Janke & Kunkel, IKAWerk, Staufeb, Germany) for 1 min at 14000 rpm. Another 15 mL of dicholoromethane was added and then homogenized for 30 s, and finally 15 mL of water (pH $\leq$2) was added and homogenized for 30 s. The tubes were centrifuged at 3500 rpm using a centrifuge (Rotina 380R Heltich Zentrifugen, Germany) for 10 min, the pH of the aqueous layer was checked to be < 2; if not, it was adjusted by using 5 M hydrochloric acid.

The top aqueous layer was siphoned off, and the organic layer was dried on sodium sulfate over a filter paper (Whatman no.1). The solvents were removed using a rotary evaporator and finally dried using nitrogen.

3.4.1.2 Base catalyzed-transmethylation with sodium methoxide at room temperature

The transmethylation method used for the epoxy fatty acids was the base-catalyzed-transmethylation with sodium methoxide at room temperature as already described by other studies (Dobarganes & Velasco, 2002; Mubiru *et al.*, 2013). The residue after extraction was dissolved in 5.0 mL of *tert*-butyl methyl ether (tBME) and vortexed for few seconds. Then 2.5 mL of 0.2 M sodium methoxide solution in methanol was added to the sample. The tube was vortexed for 1 min. Then, it was allowed to stand at room temperature for 2 minutes. The reaction was stopped by adding 0.17 mL of 0.5 M sulfuric acid. The mixture was vortexed for a few seconds. Eventually 5.0 mL of water was added and the mixture was vortexed for 30 seconds. The organic layer was collected after separation of phases and the extraction was repeated with 5.0 mL of tBME. The organic layer was dried under nitrogen and the resulting fatty acid methyl esters (FAMEs) were dissolved into 2.0 mL of n-hexane-diethyl ether (98:2, v/v) and loaded on the silica column.

For the oil samples, 500 μL of methyl *cis*-10,11- epoxyheptadecanoate (IS) solution in hexane (100 μg/mL) was accurately pipetted into a test tube and was dried under nitrogen using a nitrogen generator (Domnick Hunter, Parker, Cleveland, USA). The oil sample (200 mg) was weighed in the same test tube and transmethylation was done as described above.

3.4.1.3 SPE fractionation of fatty acid methyl esters (FAMEs)

Silica gel was dried in a muffle furnace at 450°C for 12 h and then cooled in a desiccator. Finally the moisture content was adjusted to 10% and equilibrated on a shaker for 1 hour before use. An empty SPE cartridge column (6 mL, 6.5 cm × 1.3 cm) was filled with 2.0 mL of the elution solvent; n-hexane–diethyl ether (98:2, v/v). The activated silica (1 g) was mixed with 3.0 mL of the elution solvent and poured into the column; care was taken to avoid trapping of air by tapping the column slightly to ensure uniform packing, and finally a small amount of sand was added to protect the column and the excess of the solvent was removed.

The FAMEs dissolved in 2 mL of elution solvent was loaded onto the prepared SPE column and the non-polar fraction was then eluted with 15 mL of n-hexane-diethyl ether (98:2, v/v). Subsequently the polar fraction, which contains the epoxy FAMEs, was eluted with 15 mL of n-hexane-diethyl ether (90:10, v/v) and collected in a 15 mL glass tube. The solvent was then

removed by drying under nitrogen and the resulting epoxy FAMEs was dissolved in 200 μL of isooctane for GC analysis. Separation of the fractions was confirmed by thin-layer chromatography (TLC) according to Marmesat *et al.* (2008) using a plate of silica gel 60 (5 cm × 10 cm plates, 0.25 mm thickness). The TLC plate was spotted with polar and non-polar fractions. Afterwards, it was developed with hexane–diethylether–acetic acid (80:20:1, v/v/v) and visualized with iodine vapor. Results from TLC showed clear separation between the non-polar fraction and polar fraction of FAMEs.

3.4.1.4 Gas chromatography and flame ionization detector

The polar fraction of FAMEs obtained after SPE fractionation was analyzed by gas chromatography and flame ionization detection (GC-FID) using an Agilent 6890N series gas chromatography (Agilent Technologies, USA). The samples were evaporated under nitrogen and re- dissolved in isooctane, and 0.1 μL was injected directly into the column using a cold on column injector (COC); separation was performed in a CP-Sil 88 for FAME (60 m × 0.25 mm i.d.) capillary column coated with a 0.2 μm film. A deactivated fused silica pre-column 3 m × 0.25 mm i.d. (Agilent Technologies, USA) was fitted to protect the column. The oven temperature program was set as follows: 50°C hold for 4 min, then ramp to 225°C at 12°C/min, and hold for 25 min. The flame ionization detector temperature was set at 300°C. The detector flow rates for hydrogen, air and helium (makeup) were 40, 400 and 20 mL/min respectively. The column flow rate of helium as a carrier gas was 1 mL/min. Identification of individual epoxy FAMEs was carried out by comparison of retention times with those of epoxy FAMEs mixture from C18:1, C18:2 and C18:3 synthesized and identified using GC-MS by Mubiru *et al.* (2013).

3.4.2 Determination of peroxide value

3.4.2.1 Principle

The PV value was determined using the International Dairy Federation, 1991, spectrophotometric method with modifications according to Shantha & Decker (1994), based on the ability of hydroperoxides to oxidize Fe^{2+} (ferrous) to Fe^{+3} (ferric) ions that once formed, react with ammonium thiocyanate in an acidic medium to form ferric-thiocyanate (chromophores), a red-violet complex with absorption spectra at 500-510 nm. It comprises a weighted amount of sample in a mixture of dichloromethane/methanol and addition of iron (II) chloride and ammonium thiocyanate. After a fixed reaction time, the optical density is measured.

3.4.2.2 Preparation of reagents

The iron (II) chloride stock solution was prepared under indirect, dimmed light as follows: 0.4 g of barium chloride ($BaCl_2.2H_2O$) dissolved in 50 mL of distilled water was gently mixed with a solution of 0.5 g of ferrous sulphate ($FeSO_4.7H_2O$). Then 2.0 mL of concentrated hydrochloric acid solution (10 N) was added to the resulting solution which was precipitated and filtrated until a clear liquid was obtained. The iron (II) chloride solution was poured in a brown bottle and stored until further use. The solution of ammonium thiocyanate (NH_4SCN) was prepared by weighing 30 g of NH_4SCN in a volumetric flask and diluted until 100 mL with distilled water.

Iron (III) stock solution was prepared as follows: 0.5 g of ferrous sulphate ($FeSO_4.7H_2O$) was dissolved in about 50 mL of 10 N hydrochloric acid, and then 2.0 mL of 30% (w/w) hydrogen peroxide solution was added. The excess of hydrogen peroxide was removed by boiling for 5 minutes. Afterwards the solution was cooled down at room temperature (20°C) and diluted with distilled water until 500 mL. From the previous solution 1.0 mL was transferred to a 100 mL volumetric flask and diluted up to the mark with a mixture of dichloromethane: methanol (70:30, v/v) (Fe (III) concentration 10 mg/mL).

3.4.2.3 Calibration curve for peroxide value (PV) determination

The Fe (III) standard was used to prepare a calibration curve with concentrations ranging from 1 – 40 μg of Fe^{3+}. In order to generate the chromophores, the final step comprised of the addition of 50 μL of ammonium thiocyanate to each sample. Then the samples were vortexed (2 – 3 s) and after exactly 5 minutes the absorbance was measured in a spectrophotometer at 500 nm. The solvent blank was made up by the mixture of dichloromethane: methanol (70:30 v/v).

The calibration curve was obtained in triplicates, the absorbance of samples were plotted versus Fe (III) concentrations expressed as μg Fe (III). The best fitting straight line through the points was used to calculate the slope.

3.4.2.4 Analysis of sample

A reagent blank was prepared ($E_{reagent}$ blank) as follows: 10 mL of dichloromethane: methanol (70:30, v/v) mixture were mixed with 50 μL of ammonium thiocyanate, then 50 μL of Fe (II) solution was added. This blend was mixed for 2 – 3s and the absorbance was recorded after 5 mins at 500 nm.

For the determination of PV in oil samples, 20–30 mg of oil depending on degree of oxidation was weighted in a 10 mL volumetric flask wrapped with aluminum foil, then the sample was diluted until the mark with dichloromethane: methanol (70:30, v/v). From the diluted solution 100 μL was taken and added to a test tube containing 10 mL of dichloromethane: methanol (70:30, v/v). Then 50 μL of ammonium thiocyanate were added, the mix was vortexed for few seconds and 50 μL of Fe (II) were added to trigger the reaction. Finally the absorbance was measured using a spectrophotometer after 5 min at 500 nm. All the data were recorded using a 1cm path length quartz cell.

3.4.2.5 Data calculations

The peroxide value of oils, expressed as milliequivalents/kg oil was calculated according to**:**

$$PV = \frac{(\text{Corrected absorbance - Intercept})}{55.48 \times W \times m \times 2}$$

Where:

Corrected absorbance = $E_{sample} - E_{reagent}$

m = slope of calibration curve

W = mass in grams of the sample

55.84 = atomic weight of iron

3.4.3 *p*-Anisidine value

3.4.3.1 Principle

The *p*-anisidine value is a measurement of secondary oxidation products which resulted from decomposition of fatty acid hydroperoxides. The method is based on the reaction of *p*-anisidine and aldehydes, principally 2,4-dienals and 2-alkenals. As a consequence of the aforementioned reaction a yellow-colored compound is formed which is detectable spectrophotometrically at 350 nm.

3.4.3.2 Procedure

The determination of secondary oxidation products in oxidized oils was based on AOCS (1993) method. *p*-Anisidine reagent (0.2 g) was dissolved in 100 mL of glacial acetic acid. The solution was stored at 4°C and protected from light.

The sample size was taken depending on the oil oxidation rate as follows: 0.5 g for fresh and 0.2 g for oxidized oil were weighted in a 25 mL volumetric flask previously wrapped with

aluminum foil to avoid direct exposition of sample to light. Then the sample was dissolved in isooctane and diluted to the mark.

For the spectrophotometric measurements isooctane was used as a blank. Blank was prepared by mixing 5.0 mL of isooctane and 1.0 mL of *p*-anisidine in a test tube. The mixture was vortexed and the absorbance was recorded at 350 nm exactly after 10 minutes.

A similar procedure was followed for test solution. The dissolved oil (5.0 mL) was mixed with 1.0 mL of *p*-anisidine reagent, and the absorbance was recorded at 350 nm exactly after 10 minutes of reaction.

3.4.3.3 Data calculations

All determinations including blanks readings were carried out in triplicate. The *p*-anisidine value (*p*-AV) was calculated from the expression:

$$p\text{-AV} = 25 \times (1.2A_s - A_b)/W$$

Where:

A_b = Absorbance of the solution before addition of *p*-anisidine reagent at 350 nm.

A_s= Absorbance of the solution after addition of *p*-anisidine reagent at 350 nm.

W = mass of examined oil in test solution in grams (g).

3.4.4 Microbial analysis

The growth of psychotropic bacteria in the emulsion was evaluated on the Plate count agar using the dilution plate technique at 25°C.

3.5. Statistical analysis

Analytical determinations were done in triplicates. Homogeneity of the variance was determined by Levene test. Normality was checked with Shapiro-wilk test. Statistical comparison between the results was done by one way ANOVA test at 5% level of significance. The SPSS 22 statistical package was used to process the data.

4. RESULTS AND DISCUSSION

Three sets of experiments were carried out in this study. At first, the epoxy fatty acids were analyzed in the fresh market oil samples to confirm the observations of Mubiru *et al.* (2013). The second experiment was carried out to study the stability of methyl *cis*-9,10-epoxy stearate in casein stabilized o/w emulsions at different pH levels. And the third experiment was related to the formation of epoxy fatty acids during riboflavin supported photo-oxidation in o/w emulsion at 4°C. The results of these experiments have been discussed below.

4.1 Experiment 1: comparison of the two lipid oxidation markers (epoxy fatty acids and peroxide value) in the fresh market oil samples

The C18 mono epoxy fatty acids were analyzed in the fresh market oil samples. The chromatogram of the analysis has been shown in **Figure 7**. The epoxy FAMEs mixture from C18:1, C18:2 and C18:3 (synthesized and identified by Mubiru *et al.* (2013) using GC-MS) was used as a reference for the peak identification in the chromatogram. Quantification was carried out based on the response factor of *cis*-9,10-ES with the methyl *cis*-10,11-epoxyheptadecanoate as an internal standard. All the peaks were baseline separated except the peaks of *trans*-9,10-EOL and *cis*-9,10-EOL, which were co-eluted as a single peak. The chromatogram was similar to the one observed by Mubiru *et al.* (2013).

The C18 epoxy fatty acid content of different market oil samples has been given in **Table 2**. The *cis*-9,10-ES and *trans*-9,10-ES were the epoxy fatty acids from oleic acid (C18:1), while *cis*-9,10-EO, *trans*-9,10-EO, *cis*-12,13-EO and *trans*-12,13-EO were the epoxy fatty acids from linoleic acid (C18:2).

The epoxy fatty acids were present in the varying amounts in all the samples. The *trans* isomers of epoxy fatty acids were mainly present in the refined oil samples. They were not detected in the virgin olive oils (sample 4 and 5).

The highest amount of epoxy stearate (sum of *cis* and *trans* iosmers) was found in the peanut oil (sample 11) followed by the sunflower oil samples (sample 14, 15, 16). Similarly, the highest amount of epoxy oleate (sum of *cis* and *trans* iosmers) was present in sunflower oils (sample 14, 15, 16) followed by peanuts oil (sample 11), frying oils (sample 9, 10) and mix oil (sample 12). Colza oils and olive oils contained relatively less amounts of epoxy fatty acids.

The *trans*-9,10-ES fatty acid content was higher than its *cis* isomer content in the colza oil. The percentage of *trans*-9,10-ES in relation to its *cis* isomer was around 20 % in the mix oil (sample 13). In the rest of the oil samples, the percentage of *trans* isomer content in relation to their *cis* isomer content ranged from around 1 to 15%.

It was also interesting to note that the ratio of *cis*-9,10-EO and *cis*-12,13-EO varied among the samples. The sunflower oil contained around 12 to 14 times higher amounts of *cis*-9,10-EO compared to *cis*-12,13-EO fatty acids. The formation of specific positional epoxy isomer in a higher amount might be due to the enzymatic oxidation mechanism. Similar observation was also described by Mubiru *et al.* (2013). The ratio of *cis*-9,10-EO and *cis*-12,13-EO was in the range of around 7-14 in frying oils, salad oils and mix oils possibly due to the presence of significant amounts of sunflower oil in their blend. In the peanut oil, that ratio was 4.33, while in the rest of the oils, that ratio ranged from 0.89 to 2.22.

The C18 fatty acids composition of the samples has also been given in **Table 2**. To evaluate the formation of epoxy isomers in relation to their C18 fatty acid content, the graph between molar ratio of ES to C18:1 and EO to C18:2 contents has been shown in **Figure 8**. The linearity was evaluated among the samples having similar fatty acid composition. The sunflower oil, corn oil and soybean oil were selected in one group, while rests of oils were kept in other group. In addition, the linearity was also observed between the total ES and total EO (**Figure 9**). These observations are in line with the observations reported by Mubiru *et al.* (2013).

To check the oxidation state of these fresh market oil samples, the traditional primary oxidation marker, peroxide value was additionally analyzed for these samples and the data has been given in the **Table 2**. All the samples had peroxide value (PV) below 10 meq/kg oil, which is generally the acceptable limit for the fresh market oil samples. The graph between peroxide value and the sum of EO and ES has been shown in **Figure 10**. There was no significant correlation between the peroxide value and the sum of EO and ES ($p>0.05$, $r = 0.074$, $n = 20$). Mubiru *et al.* (2013) also found no significant correlation between PV and total epoxy fatty acids ($p>0.05$, $r = -0.310$, $n = 37$).

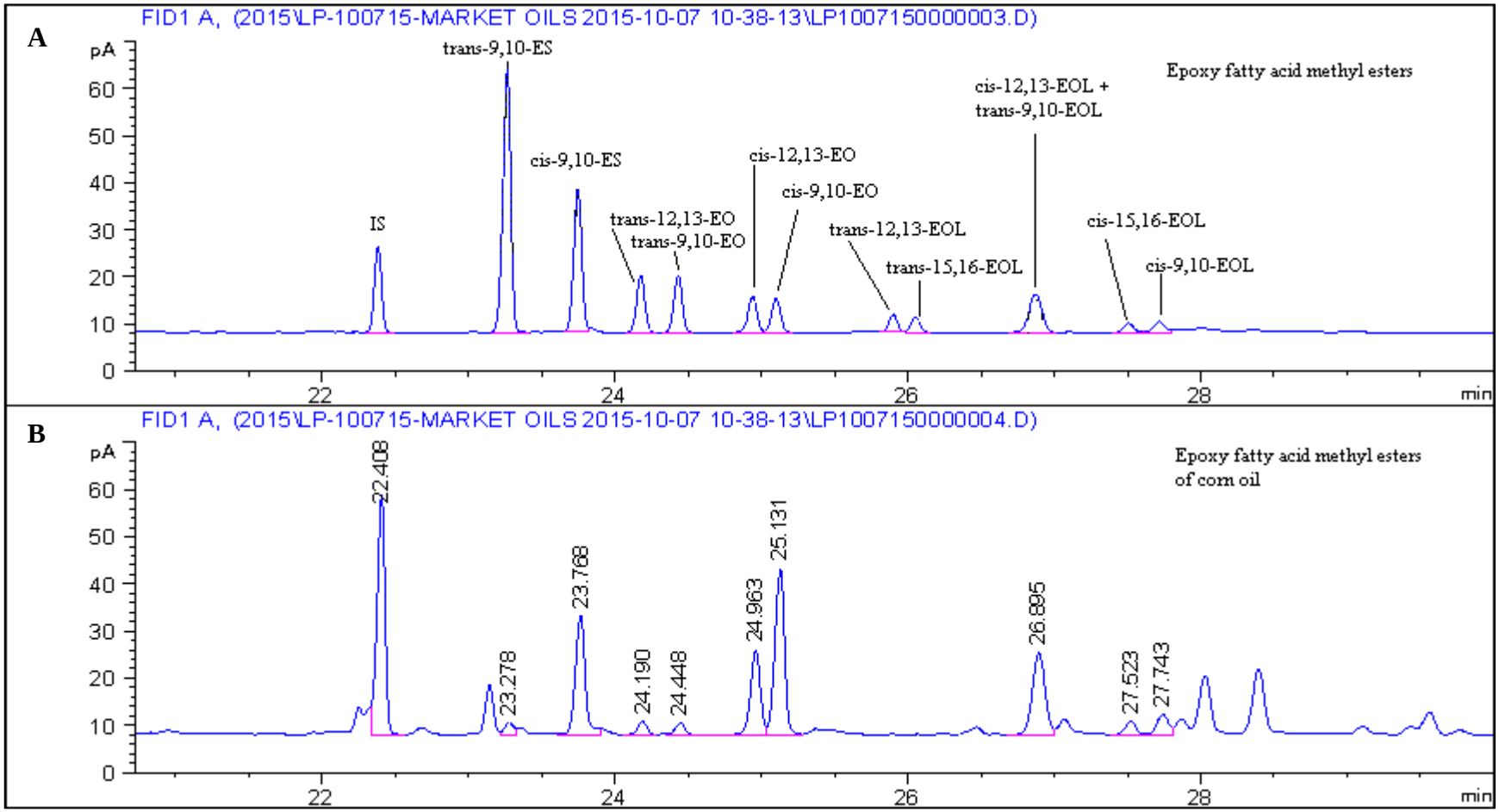


Figure 7. GC-FID Chromatograms of (A) internal standard (IS) and epoxy FAMEs mixture from C18:1, C18:2 and C18:3 identified by Mubiru *et al.* (2013) using GC-MS; and (B) polar fraction after SPE fractionation containing epoxy FAMEs from corn oil sample.

Table 2. The C18 epoxy fatty acid contents, C18 fatty acid composition and the peroxide value of 20 different market oil samples

S.N.	Samples	C18 epoxy fatty acid content (*μ*g/g oil)						Fatty acid composition (g/100g oil)			PV (meq/kg oil)
		trans-9,10-ES	*cis*-9,10-ES	*trans*-12,13-EO	*cis*-12,13-EO	*trans*-9,10-EO	*cis*-9,10-EO	C18:1	C18:2	C18:3	
1	Maize	8.64±0.24	88.25±5.64	9.64±0.26	62.84±5.48	9.35±0.10	119.19±8.57	28.39	50.76	1.06	0.31±0.03
2	Maize	9.08±0.03	248.02±4.53	11.50±1.15	164.52±1.00	11.07±1.02	365.52±0.88	25.80	49.33	0.78	0.1±0.01
3	Corn	9.70±0.78	93.52±0.77	10.40±0.87	66.77±0.11	10.06±0.33	131.88±0.48	24.98	45.51	0.80	0.43±0.01
4	Olive	ND	141.13± 4.74	ND	11.55±0.35	ND	16.34±0.38	64.27	9.19	0.67	9.72±0.55
5	Olive	ND	25.03±0.00	ND	ND	ND	ND	54.93	9.00	0.64	1.31±0.02
6	Olive	7.71±0.41	63.71±2.53	ND	7.23±0.35	ND	6.40±0.15	65.13	7.57	0.64	3.73±0.11
7	Salad oil	11.00±0.36	197.50±9.02	ND	51.76±2.49	5.51±0.44	545.87±27.60	34.67	43.62	2.44	0.73±0.02
8	Frying oil	5.71±0.06	158.64±6.93	ND	32.43±2.10	ND	454.67±9.46	30.41	40.17	0.80	2.62±0.08
9	Frying oil	23.63±1.11	276.44±2.81	ND	63.08±0.96	14.55±0.80	699.77±5.55	34.08	32.65	1.42	0.75±0.04
10	Frying oil	10.53±0.94	220.22±3.61	ND	58.25±0.91	7.76±0.14	624.95±11.27	32.87	35.62	0.80	0.64±0.03
11	Peanut oil	72.78±2.75	998.08±12.02	9.83±0.40	145.51±0.40	10.99±0.22	630.25±4.33	51.44	19.58	0.00	1.36±0.09
12	Mix oil	25.67±0.33	306.52±3.41	ND	61.16±1.86	11.51±0.74	630.32±7.12	42.80	37.64	1.27	1.26±0.12
13	Mix oil	19.20±0.81	94.80±5.18	ND	25.01±2.54	0	182.64±14.10	53.54	21.56	4.76	2.12±0.18
14	Sunflower	13.70±0.23	495.59±11.66	ND	107.36±2.37	12.50±0.22	1427.92±33.29	24.06	50.73	0.00	0.98±0.00
15	Sunflower	13.22±0.27	401.72±39.76	ND	91.17±0.71	7.67±0.06	1142.43±3.05	23.37	52.61	0.12	1.45±0.02
16	sunflower	13.40±0.28	383.93±3.08	ND	93.2±0.47	8.38±0.12	1151.82±9.29	24.50	55.08	0.15	1.89±0.06
17	Colza	ND	6.57±0.30	ND	ND	ND	ND	56.21	18.09	8.53	2.47±0.12
18	Colza	23.99±1.51	12.77±0.52	6.38±0.57	8.29±0.30	ND	8.48±0.67	54.37	16.92	7.34	2.37±0.18
19	Colza	10.48±0.91	9.78±0.87	ND	ND	ND	ND	56.50	17.22	7.17	0.29±0.12
20	Soybean	9.82±0.42	93.50±0.47	10.47±0.15	115.07±0.97	ND	203.05±0.95	21.50	45.41	5.20	2.71±0.11

ND = not detected

- Experimental data are presented as mean of three independent replicates ± standard deviation

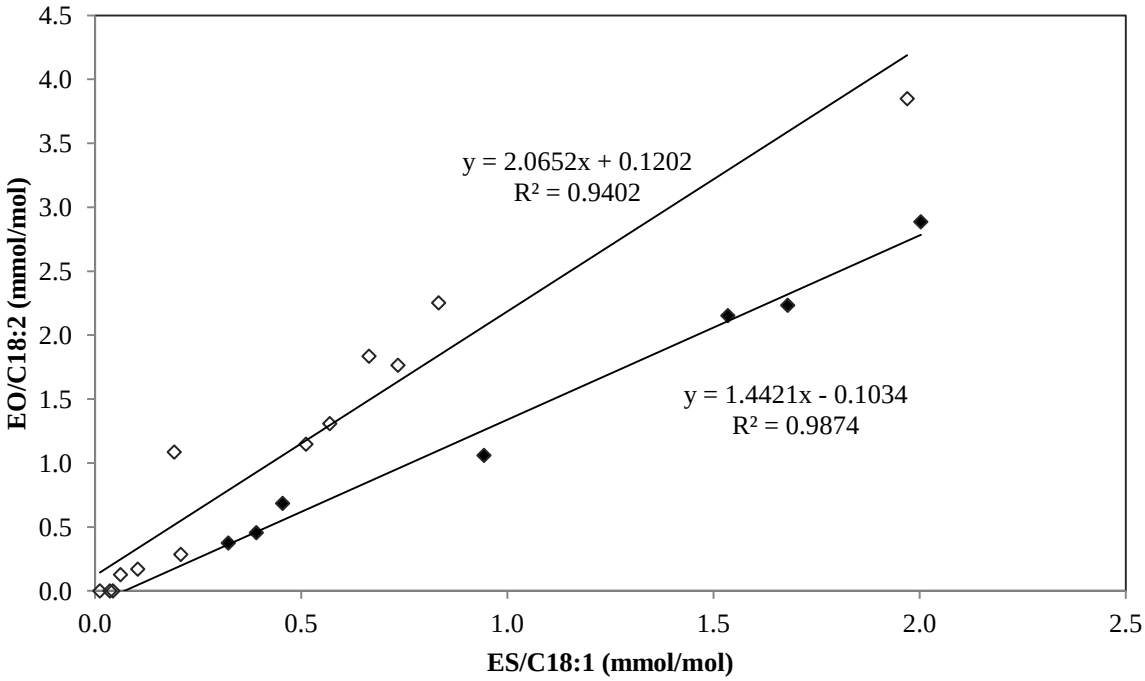


Figure 8. The graph between the molar ratio of epoxy stearate to oleic acid content and epoxy oleate to linoleic acid content of market oil samples (filled diamonds represents data of maize, soybean and sunflower oils and open diamonds represents rest of the oils)

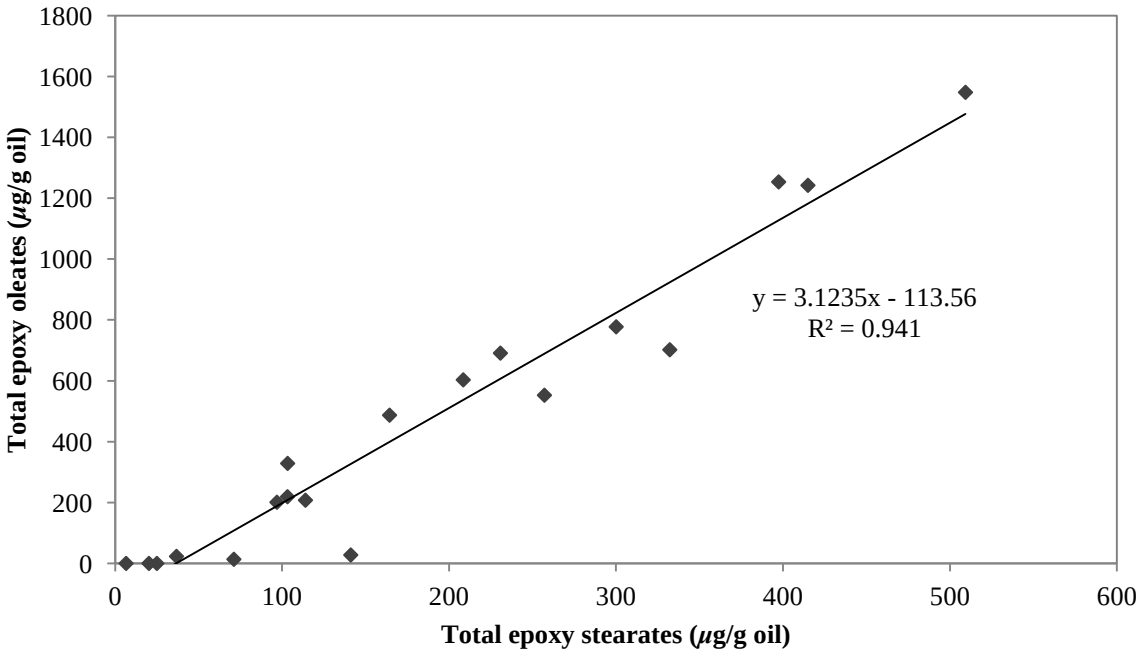


Figure 9. The graph between the total epoxy stearate and total epoxy oleate of market oil samples except that of peanut oil

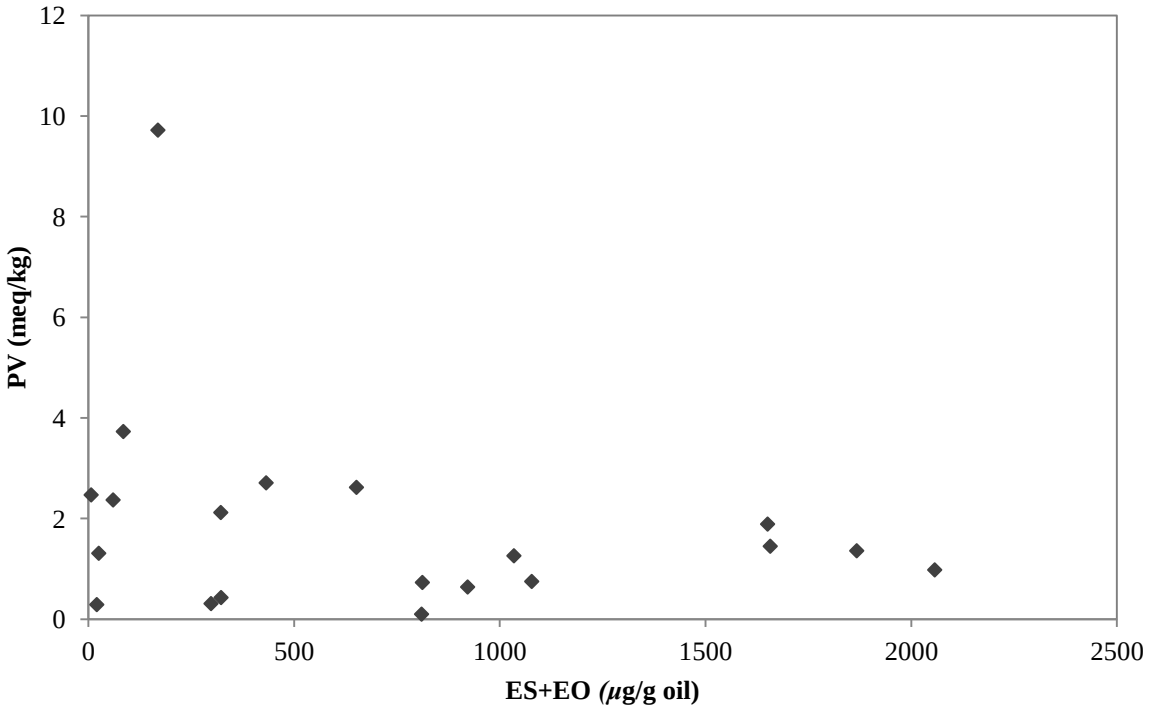


Figure 10. The graph between the peroxide value and the sum of epoxy stearate and epoxy oleate of 20 market oil samples

It is interesting to note that even though the sum of EO and ES in the oil was around 2000 μg/g of oil, the peroxide values were generally below 4 meq/kg oil. Similar observation was also made by Mubiru *et al.* (2013). It might be that lipid oxidation occurred even before the refining step. The refining process can decrease the peroxide value; however epoxy fatty acids might remain stable. Hence, it might serve as an important indicator of the oxidation history of the oil, even before the refining step.

4.2 Experiment 2: Stability of epoxy fatty acids in protein stabilized emulsion

Since the epoxy fatty acids seemed to be a potential lipid oxidation marker, it is important to understand its formation and stability in the food. Food being a complex matrix, it is better to start with a simple model system to study its formation during lipid oxidation. At first, it is necessary to confirm its stability before the study on the formation in the model system can be carried out. Therefore, this experiment was carried out in the casein stabilized o/w emulsions using saturated oil spiked with methyl-*cis*-9,10-ES. Initially, the experiment was performed using the emulsion prepared with two buffer systems, phosphate buffer at pH 7.4

and 12.0 and citrate buffer at pH 5.0 and 7.0. The two buffer systems were chosen so as to cover the wide pH range from 5.0 to 12.0. The spiked epoxy compound was monitored during storage in the dark at 25^{o}C. The stability of the spiked epoxy compound in those emulsion systems is shown in the **Figure 11**.

The spiked epoxy FAME was stable for two weeks in an o/w emulsion with the citrate buffer at pH 5.0, while its amount was reduced after two weeks at pH 7.0 in o/w emulsion prepared using that buffer. In the case of o/w emulsions prepared using phosphate buffer, the spiked epoxy FAME was stable at pH 7.4 for two weeks, while significant reduction in its amount was observed at pH 12.0 during storage for two weeks. The reduction in the spiked epoxy FAME could be due to saponification of ester to form salts of fatty acids, which is not methylated by the transesterification procedure used in the experiment. The reduction could also be due to the base catalyzed hydrolysis of oxirane ring. Similarly, free amino groups of casein can also react with epoxy compounds. At high pH, the free amino groups are deprotonated and hence could have higher reactivity with the epoxy compounds.

The spiked epoxy FAME was relatively stable in o/w emulsion in the phosphate buffer at pH 7.4 for two weeks. Since the spiked epoxy FAME is apolar in nature, it can partition more in the oil phase compared to aqueous phase of the emulsion. Therefore, it was necessary to understand if the oil could have played the protective role in decreasing its interaction with casein and aqueous phase. Hence, the stability of spiked epoxy compound was further studied in the casein solution using the phosphate buffer at pH 7.4 and 12.0 without oil (**Figure 11**). The significant reduction of the spiked epoxy FAME at pH 12.0 in the casein solution was observed within a week, which was not observed in the emulsion using the same buffer at same pH level. This supports the hypothesis that oil in the emulsion could play a protective role for the stability of the epoxy FAME. The significant reduction of the spiked epoxy FAME was not observed even in the casein solution using phosphate buffer at pH 7.4 for two weeks, even though the protective effect of oil was removed. Therefore, the reaction between casein and epoxy compound at pH 7.4 in a phosphate buffer is minimal. In addition, the oil could also play a protective role for the epoxy FAMEs in the emulsion. Hence, the casein stabilized emulsion system using phosphate buffer at pH 7.4 was chosen to further study the formation of epoxy fatty acids in the triglycerides during the riboflavin supported photo-oxidation of sunflower oil in the next experiment.

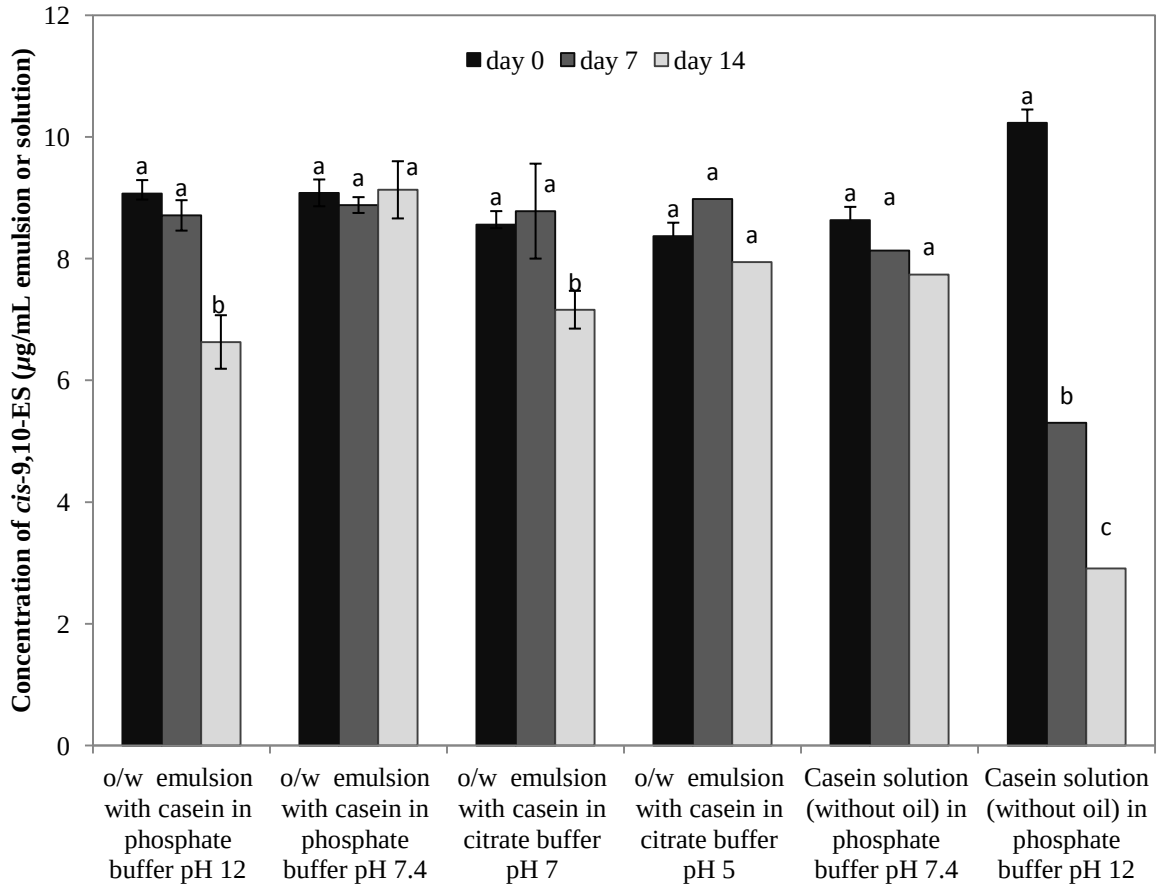


Figure 11. The stability of methyl *cis*-9,10-epoxyoctadecanoate (*cis*-9,10-ES) in different model conditions during storage in the dark at 25°C.

4.3 Experiment 3: Formation of C18 epoxy fatty acids in oil in water emulsion during riboflavin supported photo-oxidation

This experiment was carried out to study the formation of epoxy fatty acids in sunflower oil during riboflavin supported photo-oxidation in casein stabilized o/w emulsion using phosphate buffer at pH 7.4, stored at 4^{o}C. Initially, the peroxide value and *p*-anisidine value of sunflower oil was found to be 2.5 meq/kg and 5.1 respectively. The control samples were kept at same temperature under dark. The oil was extracted from the emulsion and C18 epoxy fatty acids were analyzed in GC-FID after transesterfication and SPE fractionation.

The formation of different isomers of epoxy fatty acids during storage in light and dark conditions are shown in **Figure 12** (data has been provided in **Appendix 1**). The formation of *cis*-9,10-ES was comparable in the samples stored under both light and dark conditions. The *trans*-9,10-ES was formed during photo-oxidation, while its formation was not significant in the control samples kept in dark.

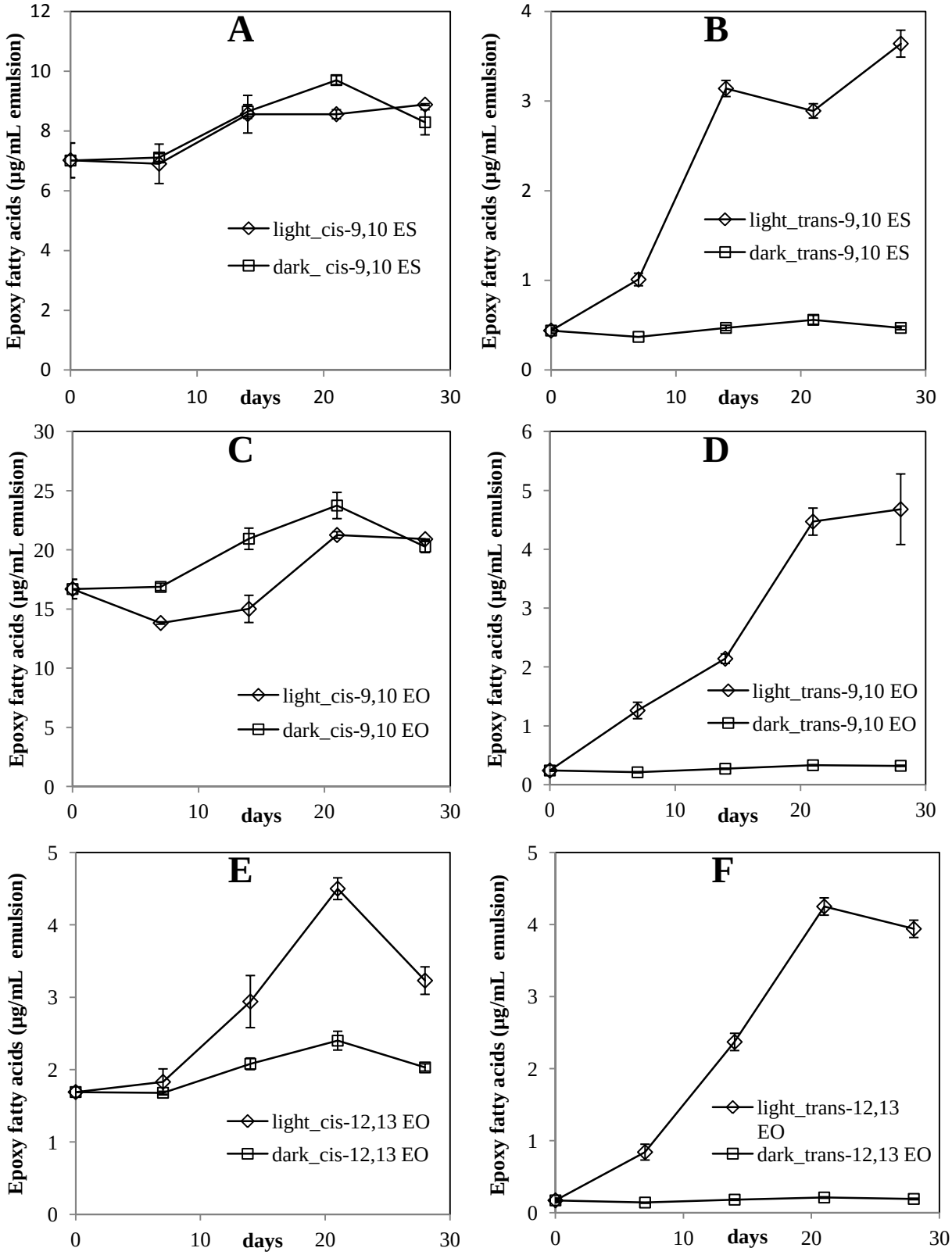


Figure 12. Formation of different epoxy fatty acids ((A) *cis* -9,10-ES; (B) *trans*-9,10-ES; (C) *cis*-9,10-EO; (D) *trans*-9,10-EO; (E) *cis*-12,13-EO; (F) *trans*-12,13-EO) during storage of o/w emulsion with casein (6 mg/mL) and riboflavin (2 μg/mL) at 4^{o}C. The open diamonds represent storage at light and open squares represent storage at dark.

Similarly, *cis*-9,10-EO was formed in the samples stored under both light and dark conditions. However, the *trans*-9,10-EO was formed only in the samples stored under light. Moreover, the formation of *cis*-12,13-EO was higher in the samples stored under light compared to the control samples stored under dark conditions. The formation of *trans*-12,13-EO was only observed in the samples stored under light.

In general, the epoxy fatty acids were formed till the 21 days of storage. Therefore, 21 days was taken as the reference point for the comparison of the formation of different isomers of epoxy fatty acids. The differences in the concentration of each isomer between 21 day and 0 day were calculated and the values were converted in μmol/mol of fatty acids, using the fatty acid composition data of sunflower oil and has been shown in **Table 3**.

Table 3. Formation of different epoxy fatty acids during storage of o/w emulsion with casein (6 mg/mL) and riboflavin (2 μg/mL) at 4^{o}C for 21 days (difference in the concentration between 21 days and 0 day) expressed in μmol/mol of fatty acids

Storage conditions	Difference in the epoxy fatty acid content between 21 days and 0 day					
	***trans*-9,10-ES (μmol/mol of C18:1)**	***cis*-9,10-ES (μmol/mol of C18:1)**	***trans*-12,13-EO (μmol/mol of C18:2)**	***cis*-12,13-EO (μmol/mol of C18:2)**	***trans*-9,10-EO (μmol/mol of C18:2)**	***cis*-9,10-EO (μmol/mol of C18:2)**
light	3.31	2.08	2.45	1.68	2.54	2.74
dark	0.16	3.63	0.02	0.43	0.05	4.23

The tabulated data supports the above observation that *trans* isomers were formed in significant quantity during photo-oxidation compared to autoxidation in the control samples. This observation could be due to the differences in the mechanism of oxidation. The autoxidation of lipids results via radical mediated oxidation mechanism, while photo-oxidation results via the formation of different reactive oxygen species such as singlet oxygen which is highly reactive and can react with lipid molecules directly without the formation of radicals that can generate different oxidation products compared to that during autoxidation. Further confirmation of the results and the mechanistic study to understand the differences in the mechanisms of the formation needs to be carried out in future.

In total, the formation of total epoxy stearate was 5.4 and 3.8 μmol/mol of C18:1 in the samples stored under light and dark conditions, respectively. Similarly, the formation of total

epoxy oleates was 9.4 and 4.7 μmol/mol of C18:2 in the samples stored under light and dark conditions, respectively. In general, the epoxy fatty acids from linoleic acids were formed in the higher molar ratios compared to that from oleic acids, which could be due to the fact that linoleic acid is more prone to oxidation compared to oleic acid (Damodaran *et al.*, 2007). Moreover, the formation of epoxy fatty acids was higher in the photo-oxidised samples compared to that in the control samples. Lee & Decker (2011) also found that riboflavin photosensitization accelerated the oxidation of lipids in o/w emulsion. Several other scientists have reported that riboflavin photosensitization increases the rate of lipid oxidation in free fatty acids, oil systems and dairy foods including milk and cheese (Kim & Lee, 2003; Lee & Chang, 2010; Lee & Min, 2009a, 2009b, 2010).

5. CONCLUSIONS

Epoxy fatty acid contents of different market oil samples were analyzed to confirm the observations of Mubiru *et al.* (2013). The market oil samples contained significant amounts of epoxy fatty acids (6.6 to 2057.1 μg/g oil), despite having low peroxide value. This result confirmed the observations made by Mubiru *et al.* (2013) and showed the possibility of epoxy fatty acid content as a marker of the history of lipid oxidation. Therefore, the research was planned to study the formation of epoxy fatty acids during lipid oxidation. At first, the stability of epoxy fatty acids in the different model solutions was studied. It was concluded that the epoxy fatty acids could be stable for around two weeks in the casein stabilized o/w emulsion system using phosphate buffer at pH 7.4, stored at 25^{o}C in the dark. Hence, same model system was chosen for the study on the formation of the epoxy fatty acids during riboflavin supported photo-oxidation at 4^{o}C, with the control samples stored in dark condition. The epoxy fatty acids from linoleic acids were formed in the higher molar ratios compared to that from oleic acids. It was also observed that different isomers could be formed during photo-oxidation compared to autoxidation. The *trans* isomers of the epoxy fatty acids were formed during photo-oxidation in comparison to autoxidation. Further confirmation of the results and the mechanistic study to understand the differences in the mechanisms of the formation needs to be carried out in future.

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APPENDIX

Appendix 1. Formation of different epoxy fatty acids from sunflower oil during storage of o/w emulsion with casein (6 mg/mL) and riboflavin (2 μg/mL) at 4^{o}C under light and dark conditions.

Condition	Time (days)	Epoxy fatty acids content (μg/mL of emulsion)					
		***trans*-9,10-ES**	***cis*-9,10-ES**	***trans*-12,13-EO**	***trans*-9,10-EO**	***cis*-12,13-EO**	***cis*-9,10-EO**
Dark	0	0.44±0.03^{ab}	7.01±0.58^{a}	0.17±0.03^{b}	0.24±0.01^{b}	1.69±0.07^{a}	16.69±0.82^{a}
	7	0.37±0.00^{a}	7.11±0.13^{a}	0.14±0.00^{a}	0.21±0.01^{a}	1.68±0.02^{a}	16.87±0.31^{a}
	14	0.47±0.03^{b}	8.65±0.23^{b}	0.18±0.01^{bc}	0.27±0.01^{b}	2.08±0.08^{b}	20.94±0.90^{b}
	21	0.56±005^{c}	9.70±0.15^{c}	0.21±0.01^{c}	0.33±0.01^{c}	2.40±0.13^{c}	23.75±1.11^{c}
	28	0.47±0.02^{b}	8.29±0.42^{b}	0.19±0.01^{bc}	0.32±0.01^{c}	2.03±0.05^{b}	20.27±0.50^{b}
Light	0	0.44±0.03^{a}	7.02±0.58^{a}	0.17±0.03^{a}	0.24±0.01^{a}	1.69±0.07^{a}	16.69±0.82^{b}
	7	1.01±0.07^{b}	6.90±0.66^{a}	0.84±0.11^{b}	1.26±0.14^{b}	1.83±0.18^{a}	13.82±0.10^{a}
	14	3.14±0.09^{c}	8.56±0.63^{b}	2.37±0.12^{bc}	2.14±0.08^{c}	2.94±0.36^{c}	15.01±1.15^{c}
	21	2.89±0.08^{d}	8.56±0.15^{b}	4.25±0.12^{bc}	4.47±0.23^{d}	4.50±0.15^{c}	21.26±0.28^{c}
	28	3.64±0.15^{e}	8.88±0.03^{b}	3.94±0.12^{c}	4.68±0.60^{d}	3.23±0.19^{b}	20.91±0.01^{c}

- Experimental data are presented as mean of three independent replicates ± standard deviation.
- Values with different alphabets in the superscript in the same column under same condition are significantly different (p-value<0.05)