A dedication to all my Family members

"Someone is sitting in the shade today because someone planted a tree a long time ago".

Warren Buffett

EPOXY FATTY ACIDS IN FOODS: ANALYTICS, FORMATION AND RISK ASSESSMENT

Thesis submitted in fulfillment of the requirements for the Degree of Doctor (PhD) in Applied Biological Sciences

Promoter: **Prof. dr. ir. Bruno De Meulenaer**
Food Technology, Safety and Health
Faculty of Bioscience Engineering, Ghent University

Examination committee:

Chairman: **Prof. dr. Ann Dumoulin**
Department of Green Chemistry and Technology
Faculty of Bioscience Engineering, Ghent University

Secretary: **Prof. dr. ir. Stefaan De Smet**
Department of Animal Sciences and Aquatic Ecology
Faculty of Bioscience Engineering, Ghent University

Prof. dr. Charlotte Jacobsen
National Food Institute, Technical University of Denmark

Prof. dr. ir. Koen Dewettinck
Food Technology, Safety and Health
Faculty of Bioscience Engineering, Ghent University

Prof. em. dr.ir. Roland Verhé
Food Technology, Safety and Health
Faculty of Bioscience Engineering, Ghent University

Dean: **Prof. dr. ir. Marc Van Meirvenne**

Rector: **Prof. dr. ir. Rik Van de Walle**

Dutch translation of the title:

EPOXYVETZUREN IN VOEDINGSMIDDELEN: ANALYTIEK, DE VORMING EN RISICOBEOORDELING

Printed by: University Press BVBA

To refer to this thesis:

Mubiru, E. (2018). Epoxy fatty acids in foods: Analytics, formation and risk assessment. PhD dissertation, Faculty of Bioscience Engineering, Ghent University, Belgium.

ISBN: 978-94-6357-104-3

MSc Edward Mubiru was financially supported by Geconcerteerde Onderzoeksacties/ Concerted Research Actions (GOA), Ghent University.

This research was performed in the laboratory of the Research Group Food Chemistry and Human Nutrition, Department of Food Technology, Safety and Health, Faculty of Bioscience Engineering, Ghent University.

ACKNOWLEDGEMENTS

The completion of my research work has been a very long journey. It would not have been possible without the help and support of many people, colleagues, friends and family. To my promoter, Bruno (as he prefers to be called), I thank you for giving me the opportunity to do a PhD. Guiding me through even the simplest of concepts surely has seemed like a struggle at times, but I will forever be grateful for your persistence and all you have helped me learn. Thank you for being patient with me all the time. You are one of the most passionate and serious educators I have met. I look forward to further engagements in your future research. Your patience with this new direction in life and your ability to help me reason through things when I thought all rational thought had abandoned me have made this whole crazy endeavor possible.

I would like to thank the members of the *examination committee* for their time, evaluation and constructive comments. Thank you both for your constructive suggestions and for taking the time from your extremely full schedules to assist me. Your advice improved not just my thesis but also my understanding of my topic.

I acknowledge the funding of Ghent University through the Geconcerteerde Onderzoeksacties /Concerted Research Actions (GOA) project, which has made my PhD work possible.

I thank Prof. Noble Banadda through his networks I was able link with Professor. dr. ir. Bruno De Meulenaer and hence secure this scholarship.

During my stay at the faculty I met nice people: *Bao, Mery (it is not working), Monica, Barbara, Gustavo, Bea, Evelien, Helena, Laurence, Bryan, Charlotte, Tania, Sofie, Imen, Raquel, Angelique, Kimberley, Nanou, Evelien and Evelien, Agnieszka, Dries, Pratheeba, Johana, Dr Amr, Phoung, Karin, Kathy, Bea, Mansonia, Senem, Nathalie, Laurent and the* other colleagues in the laboratory, thank you very much for your friendship and the joyful times at work and social activities that we shared. *Kshitij* (Edward it is easy) and *Antonios*, my other 'promotors' other than *Bruno*, you made all this possible and I am glad I worked with you. Thank you for the great contribution on the quality of this work. You have enriched my experience in this program far beyond lab work.

Anne, thank you for the administrative work you have done, starting with the paper work to enable me travel from Africa, believe me you have done a lot. *Dieter* thank you for fixing my computer problems always even when I was in Uganda I would ask you and you respond promptly.

Liesbeth and *Carl* thank you for your contribution to the exposure assessment study and the publication that we got out of it. *My other friends Ronnie, Caroline, Aisha, De Jonckheere Frank, Dr Bonamie, Jessica Nannyunja, Brenda, Anneke, Haliima, Dave, Laz* and other Ugandans in Ghent thank you for the orientation into the European setting.

I would like to thank my students: *Ziggy*, *Tamara* and *Rubel* who contributed to this thesis as well. I thank Makerere University for its support and especially my colleagues Prof. M. Ntale, Prof. JYT Mugisha and Dr J. Wasswa, thank you so much.

I would also like to acknowledge my family and especially my parents for the endless support they have provided me throughout my life. All those people who I have not mentioned and yet you contributed to the success of this work, May God reward you all.

Finally, I would like to thank my family especially Prossie my wife and my children, for the endless support. Thank you for supporting my decision of going abroad even when it was hard for you to handle the family. To my brother in law Mr Mulindwa Frank and my sister Resty thank you for taking care of my family during my absence. Thank you and may God bless you all.

Mwebale nnyo, Mukama abaweere.

Above all, I thank the Almighty God, for enabling me achieve this prestigious award.

Edward Mubiru

June 8th, 2018
Ghent, Belgium

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADI	average daily intake
BMD	Benchmark Dose
BNFCS	Belgian National Food Consumption Survey
CD	conjugated diene
CT	conjugated triene
CV	coefficient of variation
EFA	epoxy fatty acid
FA	fatty acid
FAME	fatty acid methyl esters
GC-FID	gas chromatography- flame ionization detector
GC-MS	gas chromatography–mass spectrometry
IS	internal standard
LOD	limit of detection
LOQ	limit of quantitation
MOE	margin of exposure
MSD	mass selective detector
MSM	multiple source method
TBME	tert-butyl methyl ether
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
PDF	probability density functions
PTV	programmed temperature vaporizing
PUFAs	poly unsaturated fatty acids
PV	peroxide value
SPE	solid phase extraction
StDev	standard deviation
TDI	tolerable daily intake
TTC	threshold of toxicological concern

INTRODUCTION AND OBJECTIVES

Lipids, when exposed to air, become rancid and the known reaction responsible for this is oxidative degradation. Lipid oxidation mainly occurs in unsaturated fatty acids. On the other hand, use of unsaturated fatty acid oils has gained much attention with a view of their added health benefits. These oils are more prone to lipid oxidation; thus, a thorough understanding of lipid oxidation especially secondary lipid oxidation products formation is important. The reaction proceeds by production of hydroperoxides which are the primary products of lipid oxidation. They are relatively unstable such that they enter into numerous complex reactions involving substrate degradation and interaction, resulting in several compounds some of which have off-flavours at low thresholds and are toxic.

Among the secondary oxidation products are the epoxy fatty acids (EFAs). These contain at least one epoxide group and have typically one double bound less compared to the parent fatty acid, while the number of carbon atoms is retained. Their natural occurrence in some seed oils is reported, although their biological function is not known. It is hypothesized that enzymatic processes in these plants are responsible for their biosynthesis. The formation of EFAs in foods has previously been shown in oil, but especially during high temperature frying. Remarkably, apart from those early reports, very limited information is available with respect to the formation of EFAs in foods via lipid oxidation. Similarly, limited data on the concentration levels at which this group of underinvestigated secondary lipid oxidation compounds occur in foods are available. **Therefore, the main goal of this PhD was to explore these less known lipid oxidation products focusing on their analytics, occurrence, formation, reactivity and potential food safety risks.**

EFAs were identified by Gunstone in 1954 and research in this area has been ongoing. There have been reports of natural occurrence of these compounds in many seed oils but the known route of formation is through lipid oxidation. It was observed that there is a lot of old literature on EFA research and recent research seems to be missing. Therefore, in **the first chapter**, a review of the available scientific literature of full research papers published in peer reviewed journals so as to show the current knowledge and also point out the missing links in this important area of lipid chemistry was done (**chapter 1**).

During this review of literature, it was observed that the analysis of EFAs was challenging. It was complicated due to the presence of many structurally similar fatty acids which are polar, thus individual separation was sometimes impossible. Although gas chromatography flame ionization detector quantification methods were available, they were not well optimized to analyze EFAs in less oxidized oils like the fresh ones. On the other hand, no method was available to analyze EFAs in food matrices. This lead to our **first objective**, to develop and optimize a method for analysis of EFAs in oils (**chapter 2)** and another method to analyze EFAs in food matrices (**chapter 3**) since these two matrices require different techniques for the analyte extraction.

Toxicity of some EFAs has been documented in different systems, although not enough data is available for other EFA isomers. Because of this we cannot estimate toxicity correctly however, we can extrapolate that the other EFAs exhibit similar toxicity. Despite the lack of enough toxicity data, concerns about the impact of the presence of these potentially reactive epoxy compounds on human health are raised. As no risk assessment with respect to the presence of these compounds has been done to date, our **second objective** was to do a risk assessment of these fatty acids in foods on the Belgian market (**chapter 4**).

To date no research has been done about the behavior of these compounds in foods which is important if we must know the impact of EFAs in human bodies. This lead to our **third objective** of mechanistic studies to follow the formation of these compounds in different food system models. Since literature was missing in this area, the best system to begin with was the simplest model of bulk oil. Oils of different fatty acid composition were subjected to different oxidation conditions and the formation of EFAs was followed with time (**chapter 5**). Due to the nucleophilic character and the strained geometry of the epoxide ring, it is known that epoxides show a relatively high reactivity towards a variety of nucleophiles. In foods, proteins are suitable nucleophiles, which moreover can be present in the interface between the aqueous and lipid fraction of emulsified food systems. It can be hypothesized that in this interface the produced epoxy fatty acids might (partially) react with the nucleophilic amino acid residues present in the protein. Such potential reactions are relevant with respect to i) the use of EFAs as indicators for lipid oxidation, ii) the potential loss of protein bioavailability and iii) the potential toxicity of EFAs. To find out possible interactions

of EFAs in foods, their stability was evaluated in emulsions formed with whey and casein proteins and soybean oil (**chapter 6**).

Finally, **Chapter 7** presents the general discussions and conclusion, as well as the future perspectives in the analysis of EFAs and the gaps in our current knowledge in understanding EFAs formation and interactions with proteins.

The general scheme of this PhD study is shown in **Figure I**.

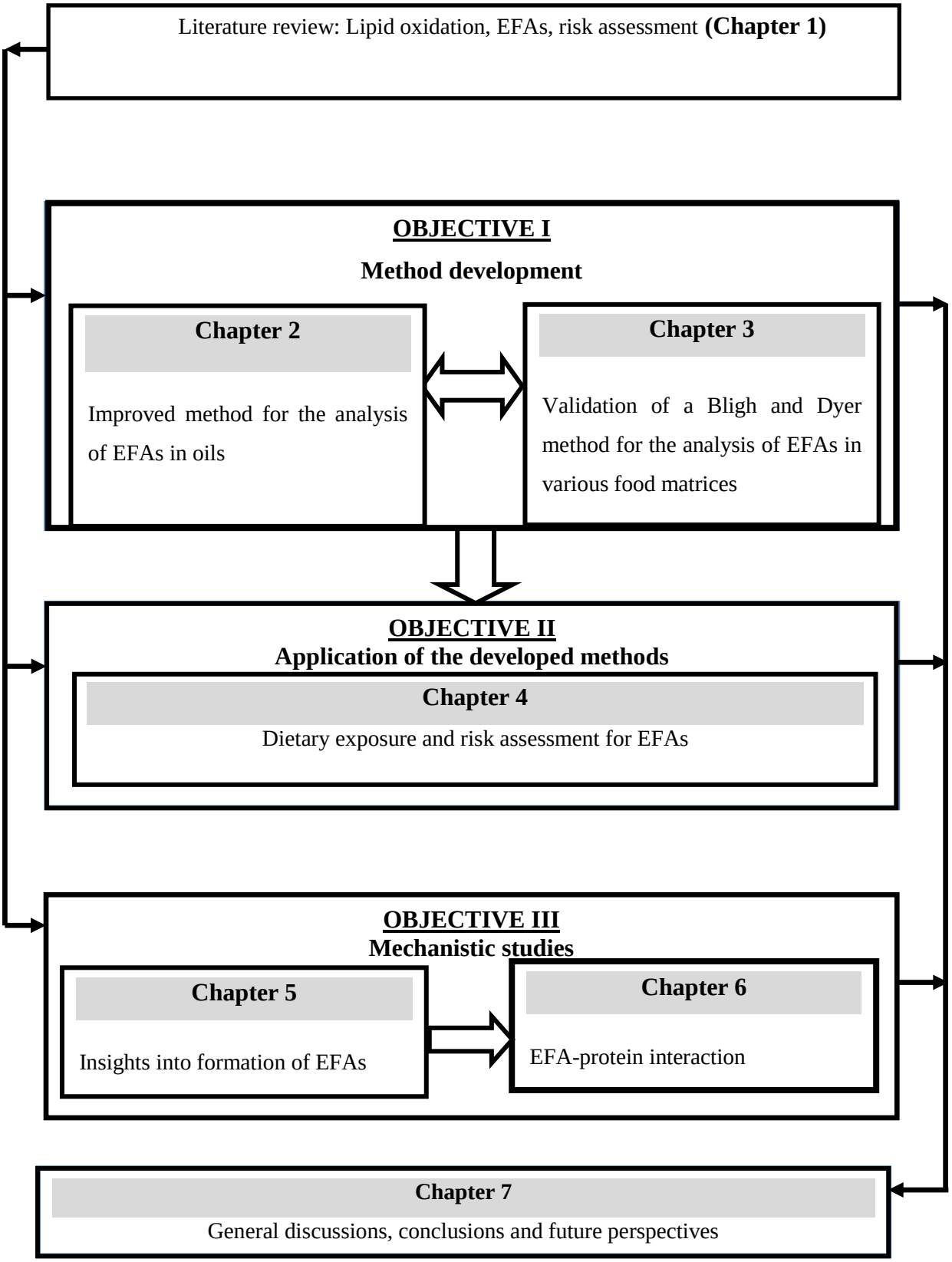


Figure 1: Schematic outline of the objectives of the PhD study

SUMMARY

This PhD thesis contributes to lipid chemistry in general and specifically to the chemistry of EFAs through, (1) developing robust analytical methods, (2) a risk assessment study due to the presence of EFAs in foods and (3) elucidating the formation and interaction of EFAs.

In **chapter 1, in a** literature review**,** a short background to EFAs is presented and a review on the classical mechanism of lipid oxidation is given plus the different factors affecting lipid oxidation have been explained. In the first part of **chapter 1**, an extensive literature review has been done and most of the recent advancements in this area of EFAs are discussed. The different gaps in the knowledge and especially in the analysis of EFAs were identified. The problem of coelution of EFAs during GC analysis was the main source of hindrance to research in this area. Finally, in the last part, an overview of the risk assessment procedure has been presented including the risk characterization using the Threshold of Toxicological Concern (TTC) concept.

Chapter 2, includes the development of a gas chromatography flame ionization detector (GC-FID) analytical method. The method was specifically developed to analyse for C18 EFAs in oil samples and it used a fatty acid methyl ester (FAME, C19:0) as an internal standard. The compounds were separated on a polar capillary column. This method was based on pre-separation of the fatty acids into polar and non-polar fraction and later partition the polar fraction by SPE to obtain the EFA fraction and the hydroxy-oxo fraction. The method was applied to fresh vegetable oils and it was seen to be accurate and repeatable. Regarding this method, it should be noted that because it uses a polar column and the analytes of interest are polar, use of a pre-column is important to guard the column and prolong its capacity.

Chapter 3 presents yet another method which was specifically developed to analyse C18 EFAs in food matrices based on the Bligh and Dyer oil extraction method. The method had an innovation in that instead of using a FAME as internal standard (IS), an epoxy FAME was synthesized by epoxidation reaction and used instead. This decreased the uncertainties in the analytical procedure because the IS could be introduced immediately at the start of the analysis. The method was validated in four food matrices, vegetable oils, potato chips,

unprocessed meat and the milk powder and low limits of detection were obtained at 2.8, 10.2, 5.2 and 1.7 µg g^{-1} of sample respectively.

In chapter 4, results of the analysis of the concentrations of EFAs in seventeen food categories (total samples = 390) available on the Belgian market are presented. By combining this information with the consumption data obtained from the Belgian national food consumption survey 2004 (BNFCS) of the Belgian population, a quantitative exposure assessment was performed. An evaluation of any potential risk related to the intake of the EFAs for the consumers of the specific food categories analyzed was made by applying the Threshold of Toxicological Concern (TTC) concept. Consumption of mayonnaise, butter-margarine and ready to eat meals was found to contribute the most to the intake of EFAs. The lowest contribution to EFA intake was from consumption of cooked meat, smoked salmon and raw cured ham. It should be emphasized that a risk may be probable because of consumption of fourteen food groups out of those analyzed.

A study to get insights into the formation of EFAs is presented in **chapter 5**. Therefore, three common unsaturated fatty acids (oleic (18:1), linoleic (18:2) and linolenic (18:3)), present in stripped oils were subjected to three different oxidation mechanisms: oxidation in the dark (autoxidation at 6°C), accelerated autoxidation (at 70°C) and oxidation under illumination (photooxidation at 6°C). These fatty acids were represented by olive oil, sunflower oil and linseed oil respectively. Because photooxidation requires a photosensitizer and yet the price of chlorophyll is prohibitive, the different oils were blended with virgin olive oil at a fixed ratio to obtain chlorophyll. This however necessitated control blends without chlorophyll but which had the same fatty acids composition. This was achieved by blending the same oils with refined olive oil. Results indicated that EFAs form in high amounts especially in these oils since they had low stability because of stripping. There was a *cis* isomers preference before the study which continually changed to the *trans* isomers with storage especially in the control blends. However, there was a faster decrease in the *cis* isomers during accelerated autoxidation probably implying heat induced isomerisation.

Chapter 6 investigated possible interactions between EFAs with casein and whey proteins. Soybean oil at three oxidation levels (fresh, medium and highly oxidized) was used to prepare emulsions. Whey and casein proteins were used as the proteins in this study to investigate any possible interaction. The emulsions were incubated at 5°C for 24 hrs. and

were analysed for EFA content. In another experiment setup, pure EFA analyte (*cis*-9,10-epoxystearate) was spiked into a C7-triglyceride (triheptanoin) and emulsions were made in the same way, incubated also for 24 hrs. at 5°C and analysed for the EFA content. Preliminary results did not show high reactivity that was expected, however some interaction appeared to have occurred.

The general discussions, conclusions and future perspectives have been discussed in **chapter 7**.

SAMENVATTING

Deze doctoraatsthesis draagt bij tot de algemene vetzuurchemie en meer specifiek tot deze van de epoxyvetzuren door (1) de ontwikkeling van een robuust analytische methode (2) een risicobeoordeling op de aanwezigheid van epoxyvetzuren in voedingsmiddelen en (3) het ophelderen van de vorming en de interactie van epoxyvetzuren in voedingsmiddelen.

Hoofdstuk 1 omvat een korte achtergrond over epoxyvetzuren en beoordeelt door middel van een review de klassieke vetzuuroxidatie mechanismen. Vervolgens worden de verschillende factoren die een invloed hebben op de vetzuuroxidatie toegelicht. Als laatste wordt een overzicht gegeven van de risicobeoordeling inclusief de karakterisering van het risico met behulp van het toxicologische drempelwaarde (TTC) concept. In het eerste deel van **hoofdstuk 1** wordt een grondige literatuurstudie uitgevoerd waarin onder andere de meest recente ontwikkelingen omtrent de epoxyvetzuren besproken wordt. Het tweede deel beschrijft de verschillende gaten in de kennis, meer specifiek in de analytiek inzake de epoxyvetzuren. Tijdens de GC methode co-eluren de epoxyvetzuren, de grootste hindernis inzake de analytiek binnen dit vakgebied.

Hoofdstuk 2 beschrijft de ontwikkeling van een gaschromatografie vlamionisatiedetector (GC-FID) analytische methode. De methode is speciaal ontwikkeld voor de analyse van epoxyvetzuren aanwezig in olie en gebaseerd op de scheiding van de vetzuren in een polaire en niet polaire fractie. De methode maakt gebruik van een vetzuurmethylester (FAME, C19:0) als een interne standaard. De componenten worden middels een polaire capillaire kolom van elkaar gescheiden. Voor de gaschromatografische scheiding is het noodzakelijk dat de polaire fractie van de olie wordt opgezuiverd via SPE om de epoxyvetzuren te scheiden van de hydroxy- en oxo-fractie. De methode werd toegepast op verse plantaardige oliën en bleek accuraat en herhaalbaar te zijn. Door het gebruik van een polaire kolom en de polaire karaktereigenschappen van de componenten is het gebruik van een pre-kolom noodzakelijk om de levensduur en de kwaliteit van de kolom te bewaken.

Hoofdstuk 3 beschrijft de ontwikkeling van een andere methode die speciaal voor epoxyvetzuren in voedingsmiddelen ontwikkeld werd op basis van de Bligh & Dyer olie extractie methode. De innovatie in deze methode is het gebruik van een epoxy FAME in plaats van de standaard FAME als interne standaard. Deze interne standaard werd

gesynthetiseerd met behulp van een epoxidatie reactie. Door het gebruik van een epoxy FAME als interne standaard daalde de onzekerheden gedurende de analytische procedure. De methode werd gevalideerd in vier voedselmatrices: plantaardige oliën, chips, onverwerkt vlees en melk poeder. De verkregen detectielimieten waren zeer laag en bedroegen respectievelijk 2.8, 10.2, 5.2 en 1.7 µg g^{-1} monster.

In **hoofdstuk 4** worden de concentraties van epoxyvetzuren in zeventien voedselcategorieën (totaal aantal stalen = 390) beschikbaar op de Belgische markt beschreven. Door deze resultaten te combineren met de verbruikersgegevens van de "Belgian national food consumption survey 2004" (BNFCS) kon een kwantitatieve blootstellingsanalyse uitgevoerd worden. Daaraan gekoppeld werd een evaluatie gedaan met behulp van het 'Threshold of Teoxicological Concern' (TTC) concept van de potentiële risico's voor de consumenten bij het innemen van epoxyvetzuren in gespecifieerde voedselcategorieën. Om deze informatie te kunnen gebruiken voor de gehele populatie werd gebruik gemaakt van het 'IF scenario' als consumptie data. Consumptie van mayonaise, boter, margarine en kant-en-klare maaltijden bleek bij te dragen tot de hoogste inname van epoxyvetzuren terwijl de laagste epoxy waarden terug gevonden werden in gekookte vleeswaren, gerookte zalm en rauwe ham. Bij de inname van de veertien andere voedselcategorieën is de kans op inname van epoxyvetzuren zeer waarschijnlijk.

Hoofdstuk 5 beschrijft de studie waarin de vorming van epoxyvetzuren onderzocht werd. In deze studie werden de mechanismen auto-oxidatie, 'versnelde' auto-oxidatie en foto-oxidatie gevolgd voor de drie veel voorkomende onverzadigde vetzuren; oliezuur (C18:1), linolzuur (C18:2) en linoleenzuur (C18: 3) afkomstig uit gestripte oliën. Deze vetzuren werden vertegenwoordigd door respectievelijk olijfolie, zonnebloemolie en lijnzaadolie. Als fotosensitizer werd extra vierge olijfolie in een vaste verhouding toegevoegd, omdat zuiver chlorofiel zeer duur is. Hierdoor werden controlestalen meegenomen met een zelfde hoeveelheid olijfolie, waaruit het chlorofiel verwijderd werd. Resultaten toonden een sterke epoxyvetzuurvorming aan in deze oliën aangezien deze een lage stabiliteit hebben door de 'stripping' van deze oliën. Er was een *cis* isomeren voorkeur voor de studie die voortdurend verandert in de *trans* isomeren met opslag, vooral in de controle mengsels. Er was echter wel een verlaging aan cis-isomeren te vinden gedurende de auto-oxidatie, mogelijk afkomstig door temperatuur geïnduceerde isomerisatie.

In **hoofdstuk 6** werd de mogelijke interactie tussen de epoxyvetzuren met caseïne-en wei-eiwitten onderzocht. Soja olie met drie verschillende oxidatie niveaus (laag, matig en hoog geoxideerd) werd gebruikt om de emulsies aan te maken. De emulsies werden gedurende 24u bij 5°C geïncubeerd waarna de epoxyvetzuurconcentratie bepaald werd. In een ander experiment, werden een zuiver epoxyvetzuur (*cis*-9,10-methyl-epoxystearate) gedopeerd in C7-triglyceride (triheptanoïne) welke geëmulgeerd werd in de aanwezigheid van caseïne- en wei-eiwitten. De emulsies werden nadien getest op hun EFA hoeveelheden. Pre-eliminaire data konden de verwachte hoge reactiviteit niet bevestigen, maar er werd wel interactie gevonden.

In **hoofdstuk 7** wordt deze thesis afgerond met de algemene discussie, de conclusies en de toekomstperspectieven.

CHAPTER 1

LITERATURE REVIEW ON LIPID OXIDATION, EPOXY FATTY ACIDS IN FOODS AND RISK ASSESSMENT

1.1 GENERAL INTRODUCTION

Lipids are any of a class of organic compounds that are fatty acids or their derivatives and substances related biosynthetically or functionally to these compounds. They are biological substances that are generally hydrophobic in nature and in many cases soluble in organic solvents. This group includes natural oils, waxes, terpenes, phospholipids, sphingolipids, and sterols (Akoh & Min, 2008). Naturally, fatty acids are the basic building blocks of lipids and they are carboxylic acids with long hydrocarbon chains attached (Damodaran, Parkin, & Fennema, 2007). When the fatty acids have an oxygen molecule incorporated into their structure, they are called oxygenated fatty acids. Oxygenated fatty acids are common in nature and are important industrial materials. The classes of oxygenated fatty acids well known are epoxy acids, hydroxy acids and keto acids. In **Figure 1.1** examples of a non-oxygenated fatty acid (normal) and three representative oxygenated fatty acids are shown. Epoxy fatty acids (EFAs) are straight-chain aliphatic mono carboxylic acids containing one or more oxirane rings in the fatty acid chain. The basic structure of EFAs is the oxirane ring shown in **Figure 1.2** (Swern, 1955). This three-member ring consists of two carbon atoms joined to an oxygen atom. Hence they can be defined as cyclic ethers with three ring atoms (Chow, 2007).

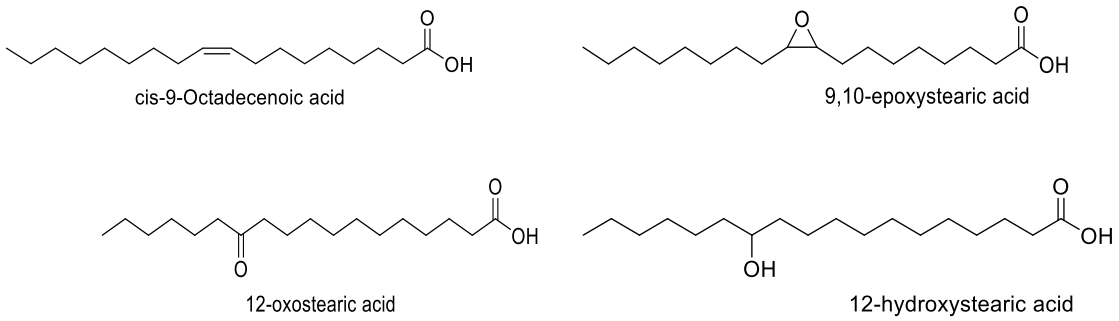


Figure 1.1: Structural formulas of selected fatty acids

The carbons in an epoxide group are very reactive electrophiles, since substantial ring strain is relieved when the ring opens upon nucleophilic attack. Epoxide's high reactivity is due to this ring-strain, inherent in the three-membered oxirane ring.

Figure 1.2: Oxirane ring structure

Oxygenated fatty acids are attracting a lot of industrial interest since they provide renewable raw materials. The advantage of most oxygenated fatty acids is that they have higher melting points and are easier to crystallize than their corresponding non-oxygenated or unsaturated analogues, thus rendering them relatively easy to isolate. They are attractive substances for studies of position, geometrical and optical isomerism and the effect of position, type and number of functional groups on physical and chemical properties (Swern, 1955).

Lipid oxidation is a major cause of chemical food deterioration which has been well studied and it is mainly associated with the loss of quality of foods. It is the process by which oxygen reacts with unsaturated lipids present in the foodstuff (Steele, 2004). Lipid oxidation may result in production of colour changes, undesirable flavours and toxic compounds which could represent a food safety problem. However, lipid oxidation (rancidity) flavours in some cases may be desirable (as in aged cheeses). Lipid oxidation includes oxidation of bulk oil (edible oil) and oxidation of lipids in emulsion type products (w/o emulsion like butter and margarine; o/w emulsion like milk, ice-cream, soup, sauces, mayonnaise, etc.). However, in some foods like biscuits, chocolate and French fries, part of the fat is in free form (free fatty acids) thus lipid oxidation is different. Free fatty acids are more susceptible to autoxidation than esterified fatty acids (Choe & Min, 2006). The free fatty acids can act as substrates for enzymes responsible for oxidative deterioration, e.g. lipoxygenase (Steele, 2004). Depending on the type of food system where lipid oxidation occurs, its contribution to food deterioration may differ.

The drive recently has been to use polyunsaturated fatty acids because of their health benefits, but they are more prone to oxidation (Ruxton, Calder, Reed, & Simpson, 2005). To monitor the extent of lipid oxidation in foods, many biomarkers have been measured by well-established analytical protocols. Among these lipid oxidation biomarkers, malondialdehyde (MDA), hydroxylated alkenals, hexanal and others, have attracted a lot of research in the recent past. EFAs as products of lipid oxidation could as well be measured to give an understanding of lipid oxidation in foods. Epoxides are not commonly mentioned by researchers, partly because epoxides are seldom measured due to lack of sensitive methods

and difficulty in detecting them (Schaich, 2013). Because lipid oxidation is a complex phenomenon (Kubow, 1992), the question is whether the focus on the short chain off-flavored volatile compounds (Aidos et al., 2002) is not an underestimation of lipid oxidation. This may require more lipid oxidation biomarkers to be able to realistically measure the extent of lipid oxidation reaction in food systems. Moreover, many researchers who have used the classical procedures to determine peroxide value (PV), p-anisidine value, MDA, 4-hydroxy-2-hexenal (HHE), 4-hydroxy-2-nonenal (HNE), acrolein and 2-butenal do agree that not always conclusive results can be obtained especially in complex food systems. This is mainly because of the alternative pathways that are involved in lipid oxidation as reported by some researchers (Frankel, 2005; Laguerre, Lecomte, & Villeneuve, 2007; Schaich, 2012). In addition, however, it was revealed that classical oxidation indicators such as MDA, used already for decades have shortcomings. For instance, MDA is very reactive hence its determination in food can be questioned whether it is a reliable indicator for the actual oxidative status of the food (Vandemoortele, Babat, Yakubu, & De Meulenaer, 2017; Vandemoortele & De Meulenaer, 2015).

Therefore, the purpose of this section is to summarise the available literature on lipid oxidation and EFAs. Emphasis has been given to the formation, analytics, occurrence, the toxicity of EFAs and risk assessment all aimed at giving recent knowledge in this area. Giving an understanding of EFA science may help to increase research on this important topic of lipid chemistry and may also attract the attention of international standard bodies especially in food toxicology and safety to focus on this area. Although EFAs may be toxic, this evidence remains scanty (IARC/WHO, 1987) to merit their wider application in the sphere of toxicological and risk assessment studies. It is hoped that the exposure of such gaps will prompt more research on toxicological and analytical components as well. It should be emphasized that EFAs of differing chain length exist, but this study mainly reviews the C18 chain family. This is mainly because their methods of analysis have been optimized and they occur more widely than other EFAs.

1.2 LIPID OXIDATION MECHANISMS

It has been proposed that lipid oxidation can be initiated and/or promoted by several mechanisms. These include the production of singlet oxygen via absorption of light by a photosensitizer, the enzymatic mechanism such as lipoxygenase and non-enzymatic

generation of partially reduced or free radical oxygen species (i.e. hydrogen peroxide, hydroxy radicals), active oxygen iron complexes and thermal or metal-mediated homolytic cleavage of hydroperoxides (Steele, 2004).

1.2.1 Autoxidation

The spontaneous reaction of molecular oxygen with radicals is what we normally refer to as autoxidation. The classical theory considers lipid oxidation, more specifically autoxidation as a free radical mechanism. This reaction between unsaturated fats and oxygen requires initiators such as metal, light and heat, but once started it is self-propagating. It involves three steps of development: initiation, propagation and termination as shown in **Scheme 1-1**.

Initiation: Formation of free radicals

$$RH \longrightarrow R^{\bullet} + H^{\bullet}$$

Propagation: Free radical chain reaction

$$R^{\bullet} + 3O_2 \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$$

Termination: Formation of non-radical products

$$ROO^{\bullet} + R^{\bullet} \longrightarrow ROOR$$

$$R^{\bullet} + R^{\bullet} \longrightarrow RR$$

(R: lipid alkyl)

Scheme 1-1: The classical lipid autoxidation mechanism

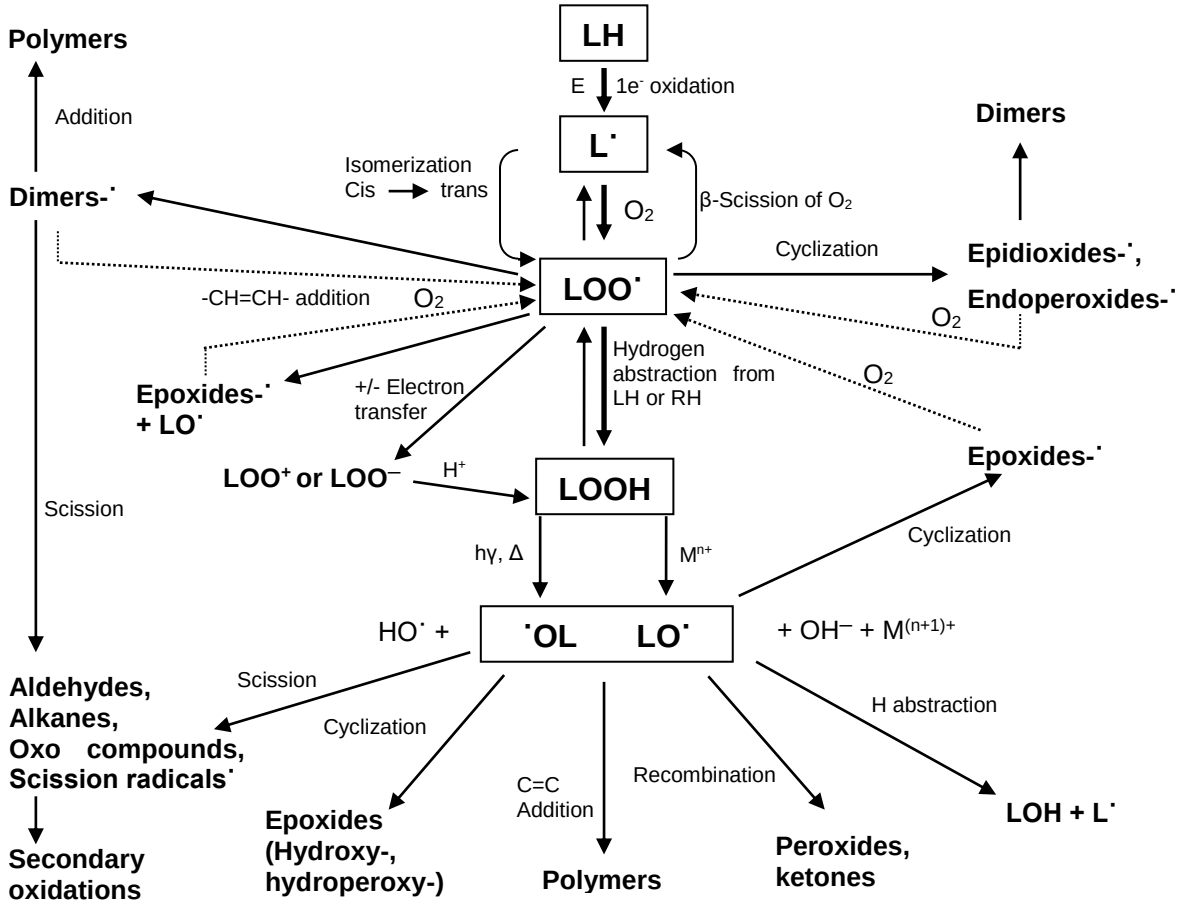
The initiation stage of autoxidation of an unsaturated fatty acid involves the formation of the first free radical (*ab initio* radical). Free radical formation starts the chain reaction of lipid oxidation with hydrogen abstraction being the driving force. The free radicals then transfer radicals to new lipids and thereby propagate the chain. The process starts with loss of a hydrogen atom, usually from an α-methylene group ($-CH_2-$), adjacent to a double bond (allylic position) which leads to formation of a free alkyl radical ($R^{\bullet}$). Although the process of lipid oxidation is highly favourable thermodynamically, the direct reaction between oxygen and even highly unsaturated lipids is kinetically hindered (Labuza & Dugan Jr, 1971). Ground state oxygen is in a triplet state when two free electrons in separate orbitals have the same spin direction, thus have a net positive angular momentum. The double bond is in a singlet state when no unpaired electrons exist, hence paired electrons are in the same orbital and have

opposite spin, thus have no net angular momentum. Quantum mechanics requires that spin angular momentum be conserved in reactions, so triplets cannot invert (flip spins) to singlet states. Reaction then demands that the double bond be excited into a triplet state, which requires prohibitive amounts of activation energy (E_a = 35–65 kJ/mole) (Schaich, 2005). Therefore, lipid oxidation is not a spontaneous reaction, and it requires initiators or catalysts to overcome this spin barrier. Hence an activating reaction is necessary to initiate free radical chain reactions. Initiation is difficult to control due to the multiple ways in which a free radical can be created.

Propagation is the second stage of autoxidation in which the free radical chain reaction is fully developed. The formed radicals can directly react with triplet oxygen to form a lipid peroxy radical, which can abstract hydrogen from another lipid molecule to form a hydroperoxide (ROOH) and an alkyl radical (Frankel, 2005; Schaich, 2005). The triplet oxygen can react with the radical state of lipids (Choe & Min, 2006). Lipid hydroperoxides are readily decomposed in the presence of suitable conditions such as heat and metals. The homolytic cleavage of hydroperoxides (ROOH) between the two oxygen molecules is the most likely decomposition pathway (Schaich, 2005), yielding an alkoxyl (RO•) and a hydroxy radical (•OH). As illustrated in **Scheme 1-2**, the alkoxyl radical (RO•), which is more energetic than either the alkyl (R•) or peroxyl radical (ROO•), can enter into several different reaction pathways (Chaiyasit, Elias, McClements, & Decker, 2007; Schaich, 2013). The alkoxyl radicals can attack another unsaturated fatty acid, a pentadiene group within the same fatty acid or the covalent bonds adjacent to the alkoxyl radical. Hydrogen abstraction by lipid peroxyl and alkoxyl radicals occurs only at allylic or doubly allylic positions of unsaturated lipids, where the C-H bond energy is sufficiently weak. Propagation results in a buildup of the hydroperoxides. Rancidity in foods will occur when the alkoxyl radicals undergo homolytic decomposition and this is known as β-scission reaction. It is important to food quality as it can cause fatty acids to decompose into low molecular weight, volatile flavoured compounds like aldehydes, alkyl and olefinic compounds (Frankel, 2005).

An expanded integrated scheme of lipid oxidation showing multiple pathways competing with the classical radical chain reaction is presented in **Scheme 1-2**. The different pathways show how complex the lipid oxidation reaction can be, which increases the complexity of both kinetics and product mixes. This eventually impact on how the lipid oxidation process is

organized, how products are generated and how lipid oxidation should be measured beyond hydrogen abstractions (Schaich, 2013).

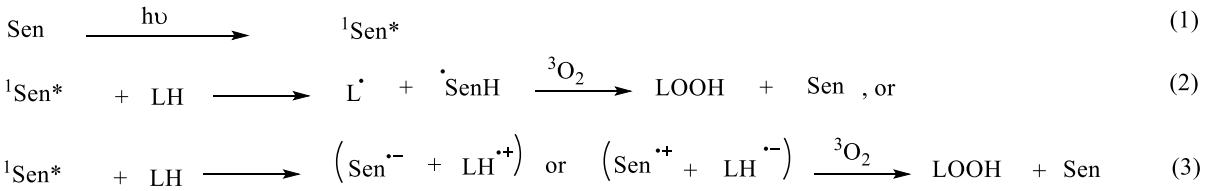


Scheme 1-2: An integrated scheme for lipid oxidation showing multiple reactions pathways. Dotted lines indicate paths for oxygen addition to secondary radicals formed in cyclic and addition products, with formation of new peroxyl radicals (Schaich, 2013)

1.2.2 Photooxidation

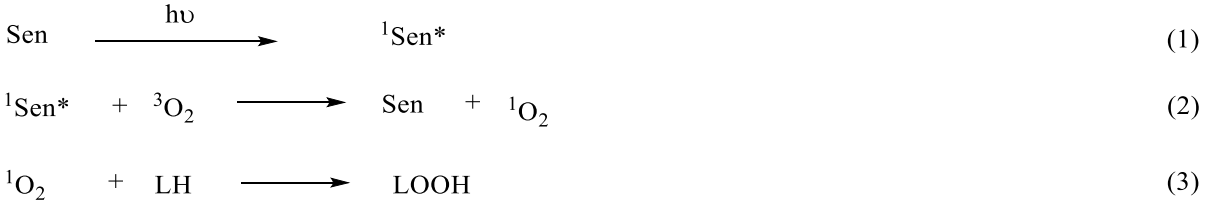
Photooxidation is the oxidation under light in the absence of photosensitizers while photosensitized oxidation is the oxidation under light in the presence of photosensitizers. The photooxidation mechanism seems to be a free radical chain reaction like autoxidation. Direct photooxidation is due to free radicals produced by ultraviolet light irradiation, which catalyses the decomposition of hydroperoxides (ROOH) and other compounds such as peroxides (ROOR), carbonyl compounds (RCOR), or other oxygenated unsaturated lipids.

Sensitized photooxidation requires the presence of a sensitizer such as chlorophyll, hematoporphyrins, myoglobin, reduced pyridine and riboflavin. Dyes, including erythrosine, tetrapyrroles (bilirubin), rose bengal and methylene blue also act as sensitizers as do heavy metals. The sensitizer must be activated by either visible or ultraviolet light (**Scheme 1-3, reaction 1**). It can then either react directly with the substrate in a one-electron transfer reaction, to produce a radical or radical ion in both the sensitizer and the substrate known as type I sensitizer (**Scheme 1-3**) or activate oxygen to the singlet state as type II sensitizer (**Scheme 1-4**) (Chacon, McLearie, & Sinclair, 1988; deMan, 1999; Frankel, 2005).



Scheme 1-3: Type I photooxidation reaction (Shahidi & Zhong, 2010)

Chlorophyll, for instance, enhances photooxidation by both mechanisms. A Type I sensitizer serves as a photochemically activated free radical initiator. The sensitizer in the triplet excited state reacts with the lipid substrate by hydrogen atom or electron transfer to form radicals, which can react with oxygen (Frankel, 2005). Formation of hydroperoxides during Type I photooxidation proceeds via a free radical or free radical ion route (**Scheme 1-3, reaction 2**).



Scheme 1-4: Type II photooxidation reaction (Shahidi & Zhong, 2010)

A Type II sensitizer in the triplet state interacts with oxygen by energy transfer to give a non-radical singlet oxygen ($^1O_2$) (**Scheme 1-4, reaction 2**). This is a highly reactive species of molecular oxygen which reacts further directly with unsaturated lipids double bonds by addition (**Scheme 1-4, reaction 3**). Hydroperoxides are generated during singlet oxygen attack and this is accompanied by a shift of the double bonds in the molecules (Shahidi & Zhong, 2010). This type of photosensitized oxidation is not inhibited by chain-breaking antioxidants (Frankel, 2005).

1.2.3 Enzymatic oxidation

Enzymes like lipases and lipoxygenases native to plants and animals can initiate oxidation reactions. When plant or animal tissues are disrupted or injured, the endogenous hydrolytic and oxidative enzymes effect lipid oxidation in the raw materials. The most important and best known of these enzymes is lipoxygenase (linoleate: oxygen oxidoreductase, EC.1.13.11.12) (LOX). Enzymatic oxidation reactions generally proceed with great stereospecificity. For instance, lipoxygenase uses molecular oxygen to catalyze the oxidation of only lipids containing a *cis*, *cis*-1,4-pentadiene moiety. Thus, the preferred substrates for LOX are linoleic and linolenic acid for the plant enzyme and arachidonic acid for the animal enzyme. The enzyme-catalyzed oxidation start with the hydrolysis of the triacylglycerols to produce the polyunsaturated free fatty acids (pentadiene) which are then oxidized by lipoxygenases (Frankel, 2005; Shahidi, 2005). Non-specific LOX may occur for instance in legumes and they react directly with esterified substrate fatty acids. In contrast to specific LOX, they do not require prior release of fatty acids from the triglyceride by a lipase enzyme (Belitz, Grosch, & Schieberle, 2009). Both LOXs oxidizes the polyunsaturated fatty acids into hydroperoxides. The produced hydroperoxides may undergo non-enzymatic or enzymatic cleavage by hydroperoxide lyases to yield a variety of breakdown products, which are often responsible for the characteristic flavors in some foods (Fennema, 1996). Hydroperoxides degradation in animals and plants fatty acid differs significantly. In animal tissue, the enzyme glutathione peroxidase catalyzes a reduction of the fatty acid hydroperoxides to the corresponding hydroxy acids. In plants and mushrooms, hydroperoxide lyase (HPL), hydroperoxide isomerase, allene oxide synthase (AOS) and allene oxide cyclase (AOC) are involved (Belitz et al., 2009).

1.3 FACTORS AFFECTING LIPID OXIDATION

Lipid oxidation is affected by many factors such as temperature, light, oxygen presence, fatty acid composition, antioxidants and pro-oxidants (Choe & Min, 2006; Frankel, 2005). It is not easy to differentiate the individual contribution of each factor. However, some of the important factors that are known to affect the lipid oxidation are discussed below:

1.3.1 Energy

Lipid oxidation is affected by energy input such as heat or light. High temperatures (160 – 190°C) like those used during frying of oils, have the required energy to break C-C or C-H bonds in the acyl chain. Moderate temperatures (<100°C) are only capable of breaking O-O bonds of the hydroperoxides (Schaich, 2005). Autoxidation of oils and the decomposition of hydroperoxides increase as the temperature increases (Shahidi & Spurvey, 1996). An increase in temperature causes a very strong reduction in the length of the induction period (period during which very little oxidation of fatty acid occurs). In principle the rate of oxidation increases exponentially with an increase in temperature (Schaich, 2005; Steele, 2004). However, the effect of temperature is complicated by a reduction in oxygen solubility in liquids at increased temperature and by changes in partitioning of antioxidants between phases if more than one phase is present (Steele, 2004). Higher temperatures however complicate lipid oxidation as they may steer the reactions to cause both thermolytic and oxidative reactions (Min, Smouse, & Society, 1985; Velasco & Dobarganes, 2002).

Visible, ultraviolet and γ-radiation are effective promoters of lipid oxidation, as discussed before in paragraph 1.2.2 (photooxidation). Light accelerates lipid oxidation especially in the presence of photosensitizers. Photosensitizers are normally in the singlet state (^{1}Sen) and become excited on absorption of light energy in picoseconds (Choe & Min, 2006). Light of shorter wavelengths has more detrimental effects on oils than longer wavelengths. Reportedly, the effect of light on oil oxidation becomes less as temperature increases (Velasco & Dobarganes, 2002).

1.3.2 Oxygen

The rate of oxidation is independent of the headspace oxygen concentration above 5% at moderate temperatures (Choe & Min, 2006). At higher temperatures, the dependency of oil oxidation on oxygen concentration increases due to a decrease in the solubility of oxygen in the oil (Labuza & Dugan Jr, 1971). Oxygen and lipids can react more efficiently when the sample size is small, which is due to a high lipids surface to volume ratio (Choe & Min, 2006).

1.3.3 Fatty acid composition

The rate of the reaction is strongly affected by the nature of the fatty acid (number, position and geometry of the double bonds). Abstraction of hydrogen during the propagation phase of the autoxidation takes place preferentially at carbon atoms where the bond dissociation energy is low. Lipids that are more unsaturated oxidize more quickly than the less unsaturated ones. Consequently, the rate of oxidation is much faster when mono unsaturated or poly unsaturated fatty acids are present in the food. The relative rate of oxidation of oleic acid (18:1) and linoleic acid (18:2) has been reported to be between 1:12 and 1:40 (Steele, 2004). Further increase in the rate with additional double bonds in the fatty acid is normally roughly in proportion to the number of methylene groups between pairs of double bonds. Thus, the relative rate of oxidation of polyunsaturated fatty acids, 18:2, 18:3 and 20:4 is roughly 1:2:3 respectively (Frankel, 2005; Steele, 2004).

1.3.4 Minor components present in the oil

Several minor components present in edible oils before and after their commercial processing can affect the rate of lipid oxidation. These may include components such as, free fatty acids, mono- and diacylglycerols, phospholipids, carotenoids, tocopherols, chlorophylls and metals. Some of these minor components can act as antioxidants while others have a prooxidant effect (Choe, 2008).

1.3.4.1 Metals

Metals such as iron or copper in oil are very effective pro-oxidants even if present at part per million levels or less. Redox-active metals undergoing one electron transfer such as iron and copper are of greatest importance in lipid oxidation (Schaich, 2005; Steele, 2004). These metals can directly remove an electron from a double bond, or a labile H from a C-H bond in the lipid molecule (e.g. allylic hydrogen) to generate alkyl radicals. The lower valence metals (Cu^{+}, Fe^{2+}) form a complex with oxygen and then react directly with lipid molecules to generate alkyl and alkoxy radicals. In addition, they can also generate different reactive oxygen species such as a hydroxy radical, superoxide and $^{1}O_{2}$. These reactive oxygen species are well known to further catalyze the lipid oxidation (Choe & Min, 2006; Schaich, 2005).

1.3.4.2 Antioxidants

Oils naturally contain different antioxidants such as tocopherols, tocotrienols, carotenoids and phenolic compounds and sterols (Δ^5-avenasterol and fucosterol). The phenolics with antioxidant activity include simple phenols (hydroxytyrosol, tyrosol); secoiridoids (oleuropein, the aglycone of ligstroside, and their respective decarboxylated dialdehyde derivatives) and the lignans (1-acetoxypinoresinol and pinoresinol) amongst others.

Antioxidants are compounds that can extend the induction period of oxidation or slow down the oxidation rate of a substrate. Standard one-electron reduction potentials of alkoxy, peroxy and alkyl radicals of unsaturated fatty acids are 1600, 1000 and 600 mV respectively; that of antioxidants are generally below 500 mV (Buettner, 1993). This implies that antioxidants can easily react with these radicals by donating a hydrogen because of the low reduction potential and form antioxidant radicals that are stabilized by their resonance structures. The scavenging of lipid free radicals to produce less reactive species and hence interrupt the propagation stage of lipid autoxidation is the main antioxidant mechanism (primary antioxidants) by which phenolic antioxidants such as α-tocopherol act. During lipid oxidation, tocopherols act as hydrogen donors and react with free radicals (Chen, McClements, & Decker, 2011). However, metal chelation by antioxidants such as citric acid, phosphoric acid, ascorbic acid and EDTA (ethylenediaminetetraacetic acid) is also an effective mechanism (secondary antioxidants) of antioxidant action. Maillard browning reaction products also act as antioxidants by reducing metal complexing properties and free radical scavenging capacity (Frankel, 2005). Compounds such as vitamin C contribute to the total antioxidant potential of a food as well (Choe & Min, 2006; Steele, 2004). Antioxidants like carotenoids can reduce oxidation by light filtering, quenching the $^1O_2$, inactivating the photo-sensitizer and scavenging of free radicals (Choe & Min, 2006).

1.3.4.3 Free fatty acids and mono-and diacylglycerols

Crude oil contains free fatty acids and oil refining decreases the free fatty acid content. They are removed from crude oils by neutralization and deodorization during the refining process. However, these refining processes do not remove 100% of the free fatty acids with commercial oils typically containing 0.05-0.70% depending on the type of oils and the refining process (Chaiyasit et al., 2007). Contradictions exist on the role of the free fatty acids

as prooxidants. It is believed that free fatty acids are strong prooxidants in bulk oils because of the presence of both hydrophilic and lipophilic groups in the same molecule, thus they prefer to concentrate on the surface of edible oils. The hydrophilic carboxyl groups of the free fatty acids will not easily dissolve in the hydrophobic oil (Choe & Min, 2006). When the free fatty acids concentrate at the surface, they decrease the surface tension of the oil and this in turn increases the diffusion rate of oxygen from the headspace into the oil, thus increasing lipid oxidation (Choe, 2008). It is also now known that free fatty acids' prooxidant activity is due to the ability of the carboxylic acid group of free fatty acids to form complexes with transition metals and make them more prooxidative. Furthermore, the ability of the acid group to directly promote hydroperoxide decomposition as well as co-oxidise the triacylglycerol in the oil into free radicals (Kittipongpittaya, Panya, McClements, & Decker, 2014; Waraho, 2011).

Free fatty acids could be important prooxidants in oil-in-water emulsions as well because they are surface active compounds. They are more polar than triacylglycerols due to the presence of an unesterified carboxylic acid groups. The surface activity of free fatty acids allows them to diffuse and concentrate at the water-lipid interface of the oil-in-water emulsions (Nuchi, Hernandez, McClements, & Decker, 2002). Free fatty acids in emulsions increase the negative charge of the emulsion droplets which increase metal-lipid interactions thus accelerating oxidation (Chen et al., 2011; Waraho, Cardenia, Rodriguez-Estrada, McClements, & Decker, 2009; Waraho, McClements, & Decker, 2011).

Mono and diacylglycerols exist in oils mainly because lipase activity leads to a partial hydrolysis of triacylglycerols. Monoacylglycerols and diacylglycerols are esters of glycerol in which one or two hydroxyl groups are esterified with fatty acids. Because of the surface activity property, mono and diacylglycerols are most commonly used as emulsifiers (Waraho, 2011). They occur in oils at much smaller concentrations than triacylglycerols. Mono and diacylglycerols are surface active compounds due to the presence of both lipophilic (fatty acid) and hydrophilic (hydroxyl) groups. This causes them to be partially soluble in fat and water and reduce interfacial tension of the oil. This results in an increased diffusion rate of oxygen to the oil, accelerating lipid oxidation (Choe & Min, 2006). In emulsions however, mono- and diacylglycerols act as antioxidants with diacylglycerols being stronger. Diacylglycerols are effective antioxidant in oil-in-water emulsions perhaps due to their ability to form a liquid crystal phase which could form a physical barrier that decreases interactions

between unsaturated fatty acids in the emulsion droplet core and prooxidants or oxygen in the aqueous phase of the emulsion (Waraho, 2011).

1.3.4.4 Phospholipids

Phospholipids are components of biological membranes and thus present in all living species from which foods are derived. Phospholipids are a group of fatty acyl containing lipids with a phosphoric residue. Crude oils contain common phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidic acid (Chen et al., 2011). Phospholipids may act as antioxidants or prooxidants depending on their concentration and presence of metals ions. They exercise this dual role due to the presence of the polar and nonpolar ends (Choe & Min, 2006). Phospholipids are known to play an antioxidant activity through sequestering metals by the phosphate group. A mixture of α-tocopherol with phosphatidylethanolamine exhibits higher antioxidant activity than with phosphatidylcholine. This difference is attributed to better regeneration of the tocopheroxyl radical to tocopherol by easier hydrogen transfer from the amino group of phosphatidylethanolamine to the radical (Frankel, 2005). Further still, phospholipids aid the radical scavenging activity of tocopherols via a physical mechanism that increases the accessibility of tocopherols toward chain initiating radicals in an aqueous microenvironment where lipid oxidation reactions are prevalent (Koga & Terao, 1994). Phospholipids containing an amino group (e.g. phosphatidylethanolamine (PE)) take part in Maillard type browning reactions. The reaction products of PE with the carbonyl substances are known to generate compounds with strong antioxidant activity (Shrestha & De Meulenaer, 2014; Shrestha, Gemechu, & De Meulenaer, 2013; Zamora & Hidalgo, 2005).

On the other hand, the hydrophilic groups of the phospholipids such as choline or ethanolamine are on the surface of oil while the hydrophobic hydrocarbons are in the oil. This decreases the surface tension of oil and increases the diffusion rate of oxygen from the headspace to the oil which accelerates oil oxidation (Choe, 2008). Phospholipids themselves are also susceptible to lipid oxidation due to presence of unsaturated fatty acids especially in meat and dried milk products (Cui & Decker, 2016). Phospholipids in bulk oils form association colloids such as reverse micelles. The presence of phospholipid reverse micelles in bulk oils creates oil–water interfaces where hydrophilic (e.g. iron) and amphiphilic (e.g. lipid

hydroperoxides) prooxidants and triacylglyceride substrate come into close contact with increase in lipid oxidation (Cui & Decker, 2016; Kittipongpittaya et al., 2014).

1.3.4.5 Photosensitizer

As mentioned in section 1.2.2 various photosensitizers can be present in foods. We shall in this paragraph focus only on chlorophyll as we have used this compound and its degradation products in one of the experimental chapters (chapter 5) in order to evaluate the impact of photooxidation on the formation of EFAs. Chlorophylls and its degradation products are common pigments present in edible vegetable oil which are removed during refining. Chlorophylls are composed of porphyrin rings with magnesium ion at the centre and a long phytol side chain. Two classes of chlorophylls exist namely, chlorophyll a and b. Chlorophyll a contains methyl group as a side chain at C-3 position, whereas, chlorophyll b contains aldehyde group instead. Chlorophylls could undergo chemical or enzymatic degradation which produces derivatives such as pheophytins (magnesium free derivatives) and pheophorbides (dephytyllated derivatives) (Schelbert et al., 2009). It has been reported that virgin olive oil and rapeseed contain up to 10 ppm and 5 - 35 ppm chlorophyll respectively (Psomiadou & Tsimidou, 2002), while crude soya bean oils contains up to 0.30 ppm (Jung, Yoon, & Min, 1989). Chlorophylls and their degradation products, pheophytins and pheophorbides, act as sensitizers to produce $^1O_2$ in the presence of light and atmospheric $^3O_2$ and thus accelerate the oxidation of oil. Pheophytins have a higher sensitizing activity than chlorophylls, but lower than that of pheophorbides. Although chlorophylls are strong prooxidants under light via acting as a sensitizer to produce $^1O_2$, they act as antioxidants in the dark possibly by donating hydrogen to free radicals (Choe & Min, 2006).

1.3.4.6 Water and water activity

As water is removed from a food system, lipid oxidation rates generally decrease. In some foods, continued removal of water will result in an acceleration of lipid oxidation. Increased lipid oxidation at low water activity ($a_w \leq 0.3$) is thought to be due to the loss of a protective water solvation layer surrounding lipid hydroperoxides (Damodaran et al., 2007). The water activity of a food is the ratio between the water vapor pressure of the food itself, when in a completely undisturbed balance with the surrounding air media and the vapor pressure of pure water under identical conditions (deMan, 1999). The water activity in dry foods increases with

temperature at constant moisture (Frankel, 2005). In dried foods with very low moisture contents (a_w values of less than about 0.1), oxidation proceeds very rapidly. Increasing the a_w to about 0.3 retards lipid oxidation resulting often to a minimum rate. This effect of small amounts of water may be due to the reduction of the catalytic activity of metal catalysts, quenching of free radicals and by delaying access of oxygen to the lipid (Fennema, 1996). At higher a_w, oxidation is accelerated by an increased mobilization of components (catalysts and oxygen) that are made nonreactive at low water activities by being trapped or "encapsulated" within a matrix of nonreactive food components (Karel, 1980).

1.3.5 Food structure

The structure of foods will affect the lipid oxidation process as well. In this review, this aspect is especially considered for oil-in-water emulsions, considering especially their surface area characteristics. In oil-in-water emulsions, or in foods where oil droplets are dispersed into an aqueous matrix, oxygen must gain access to the lipid by diffusion into the aqueous phase and passage through the oil-water interface. The rate of oxidation will depend on several factors like type and concentration of emulsifier, size of oil droplets, surface area of interface, viscosity of the aqueous phase, composition and porosity of the food matrix and pH (Fennema, 1996; Waraho et al., 2011). Interactions between metals and hydroperoxides at the interface of emulsion droplets is important and suggests that emulsion droplet surface area is important to lipid oxidation (Chen, McClements, & Decker, 2013). It has been shown that the rate of lipid oxidation increases proportionally to the surface area of the lipid that is exposed to air. It is also true that as the surface-volume ratio is increased, a given reduction in oxygen partial pressure becomes less effective in decreasing the rate of oxidation (Fennema, 1996). In emulsions, the specific surface area (the surface per unit volume) increases rapidly with decreasing particle size (deMan, 1999), therefore, any condition that increases the surface area is expected to increase lipid oxidation. Small droplet size corresponds to a large surface area, implying a high chance of contact between diffusing oxygen, water-soluble free radicals and antioxidants and the interface (Lethuaut, Métro, & Genot, 2002; McClements & Decker, 2000). However, conflicting reports about how surface area affects lipid oxidation exist (Chen et al., 2013). Some researchers have found lipid oxidation rates to be independent of surface area while others have reported an increase in the rate of lipid oxidation when the surface area

decreased. Others found an increase in lipid oxidation rates with increasing surface area (Lethuaut et al., 2002; Osborn & Akoh, 2004; Waraho et al., 2011).

1.4 EPOXY FATTY ACIDS

1.4.1 Nomenclature of EFAs

EFAs are named similarly to cyclopropane fatty acids with the parent acid considered to have a substituted oxirane substituent. The EFAs are characterized by the presence of a cyclic bond between two carbons in the chain and one oxygen atom. The epoxy carbons are counted in the longest hydrocarbon chain and the acids are named accordingly (Chow, 2007). The common vernolic acid (*cis*-12,13-epoxy-octadec-*cis*-9-enoic acid) for example is named (using standard IUPAC nomenclature) as 11-(3-pentyloxiranyl)-9-undecenoic acid. In older nomenclature, where the carbon chain is carried through the oxirane ring, it would be called 12,13-epoxyoleic acid or 12-13-epoxy-9 octadecenoic acid (Akoh & Min, 2008). The configuration of the oxirane ring substituents can be named in the cis/trans, E/Z, or R/S configuration systems (Akoh & Min, 2008). In **Table 1.1** some common names of selected C18 mono EFA methyl esters and their systematic nomenclature names are shown.

Epoxy carbons allow *cis* or *trans* conformations to occur, and this can be indicated as well. In common fatty acids, the *cis* or *trans* terms refer to the positions of atoms or groups connected to doubly bonded atoms. However, in EFAs, *cis* or *trans* is used to differentiate between the two geometrical (*cis* and *trans*) isomers of each oxirane ring which exist because of its inability to undergo free rotation. EFAs thus exhibit stereoisomerism. Stereoisomers differ in the spatial orientation of their component atoms and thus exist as optical isomers. Thus, the 9,10-epoxystearic acid contains two asymmetric carbon atoms, thereby furnishing two racemates: In the *cis* stereoisomer, the acyl chains lie on the same side of the reference plane in the molecule, while in the *trans* isomer they are at the opposite sides of this plane (as illustrated in **Table 1.1**, **structure**, **entry 1 and 2**) (Swern, 1955). The nomenclature of absolute configuration (R/S system) is used to identify the exact structure of the fatty acid to distinguish between stereoisomers. The "right hand" and "left hand" nomenclature is used to name the enantiomers of a chiral compound. This was originated by three chemists: R.S. Cahn, C. Ingold, and V. Prelog and, as such, is also often called the Cahn-Ingold-Prelog rules (Akoh & Min, 2008).

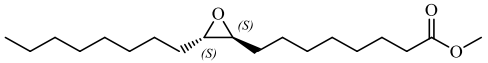
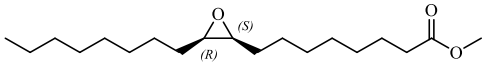
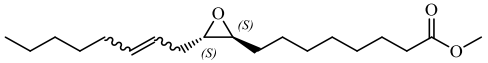
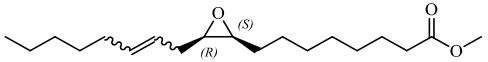
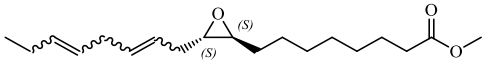
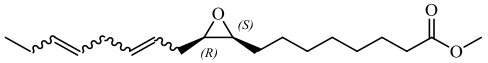
In position isomerism (regioisomerism) a functional group or other substituent changes position on a parent structure. An example is coronaric acid (*cis*-9,10-epoxyoctadec-12*Z*-enoic acid) which is a regioisomer of vernolic acid (*cis*-12,13-epoxyoctadec-9*Z*-enoic acid) (Cui, Duke, & Duke, 2008a).

1.4.2 Occurrence of EFAs

The natural occurrence of EFAs in foods especially in oil seeds, in cutins, agricultural raw material and other matrices is described (Gunstone & Jacobsberg, 1972; Gunstone & Schuler, 1975). A number of plants have been reported to have a high abundance of EFAs. The most promising prospective sources of high epoxy oils are: *Euphorbia lagascae* (family Euphorbiaceae) seed containing 42-50% oil, of which 58-62% is vernolic acid and *Stokesia laevis*, a member of the family Asteraceae, which produces an oil containing 74% vernolic acid. Occurrence of EFAs in *Vernonia anthelmintica* (Compositae) seed oil was confirmed by Gunstone and it is believed to be as high as 60 to 80% vernolic acid (Gunstone, 1954). Another EFA (which is an isomer of vernolic acid), named as coronaric acid was isolated from *Chrysanthemum coronarium* seed oil (Smith Jr, Bagby, Lohmar, Glass, & Wolff, 1960). Many researchers have further confirmed the natural occurrence of EFAs in many wild seed oils (Badami & Patil, 1980; Cahoon & Kinney, 2005; Cahoon, Ripp, Hall, & McGonigle, 2002; Earle, 1970; Gunstone, Harwood, & Dijkstra, 2007; Morris, Holman, & Fontell, 1961; Perdue, Carlson, & Gilbert, 1986; Smith, 1980; Tallent, Cope, Hagemann, Earle, & Wolff, 1966; Velíšek & Cejpek, 2006).

EFAs are also manufactured in large amounts by epoxidation of appropriate alkene esters for industrial purposes. Epoxidized vegetable oils such as epoxidized soya bean oil (ESBO), epoxidized linseed oil (ELO) are used as plasticizers and stabilisers for PVC. Similarly, seed oils that are enriched in EFAs such as vernolic acid have commercial value as components of plasticizers for polymers (Bhardwaj, Hamama, & Dierig, 2007; Carlson & Chang, 1985; Gunstone, 2004; Mungroo, Pradhan, Goud, & Dalai, 2008).

Table 1.1:Structure and nomenclature of C18:1, C18:2 and C18:3 epoxy FAMEs in order of elution on a CP-Sil 88 column

Order	EFA common name	IUPAC (Systematic) name	Chemical structure
1*	Methyl *trans*-9,10-epoxyoctadecanoate	Methyl 8-((2S,3S)-3-octyloxiran-2-yl)octanoate	
2*	Methyl c*is*-9,10-epoxyoctadecanoate	Methyl 8-((2S,3R)-3-octyloxiran-2-yl)octanoate	
3*	Methyl *trans*-12,13-epoxy-octadec-9-enoate	Methyl 11-((2S,3S)-3-pentyloxiran-2-yl)undec-9-enoate	
4*	Methyl *trans*-9,10-epoxy-octadec-12-enoate	Methyl 8-((2S,3S)-3-(oct-2-en-1-yl)oxiran-2-yl)octanoate	
5*	Methyl c*is*-12,13-epoxy-octadec-9-enoate	Methyl 11-((2S,3R)-3-pentyloxiran-2-yl)undec-9-enoate	
6*	Methyl *cis*-9,10-epoxy-octadec-12-enoate	Methyl 8-((2S,3R)-3-(oct-2-en-1-yl)oxiran-2-yl)octanoate	
7	Methyl t*rans*-12,13-epoxy-9,15-octadecadienoate	Methyl 11-((2S,3S)-3-(pent-2-en-1-yl)oxiran-2-yl)undec-9-enoate	
8	Methyl *trans*-15,16-epoxy-9,12-octadecadienoate	Methyl 14-((2S,3S)-3-ethyloxiran-2-yl)tetradeca-9,12-dienoate	
9	Methyl c*is*-12,13-epoxy-9,15-octadecadienoate	Methyl 11-((2S,3R)-3-(pent-2-en-1-yl)oxiran-2-yl)undec-9-enoate	
10	Methyl *trans*-9,10-epoxy-12,15-octadecadienoate	Methyl 8-((2S,3S)-3-(octa-2,5-dien-1-yl)oxiran-2-yl)octanoate	
11	Methyl *cis*-15,16-epoxy-9,12-octadecadienoate	Methyl 14-((2S,3R)-3-ethyloxiran-2-yl)tetradeca-9,12-dienoate	
12	Methyl *cis*-9,10-epoxy-12,15-octadecadienoate	Methyl 8-((2S,3R)-3-(octa-2,5-dien-1-yl)oxiran-2-yl)octanoate	

*Major EFAs that occur in foods

Epoxidized vegetable oil (EVO) can act as a raw material for synthesis of a variety of chemicals including polyols and glycol (Gunstone, 2004; Saurabh, Patnaik, Bhagt, & Renge, 2011). They are also used in adhesives, paints and composite materials (Hammarling, Gustavsson, Svensson, Karlsson, & Oskarsson, 1998). When ESBO and other epoxidized lipids are used in plastic food contact materials, potentially these compounds can migrate to the food. Several studies have been done to investigate the potential risk of this phenomenon (Castle, Mayo, & Gilbert, 1990; Fankhauser-Noti, Fiselier, Biedermann-Brem, & Grob, 2006; Pedersen et al., 2008).

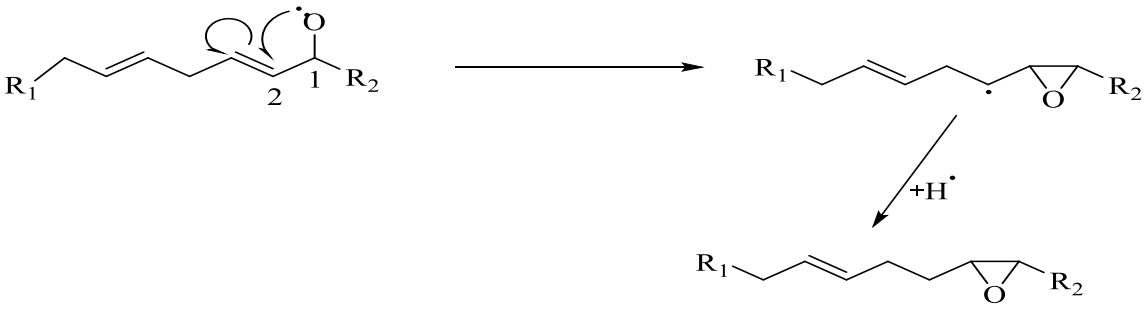
The occurrence of EFAs in foods as a result of lipid oxidation has been documented (Berdeaux, Marquez-Ruiz, & Dobarganes, 1999b; Dobarganes, 2009; Swern, 1955; Velasco, Berdeaux, Márquez-Ruiz, & Dobarganes, 2002). These reports mainly deal with the formation of EFAs at high temperatures, i.e. typically during frying operations. However, it has also been reported that EFAs form after prolonged storage of some seeds (Gunstone et al., 2007) which suggests that enzymatic oxidation by epoxygenases (a cytochrome P450 acting on linoleic acid) may be involved in their formation. More details about the formation of EFAs during lipid oxidation, will be given in **section 1.5**. The major EFAs that occur in foods are reported in **Table 1.1** indicated with an asterisk. Quantitative results on the occurrence of EFAs in vegetable oils and food matrices, are summarized in **chapters 2-5**. The quantitative data show that oils have high EFAs concentrations in the range of mg/g.

The information on occurrence of EFAs is vital and may be used by international bodies such as the European Food Safety Authority (EFSA) and the Food and Drug Authority (FDA) to make firm conclusions on the EFA occurrence in different foods. When such information is combined with consumption data, it can lead to estimation of consumers and total population exposure to EFAs which can later be used in risk assessment studies. In cases where tolerable daily intakes are known, when the amounts consumed exceed those limits, then conclusions can be drawn as to whether the population is at a risk or not.

1.5 FORMATION OF EFAs DURING LIPID OXIDATION

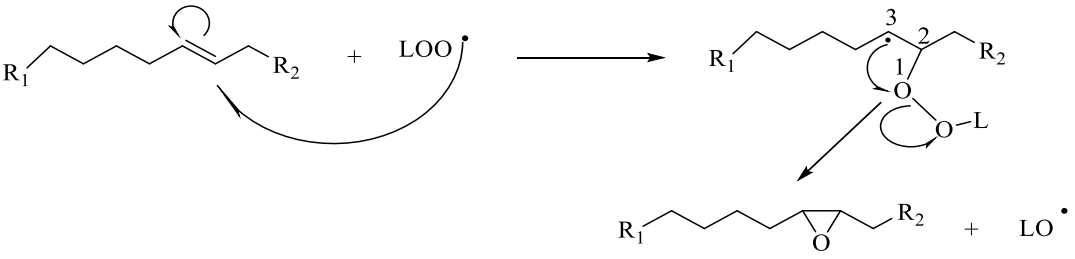
During lipid oxidation, the normal fatty acids are modified by inclusion of oxygen atoms into their structure. Two main mechanisms are postulated that lead to the formation of EFAs during lipid oxidation (Dobarganes, 2009; Giuffrida, Destaillats, Robert, Skibsted, & Dionisi,

2004). The first mechanism involves 1, 2-addition of an alkoxy radical (LO•) to the adjacent double bond with formation of an epoxyallylic radical (**Scheme 1-5**) (Gardner, 1989; Schaich, 2005). In this mechanism, the alkoxyl –O• adds to the α carbon of the immediately adjacent double bond to form an epoxyallylic radical, with transfer of the free electron to the β carbon to generate epoxides. This is the dominant reaction in aprotic solvents, when lipids are at low concentration or highly dispersed at the surface and at low oxygen pressures (Schaich, 2013). Cyclization of LO• is stereospecific whereby the configuration of epoxides is fixed by the conformation of the fatty acid alkoxyl radical at the point of cyclization rather than post cyclization isomerization (Schaich, 2005). This is because allylic radicals are resistant to rotation and the final products largely reflect the geometry of the original configuration (Gardner, 1989).



Scheme 1-5: 1,2 addition to the adjacent double bond of LO• radical (modified after Schaich, 2005)

The second mechanism which is considered to be less probable, is the direct addition of a peroxy radical (LOO•) to an isolated or non-conjugated double bond which later undergoes 1,3-cyclisation (**Scheme 1-6**) to form an epoxide with elimination of an alkoxy radical (LO•).

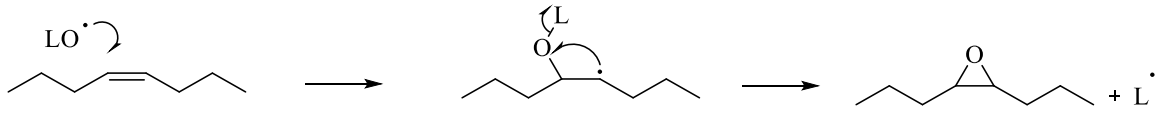


Scheme 1-6: 1,3 cyclisation of the LOO• radical to the double bond (modified after Schaich, 2005)

During this mechanism, the LOO• adds to double bonds to form an initial dimer peroxy complex. At the beginning of oxidation, LOO• adds to isolated or non-conjugated double bonds, though not easily due to steric hindrance from the long acyl chain. A radical simultaneously forms on second carbon of the double bond, which reacts immediately with the peroxide adduct. The adduct undergoes 1,3-cyclization to form an epoxide, eliminating LO• in the process (Gardner, 1989).

In a given reaction system both methods seem to contribute to the formation of EFAs. Cyclization is catalysed by metals, particularly Fe and Cu. Since presence of metals in foods is so common, Schaich hypthosesized that EFAs should always be a major product in oxidizing lipids (Schaich, 2013). However, the content of EFAs may be reduced since the same metals can cause other competing reactions to occur which yield other compounds like hydroxy compounds.

It is also possible that EFA formation may occur via alkoxy (LO•) addition to the unsaturated fatty acid (**Scheme 1-7**). It has been reported that addition of LO• to double bonds occurs particularly in the absence of allylic hydrogens and conjugation (Schaich, 2013).



Scheme 1-7: Alkoxy addition to the double bond

Propagation of lipid oxidation by LO• addition is most active in catalysing chain branching during secondary stages of oxidation. LO• addition increases with *cis* configuration and asymmetrical substitution on double bonds (Schaich, 2013).

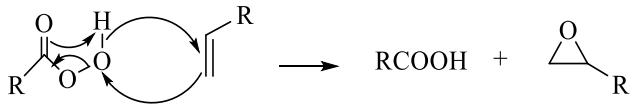
1.6 SYNTHESIS AND REACTIVITY OF EFAS

1.6.1 Synthesis of EFAs

EFAs can be synthesized by a reaction known as epoxidation by use of peroxy acid or a peroxide with active oxygen on the corresponding olefinic acid (-C=C-). This adds an atom of oxygen and converts the -C=C- bond to the three-membered epoxide or oxirane ring group. The epoxidation reaction is important to those who use EFAs as industrial raw

materials (Heath, Di, Clara, Hudson, & Manock, 2005). The epoxidation reaction is stereospecific, whereby the geometrical configuration of the resulting epoxy acid is identical with that of the olefinic acid from which it is derived (Swern, 1955). Most epoxidations are effected by reaction of the olefinic acid, alkyl ester, or glyceryl ester with peroxy acids (RCOOOH) which are often preformed and used in situ for epoxidation, although some can be stored and used as required (EFSA, 2004). The peroxy acids are made by interaction of carboxylic acids (RCOOH), anhydrides or acid chlorides with hydrogen peroxide, with the first of these requiring an acidic catalyst, such as sulphuric acid or sulphonic acid. The most commonly used peroxy acids include aliphatic (peroxyformic, peroxyacetic, peroxy trifluoroacetic, peroxylauric), aromatic (peroxybenzoic, m-chloroperoxybenzoic) species as well as monoperoxy acids based on dibasic acids (succinic, maleic, phthalic) acids (Gunstone et al., 2007). The epoxidation with peracids (RCOOOH) proceeds via an ionic synchronous or concerted mechanism whereby bond breaking and making occurs at the same time, thus preserving the stereochemical information from the alkene (E to *trans* and Z to *cis*) (**Scheme 1-8**).

In situ generation of the paracid

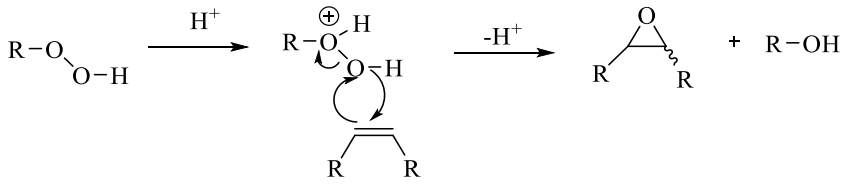


Scheme 1-8: General epoxidation reaction by peracids (RCOOOH) (EFSA, 2004)

The reaction is exothermic thus use of a concentrated acid should be avoided and the products themselves are unstable towards acids (Gunstone et al., 2007). It is a rapid reaction at room temperature with good yields above 80%.

On the other hand, epoxidation of double bonds or unsaturated fatty acids with alkyl hydroperoxides is a known reaction and may occur using the basic mechanism (**Scheme 1-9**) below. The epoxidation can be done on alkenes using molecular oxygen *via* a catalytic process. Since the direct epoxidation of alkenes with molecular oxygen, which lies in triplet

ground state, is inhibited, epoxidation using O_2 is carried out in the presence of a compound like alkyl hydroperoxides which serves as an active oxygen carrier (Iwahama, Hatta, Sakaguchi, & Ishii, 2000).

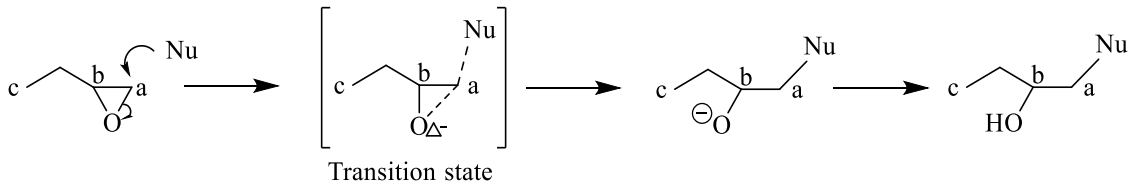


Scheme 1-9: Epoxidation of double bonds by alkyl hydroperoxides

Epoxidation can as well be carried out enzymatically which has an advantage that no epoxy ring opening occurs (Gunstone et al., 2007). Soybean oil and linseed oil have been epoxidised by hydrogen peroxide with the lipase B from *Candida antarctica* yeast, available in immobilised form (Novozym 435) (Gunstone, 2004). The mechanism of chemo-enzymatic epoxidation of methyl esters involves a two-step reaction. First the lipase enzyme catalyses the peracid formation. Alternatively, the lipase catalyses the hydrolysis of the ester into the respective acid first and then it catalyses the formation of the peracid from the acid. Finally, the peracid spontaneously donates the oxygen to a double bond, to form the epoxide via a similar mechanism as shown in **Scheme 1-8**. Both free fatty acids and the methyl esters can undergo the epoxidation (Severiano, Hagström, Hatti-Kaul, & Da Fonseca, 2008). Immobilized oat peroxygenase enzyme was successfully used as well to epoxidise linoleic and linolenic acids (Piazza, Nunez, & Foglia, 2003a).

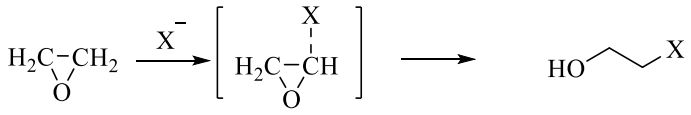
1.6.2 EFA reactivity

The highly strained geometry of this epoxy moiety with a strain energy of 114 kJ/mol accounts for its reactivity with many nucleophilic or electrophilic compounds (Gunstone, 2004; Heath et al., 2005; Kent, 2013; Mungroo et al., 2008). The epoxide ring can be opened and the reaction involves either electrophilic attack on the oxygen atom or nucleophilic attack on one of the ring carbons. All the mechanisms are ionic (anionic or cationic) in presence of water so that the highly polar carbon-oxygen bond can be broken. The important reaction mechanism of epoxides that can be used to convert them to different products are shown (**Scheme 1-10**). It should be noted that both base and acid environments can promote the ring opening (Heath et al., 2005).



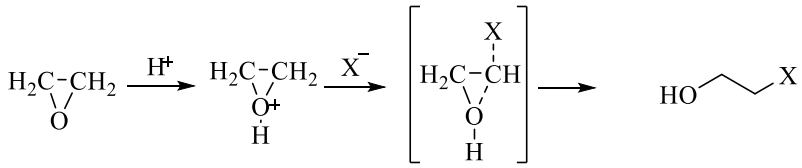
Scheme 1-10: Summary of the main reaction mechanism of the epoxy group (Roberts, Aptula, & Api, 2017)

Epoxides can easily be hydrolyzed to a diol (or its monoester) by a nucleophilic substitution reaction, especially under acidic conditions. These reactions occur stereospecifically with inversion, so the *cis* and *trans* epoxides give the *threo* and *erythro* diols, respectively. An example is during heating with acetic acid (acetolysis) followed by hydrolysis of the monoacetate (Gunstone et al., 2007). In a basic or neutral condition, all ring opening reactions are similar and involve an attack by a nucleophile on one of the carbon atoms of the oxirane group. The mechanism is regarded as S_N2 as illustrated in **Scheme 1-11**.



Scheme 1-11: Epoxy reaction under basic conditions

When an acid is involved, the reactions of most nucleophiles will be accelerated, through the formation of a reactive conjugated acid from the epoxide species, which involves proton attack on the ring's oxygen atom (**Scheme 1-12**).



Scheme 1-12: Epoxy reaction under acidic conditions

Despite the high reactivity of the epoxy group, no information about the stability of EFAs in food matrices could be found in the literature.

1.7 ANALYSIS OF EFAS

1.7.1 Introduction

Analytics in some cases have limited our advancement in the knowledge of lipid oxidation. However, this has changed with the developments in the analytical techniques such as gas chromatography (GC), mass spectrometry (MS), high pressure liquid chromatography (HPLC), ultra-high-pressure liquid chromatography (UHPLC) and nuclear magnetic resonance (NMR) which can be even further hyphenated (Wilson & Brinkman, 2003). Hyphenation like in multi-dimensional gas chromatography (MDGC) has increased the separation power and our capacity to analyse more complex secondary oxidation products. Ever since research about EFAs was done in the 60s and the 70s (Earle, 1970), the focus by lipid analysts has been to come up with more robust analytical methods that can accurately quantify EFAs.

1.7.2 Qualitative analysis of EFAs

1.7.2.1 Paper chromatography and thin layer chromatography (TLC)

Paper chromatography is a rare technique which was used in the past so it has become obsolete, while TLC is a handy technique which is frequently used as an additional separation step before GC analysis (Spitzer, 1999). The initial analysis of EFAs relied a lot on these two methods. It helps to isolate complex mixtures of compounds and to check the effectiveness of the column fractionation process during method development and optimisation. In EFA analysis, TLC application has been mainly qualitatively used to detect, separate and isolate them from other fatty acids (Morris & Wharry, 1965). Preparative TLC is used to separate the esters into FAMEs, epoxy and hydroxy fractions. Although it is not as sensitive as GC, it provides very useful information regarding the effectiveness of separation of closely related compounds.

The epoxy FAMEs can be separated using different solvent systems per their retention factor (Rf) values. Normally TLC is performed on 0.25 mm pre-coated silica gel 60 plates, eluted with hexane–diethyl ether–acetic acid (80:20:1, v/v/v) (Tallent et al., 1966) or with hexane-ether-acetone (33:4:3, v/v/v) (Gardner, Weisleder, & Nelson, 1984). TLC can reveal epoxy compounds by use of an acidic chromophore such as picric acid; presence of orange coloured spots indicate an epoxy group (Earle, 1970; Gray, 1978). Visualisation of the compounds can

be achieved by use of iodine or ultra violet light. However, to prevent alteration of the acid sensitive epoxy group, the contact time with acidic conditions must be as short as possible.

1.7.2.2 HPLC, LC-MS and NMR

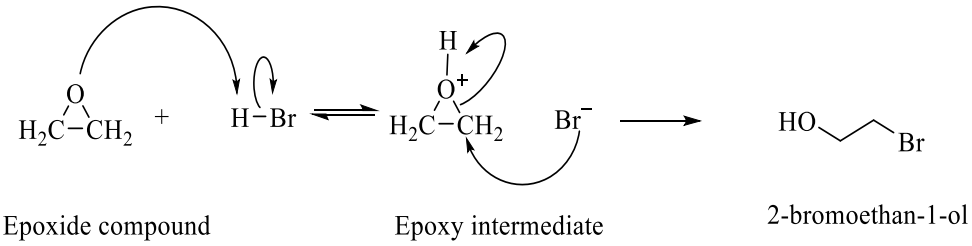
High-performance liquid chromatography (HPLC) has widely been used as a preparative step by fractionating the different EFAs in question. Although these techniques can be used to quantify analytes, in EFA analysis, they have mainly been limited to qualitative analysis. Studies have been based on HPLC pre-separation of EFAs on a C-18 column using methanol-water containing 0.05% acetic acid as a mobile phase (Gérard, Moreau, Fett, & Osman, 1992; Morales, Dobarganes, Márquez-Ruiz, & Velasco, 2010; Orellana-Coca, Adlercreutz, Andersson, Mattiasson, & Hatti-Kaul, 2005; Piazza et al., 2003a). HPLC coupled with different detectors such as refractive, evaporative light scattering detector, ultra violet and others, can be used to detect the different compounds which later can later be identified by either MS or proton nuclear magnetic resonance (^{1}H NMR) (Fer et al., 2006; Giuffrida et al., 2004). Use of liquid chromatography-mass spectrometry (LC-MS) has the added advantage that after separation, confirmation can be done on the MS. However, LC-MS and NMR are quite expensive analytical techniques although gaining a lot of applicability. Using normal-phase HPLC, EFAs were separated according to the place of the epoxy group in the carbon chain and according to the degree of unsaturation of the fatty acid (Cui et al., 2008a; Cui, Duke, Tattam, & Duke, 2008b; Cui et al., 2009).

Valuable qualitative and quantitative information can be given by NMR about lipid oxidation and the formed products. The NMR technique has the advantage that a sample is analysed by simply dissolving it in deuterated chloroform. Thus, a lipid can be studied without further alteration in its composition to give more sound information (Martínez-Yusta, Goicoechea, & Guillén, 2014). However, this technique has not received a lot of application probably because of the cost involved. During ^{1}H NMR determination, correct assignment of the signal to the corresponding proton is important. As reported by some authors, most epoxy compounds have signals for the protons attached to the epoxidized carbons at 2.7 ppm for *cis* and 2.45 ppm for *trans* (Gunstone & Jacobsberg, 1972; Knothe). It should be noted that in NMR characterization, the signals for the α-methylenes in between an epoxide and a double bond are δ_H 2.2 ppm and 2.4 ppm and δ_C 26.2 ppm on a 300 MHz instrument. This signal is a good indicator for the presence and location of the monoepoxides (Cui et al., 2008a).

Quantitative NMR (qNMR) is almost as old as NMR itself and has not been popular. Because qNMR has been living in the shadow of the multifaceted and multidimensional qualitative NMR used in structure analysis, neither has it been used as widely and routinely. The method is limited by its sensitivity and chemical shifts may not be consistently determined. Reports regarding the achievable precision of quantitation are inconsistent, and some of them even tend to deny NMR as a precision method by estimating the error to be in the 10% range. (Pauli, Godecke, Jaki, & Lankin, 2012). However, the method is convenient, sample preparation is simple and rapid as no derivatization is needed for compound analysis. Recently quantitative methods have been validated and used to quantify the epoxy fatty acids in oils with reliable results (RSD of 1-4%) down to 6.3 mmol/kg LOQ (Aerts & Jacobs, 2004; Xia, 2017; Xia, Budge, & Lumsden, 2015; Xia, Budge, & Lumsden, 2016).

1.7.3 Semi-quantitative determination epoxide by HBr uptake

This is an AOCS method used to measure lipid oxidation by determining the presence of the epoxy groups semi quantitatively. This method has not found a lot of application in the analysis of EFAs in foods because it has limitations. However, it is used mainly by those in the plastic industry. It is particularly more useful in characterizing epoxidised soybean oil which as mentioned before is industrially produced to be applied as a plasticizer (Holser, 2008; Kent, 2013; Kumarathasan, Rajkumar, Hunter, & Gesser, 1992). In this method, analysis is performed on the oil without derivatising it to methyl esters. The standard HBr titration method, AOCS Tentative Method Cd 9-57 (21) for oxirane determination was adopted after collaborative analyses conducted in five different laboratories (Gray, 1978). The epoxy groups are usually determined by reacting the compound with an excess of halogen in a suitable solvent (**Scheme 1-13**), the halogen consumed being a measure of the epoxide. The halide uptake can be measured directly, potentiometrically or by back titration. This method is only able to estimate the total epoxides present by measuring the uptake of hydrogen halide (Morris et al., 1961) and is nonspecific. Hence it cannot be used to identify and quantify the different isomers.



Scheme 1-13: A reaction of epoxide compound with hydrogen bromide

The "epoxy value" indicates the percentage of total test substance mass which is attributed to the epoxy functional group and thus indicates the degree of epoxidation of the test substance. The method however, has a drawback as it is not sensitive, lacks specificity and a large sample size is needed if the oxirane percentage is below 5% (Morris et al., 1961). The results are expressed as hydrogen bromide equivalent (HBE) and calculated as epoxyoleic acid (Earle, 1970). Sometimes other compounds such as cyclopropenoid fatty acids could account for the hydrogen bromide equivalent observed during the titration unless it was carried out in the cold (Earle, 1970). Therefore, more sensitive methods were required for EFA detection and measurement. This led to development of more specific methods such as, IR spectrophotometry, HPLC, GC and NMR to analyse for EFAs. Recent advancements in analytics have enabled the determination of the different EFA isomers.

1.7.4 Quantitative analysis of EFAs in oils and foods

1.7.4.1 Introduction

Because of their low volatility, EFAs must be methylated like any other fatty acids to be analysed quantitatively by GC methods (**Table 1.2**). Currently liquid chromatography (LC) methods are less used to analyse EFAs because of their limitations. As the epoxy group is very reactive to acids and sensitive to heat (Christie, 2011a; Kleiman & Spencer, 1973), the method of derivatisation involves room temperature base catalysed methylation in order to avoid ring opening. In the past, analysis of oils without derivatisation was tried out but it did not yield good results (Earle, 1970).

During analysis of EFAs after methylation, FAMEs need to be pre-separated by solid phase extraction (SPE) to remove the non-polar fatty acid FAMEs which comprise the biggest percentage of the fatty acids. This is required because the non-polar fatty acids can coelute with the EFAs during GC analysis on a polar column (Mubiru, Shrestha, Papastergiadis, &

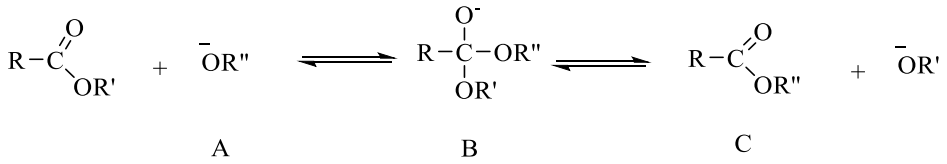
De Meulenaer, 2013). SPE pre-separation is important so that all the fatty acids are portioned as per their polarity, which is achieved by increasing the polarity of the eluting solvents. Normally hexane: diethyl ether of varying strength is the eluting solvent that is used to separate the different fatty acids. Use of diethyl ether at full strength to elute all polar fatty acids at once, is applied although it is not a good practice as this has a disadvantage of eluting even the polymeric compounds especially in frying oils. These polymeric compounds are not detectable on a GC capillary column and moreover, they stick to the column and hence reduce its capacity (Mubiru et al., 2013).

1.7.4.2 Preparation of ester derivatives of FAs and EFAs

The formation of methyl esters can be done from fatty acids (esterification) or directly from glycerol esters (ester exchange or transesterification). The common practice during ester preparation is that the triacylglycerols are saponified with a methanolic NaOH solution to liberate the fatty acids. Subsequently the fatty acids are esterified to increase the volatility during GC analysis. The ester derivatives are much less polar than the corresponding free fatty acid and do not tend to absorb or dimerize on the column, thus avoiding peak tailing, peak asymmetry and peak shouldering. Another requirement is that the derivatisation procedure should avoid artefact formation which would lead to overestimation of the analyte (Berdeaux, Marquez-Ruiz, & Dobarganes, 1999a).

During ester preparation a catalyst is generally required which may be acidic, alkaline or enzymatic although alkaline catalysts cannot be used for the esterification of free acids (Berdeaux et al., 1999a). EFAs are very sensitive to acidic conditions and they react with opening of the oxirane ring. In presence of acids as a suitable catalyst, protonation of the epoxy oxygen leads to the opening of the epoxy ring and it is hydrolysed to hydroxyl fatty acids. For example, hydrogen chloride adds across the ring to form halogen hydrins, and boron trifluoride-methanol adds methanol across the ring to give a methoxy-hydroxy product. Epoxy acids are not harmed by basic conditions under normal circumstances, thus they can be transesterified safely with alkaline reagents. Hence the best method of transesterification used for EFAs is the base catalysed sodium methoxide methanolysis at room temperature (Christie, 2011b).

Esters, in the presence of base such as an alcoholate anion (A) (**Scheme 1-14**), form an anionic intermediate (B), which can dissociate back to the original ester or form the new ester (C). With excess of the alcohol from which the anion was derived, the equilibrium point of the reaction will be displaced until virtually the sole product is the new ester.



Scheme 1-14: Base-catalysed transesterification of lipids (Christie, 2011b)

However, the base catalysed reaction is not able to esterify the free fatty acids simply because the free fatty acid is converted to a carboxylate ion ($RCOO^-$) in a basic solution. This ion is not subject to nucleophilic attack by alcohols or bases derived from them because of its negative charge. Hence, transesterification occurs by this mechanism with basic catalysis but esterification does not (Christie, 2011b). The reaction medium should be void of water to prevent dissociation to free fatty acids of the intermediate (B). In most methods, base catalysed methylation is achieved by use of sodium methoxide in methanol in excess using tert-butyl methyl ether (TBME) as a mediator solvent. The reaction is fast and it is normally achieved in 1 min. It is stopped by addition of an acid to neutralise the reaction medium and prevent potential further saponification (Suter, Grob, & Pacciarelli, 1997).

1.7.4.3 Chromatographic techniques

1.7.4.3.1 GC-FID analysis of EFAs

In quantitative analysis, the best analytical quality control approach would be to introduce the IS immediately at the start of the analysis. This has not been possible in some cases because of the limitation of the methylation procedure vis-à-vis the sensitivity of the epoxy ring. Lipid analysts had to choose between the use of a free EFA and an EFA methyl ester (FAME) as an internal standard (IS). Because free EFAs are not esterified by the base catalysed methylation, the use of a FAME standard as an internal standard is common (**Table 1.2**) and it is normally added after SPE. However, this may increase the level of uncertainty during analysis. Furthermore, a better method involving a two-step methylation procedure can be applied to overcome this problem. This starts with base catalysed transmethylation with

sodium methoxide in TBME which is followed by direct methylation of the carboxylic groups with diazomethane (Berdeaux et al., 1999b; Velasco et al., 2002; Velasco, Marmesat, Bordeaux, Márquez-Ruiz, & Dobarganes, 2004). However, this method is not preferred because of the limitations of diazomethane being explosive, toxic and the nitrosamides used to prepare it are carcinogenic (Christie, 2011a). Recently by epoxidation of FAMEs using 3-chloroperoxybenzoic acid in chloroform at room temperature to EFA methyl esters, it was possible to synthesise methyl *cis*-10,11-epoxyheptadecanoate from methyl *cis*-10-heptadecenoate (C17:1) which was used as an IS to quantify EFAs in oils and food matrices (Mubiru, Shrestha, Papastergiadis, & De Meulenaer, 2014). However, use of deuterated standards would have been an even better approach, but the cost of these stable isotopes is prohibitive. Moreover, this should require the use of GC-MS as an analytical method, while GC-FID works fine as well.

1.7.4.3.2 GC-FID conditions

GC-FID instrumentation is the most commonly used technique and provides good results for the analysis of FAMEs and EFAs (Aguirre, Dobarganes, Marmesat, & Ruiz Méndez, 2010; Dodds, McCoy, Rea, & Kennish, 2005; Gilbert, Shepherd, Startin, & Eagles, 1981). This may be attributed to its being an accessible technique to many analysts. During epoxy FAME analysis on a GC, the sample must be injected into the column where separation takes place and finally EFAs are detected by an appropriate detector. In the past, research was limited by the power of the available technology like columns. Currently, analysis of epoxy fatty methyl esters on different capillary columns by gas chromatography flame ionisation detector (GC-FID) has been made possible (Delmonte, Kia, Hu, & Rader, 2009; Earle, 1970; Hammond, 2002; Morales, Marmesat, Dobarganes, Márquez-Ruiz, & Velasco, 2012; Spitzer, 1999). For instance, Morris et al. could not separate the different epoxy isomers because of the column they used (Morris et al., 1961). However, currently there has been a lot of advancement in technology to separate different compounds using more robust columns. Many parameters can be changed to achieve the best results, more frequently the column is varied as can be seen in **Table 1.2**.

Table 1.2: Some examples of quantitative studies for EFAs analysis

Sample preparation	Analytical procedure	Sample matrix	Amounts detected	Reference
Room temperature base transmethylation using C13:0 and C15:0 FAME as IS	GC-FID; split-splitless injector and HP Innowax column	Heated Models of FAMEs and monoacid TAGs	32.3 mg g^{-1} in methyl oleate; 19.3 mg g^{-1} in methyl linoleate; 35.7 mg g^{-1}in triolein and 18.3 mg g^{-1} in trilinolein	Berdeaux et al., 1999
Room temperature base trans methylation followed by diazomethane methylation using C13:0 and C15:0 acids as IS, two step SPE	GC-FID using both split-splitless and COC injector on a DB-Wax column	Sunflower and olive oil oxidised at 180°C	10.88 mg g^{-1} in SFO and 13.52 mg g^{-1} in olive oil	Velasco et al., 2002
EFAs extracted after acidification in hexane: ether and methylated using BF3 in methanol.	GC-MS in single ion monitoring mode. Calculation as GC peak areas	Blood plasma lipid samples of women	Up to 0·08 mmol L^{-1} for diepoxy lipids and 0.18 mmol L^{-1} for mono epoxy lipids	Wilson et al, 2002
SPE silica on column transmethylation in methanol	GC-FID; split-splitless injector and Rtx 2330 column. Calculation as GC peak areas	Thermoxidised FAMEs	1.46% in methyl oleate and 1.75% in methyl elaidate	Lercker et al., 2003
According to Velasco et al., 2002	GC-FID; split-splitless injector and HP Innowax column	Themoxidised olive and sunflower oils	14.24 mg g^{-1} in olive oil and 9.44 mg g^{-1} in sunflower oil	Velasco et al., 2004
Direct sample base transmethylation	GC-FID COC injection on SP-2560 column. Calculation as GC peak areas	Food samples	40,300 mg kg^{-1} of sample (C18:1 EFA) and 10,200 mg kg^{-1} (C18:2 EFA) in biscuits oxidised for 3 days at 70°C	Fankhauser-Noti et al., 2006
Room temperature base transmethylation using C21:0 FAME as IS	GC-MS; split-splitless injector using BPX70 SGE column	Cotton seed, sunflower, palm and vegetable shortening oil heavily oxidised at 180°C	1536 µg g^{-1}in SFO fresh oils and 133.6 µg g^{-1} in CSO; 172.4 -1140.8 µg g^{-1} in heavily oxidised oils	Kalogeropoulos et al., 2007
Room temperature base transmethylation using C21:0 FAME as IS after two step SPE	GC-FID on split-splitless injector and DB-Wax column. Calculation as GC peak areas	Thermoxidised sunflower oil	LODs was 1.6, µg mL^{-1} for methyl trans-9,10 ES and detected up to 4.2 mg g^{-1}	Marmesat et al., 2008
Room temperature base transmethylation using C23 FAME as IS after two step SPE	High temperature GC-FID on split-splitless injector and VF-5ht Ultimetal column	Used frying fats and thermoxidised olive oil	Results reported as percentage polar fatty acids	Aguirre et al., 2010
Room temperature base transmethylation using C19:0 FAME as IS after three step SPE	GC-FID on COC injector and CP Sil 88 column. Calculation as GC peak areas	Fresh plant oils	LOD in µg g^{-1} of oil was 1.45 and quantified concentration was 0.03- 2 mg g^{-1} of oil	Mubiru et al., 2013
Room temperature base transmethylation using C17:0 FAME epoxy as IS after three step SPE	GC-FID on COC injector and CP Sil 88 column. Calculation as GC peak areas	Food matrices	LODs determined in µg g^{-1} sample were:2.8 (oil); 10.2 (crisps); 5.2 (pork) and 1.7 (milk)	Mubiru et al., 2014

SFO, sun flower oil; CSO, cotton seed oil; IS, internal standard

Previously the commonly used chromatographic columns during epoxy FAME analysis were the nonpolar columns (Berdeaux et al., 1999a; Marmesat, Velasco, & Dobarganes, 2008; Velasco et al., 2002). This was mainly because on a nonpolar column, polar fatty acids could be analysed without any problem of sticking to the column. However, this approach suffers from the disadvantage of failure to separate the different isomers because of coelution. Also, it was not possible to separate the FAMEs based on the number of double bonds. But now it is possible to separate isomers on a polar column after a pre-separation step on a silica pre-column. Recently a method was validated that involves the use of a polar column to analyse the different EFAs in oils and food matrices (Mubiru et al., 2013, 2014). The use of polar columns is a promising approach which will help most lipid chemists to do mechanistic studies on EFAs because various isomers can individually be separated and detected. The common injector used is the split-splitless (S/SL) injector, however because of its drawbacks such as sample discrimination the best alternative is the cool on column (COC) injector (Spitzer, 1999).

1.7.4.3.3 GC-MS conditions

Qualitative analysis is mainly based on mass spectrometry (MS) (Christie, 2012; Kleiman & Spencer, 1973). Structure elucidation has mainly been based on mass spectra using the inbuilt spectral libraries. A number of studies have done structural analysis of EFAs using GC-MS (Christie, 2012; Cui et al., 2008b; Gunstone & Jacobsberg, 1972; Kleiman & Spencer, 1973). During such studies, the mass selective detector is normally operated in electron ionization mode at 70 eV. However, to avoid excessive fragmentation of the esters, the ionization energy can be reduced accordingly to make interpretation of the mass spectra easier. Many researchers have complained about the complexity of the mass spectral data (Kleiman & Spencer, 1973; Orellana-Coca et al., 2005). For instance, at 16 eV, Gunstone and Jacobsberg obtained more information when they examined 31 isomers of EFAs than when they did it at 70 eV (Gunstone & Jacobsberg, 1972).

1.8 RISK ASSESSEMENT OF FOOD CHEMICALS

1.8.1 Elements of risk assessment

Chemical risk assessment is the process by which the potential (or probability of) adverse health effects of exposure are characterized (Williams, James, & Roberts, 2000). It is a

scientifically based process aimed at estimating the likelihood and the extent of adverse effects occurring to humans because of possible exposure(s) to hazards and it consists of the following steps: hazard identification, hazard characterization, exposure assessment and risk characterization as illustrated in **Figure 1.3** (Derelanko & Hollinger, 2001). Risk assessment is often an uncertain process requiring considerable judgment and assumptions on the part of the risk assessor. The probability of harm can be expressed either qualitatively or quantitatively (Williams et al., 2000). Hazard identification can be considered as a qualitative risk assessment, i.e., it determines whether and to what degree it is scientifically correct to infer that toxic effects observed in one setting will also occur in other settings (De Vries, 1996). On the other hand, quantitative risk assessment is based on numerical data and analysis which derives inputs from concentration and consumption data.

To assess the health risks from food components, information on the components, estimation of the exposure to the components, the consumer and the interactions between the components and the consumer is needed. Quantitative risk assessment can be deterministic or probabilistic (De Vries, 1996). However, a criticism of quantitative risk assessments, specifically, risk assessments that produce a numerical estimate of risk, is that they often convey the impression of greater precision than exists. It is vitally important that risk assessments include qualitative information as well, such as a discussion of the uncertainties associated with the risk estimate and the extent to which evidence of a true human hazard is weak or controversial (Williams et al., 2000). Each step in the process of risk assessment is briefly discussed below.

1.8.2 Hazard identification

Biological, chemical and physical agents that are capable of causing adverse health effects and which may be present in a particular food or group of foods are identified before risk assessment can be done (Codex Alimentarius, 1999; WHO/FAO, 2009). This aims at evaluating whether the chemical has the potential to cause adverse health effects in humans by reviewing all available data on toxicity and the biological mechanism that leads to toxicity (Barlow et al., 2002). To assess the toxicity of chemical compounds, studies can be performed either on a whole animal (in vivo) or on cells (in vitro) or on parts of animals with minimal alteration (ex-vivo) and the data can be extrapolated to humans. From the range of studies and observations available, the nature of any toxicity or adverse health effects occurring and the affected target organs or target tissues are identified (WHO/FAO, 2009).

The risk assessment process involves extrapolation of dose-response data from animals to humans and quantitative estimates of human exposure. For this to be accomplished, a detailed understanding of interspecies differences, population diversity and environmental factors is critical. Comparative quantitative morphological, physiological and biochemical information is required for different species. The heterogeneity of exposed populations with respect to such factors as age, lifestyle and activity patterns must be characterized (Derelanko & Hollinger, 2001).

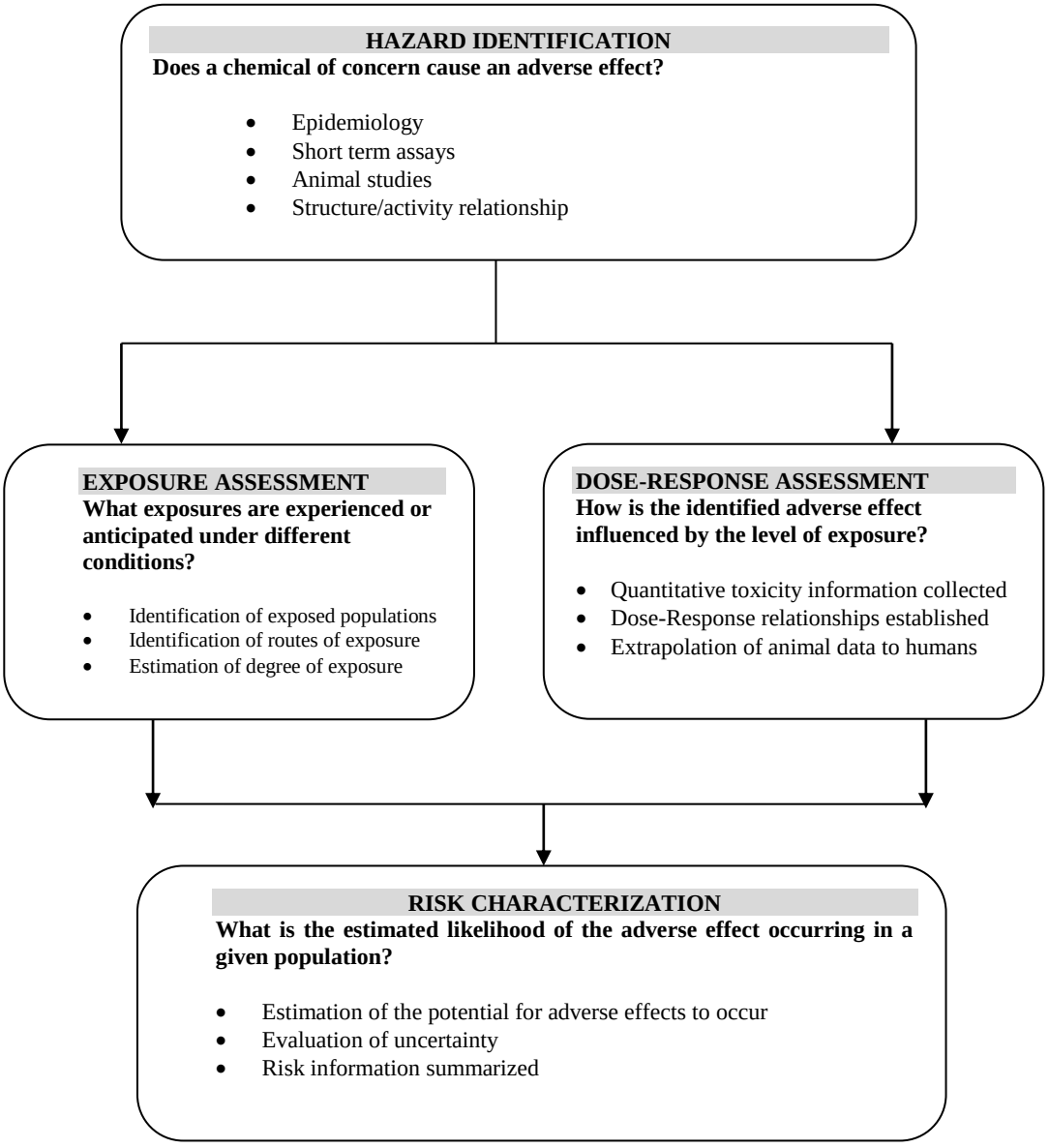


Figure 1.3: Elements of risk assessment process (Derelanko & Hollinger, 2001)

1.8.3 Hazard characterization

Hazard characterization is the qualitative and wherever possible quantitative description of the inherent property of an agent or situation having the potential to cause adverse effects. This should, where possible, include a dose–response assessment and its attendant uncertainties (WHO/IPCS, 2004). The hazard characterisation concerns the actual toxicological research to the possible adverse health effect of a compound on a living organism. Ideally it is the process of determining whether exposure to a chemical agent, under any exposure condition can cause an increase in the incidence or severity of an adverse health effect such as cancer, birth defect, neurotoxicity, etc. (Williams et al., 2000). During this stage the dose response is evaluated and toxic doses are documented. The dose-response assessment is the determination of the relationship between the magnitude of exposure to a chemical (dose) and the severity and frequency of the associated health effects (response) to humans. The dose-response curve generally takes two forms: the first displays the distribution of an effect within a population as a function of changing exposure and the second indicates the degree of change of an effect in an exposed individual of a population as a function of changing exposure.

Analysis of the dose-response curve can demonstrate average response, the degree of susceptibility within a population, and the range of exposure affecting hyper reactive individuals. The slope of the dose response curve categorizes the potency of the toxin and indicates the magnitude of effect associated with incremental increases in exposure (Derelanko & Hollinger, 2001).

Non-carcinogenic adverse effects observed in animal or humans are characterized by a threshold dose, below which no adverse effects are observed. Two thresholds are normally used, the first is the lowest observed-adverse-effect level (LOAEL) (Lowest found concentration or amount of a substance, which causes an adverse effect). The second is the no-observed-adverse-affect-level (NOAEL) and is the highest dose in the most sensitive experimental animal species which causes no toxic effects for the non-carcinogens (De Vries, 1996). The NOAEL can be divided by a safety factor to set a level which regulatory agencies have referred to as either an acceptable daily intake (ADI) or tolerable daily intake (TDI) or reference dose (RfD) (Williams et al., 2000). The ADI/TDI/RfD is an estimate of the amount of a food substance, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk (Barnes et al., 1988; De Vries, 1996). For genotoxic

carcinogens, a dose without a potential effect cannot be defined as it is assumed that there is no safe threshold (De Vries, 1996). Hence the previous advice of recommending that exposures should be reduced to As Low As Reasonably Achievable (ALARA) is considered to be of limited value (Benford et al., 2010).

1.8.4 Exposure assessment

To be able to know whether a population that consumes a certain type of food is at a risk, estimate of the chemical in question is done through exposure assessment. Exposure assessment is defined as the qualitative and/or quantitative evaluation of the likely intake of the hazard via food as well as exposure from other sources, if relevant (Codex Alimentarius, 1999). For food, the level ingested will be determined by the levels of the agent in the food and the amount consumed. Dietary exposure assessment takes into consideration the occurrence and concentrations of the chemical in the diet, the consumption patterns of the foods containing the chemical and the likelihood of consumers eating large amounts of the foods in question (high consumers) and of the chemical being present in these foods at high levels. Usually a range of intake or exposure estimates will be provided (e.g. for average consumers and for high consumers) and estimates may be broken down by subgroups of the population (e.g. infants, children, adults) (WHO/IPCS, 2004). The process of exposure assessment cannot be complete without reliable concentration data. In all analytical procedures, there are uncertainties associated with the various steps and techniques. For quantitative food chemical determination, the uncertainty is addressed by data censoring, where the criteria used to treat the “non-detects”, values which fall below the limit of detection (LOD) is well explained. Accordingly, non-detects are considered as zero (lower bound scenario), ½ LOD (medium bound scenario) or LOD (upper bound scenario) (Medeiros Vinci et al., 2012; WHO/FAO, 1985). A similar approach is use for samples which fall below the limit of quantification (LOQ), but above the limit of detection. Estimation of exposure to a compound is obtained after combining concentration data obtained in foods with the data related to their consumption by a given population. The commonly applied methodologies for exposure assessment are: single-point estimates or deterministic approach and probabilistic analysis.

The single-point or deterministic modeling involves using a single "best-guess" estimate of each variable within a model to determine the model's outcome(s). A deterministic approach to risk calculation is used where a single value is selected for each exposure variable and a

single risk estimate is produced. Sensitivities are then performed on the model to determine how much that outcome might vary from the model outcome. This is achieved by selecting various combinations for each input variable. These various combinations of possible values around the "best guess" are commonly known as "what if" scenarios. The model is often also stressed by putting in values that represent the best-case scenario and worst-case scenarios (Vose, 2008). The exposure assumptions are chosen to represent the plausible upper bound of exposure and the risk estimate is said to be associated with reasonable maximal exposure (RME) or high-end exposure (Williams et al., 2000). Normally the mean, the 95^{th} or the 97.5^{th} percentile of food consumption data is multiplied with the corresponding mean, 95^{th} or 97.5^{th} percentile of contamination data to obtain it. Conclusions from this approach are not always very accurate due to possible over or underestimation of the exposure levels.

Probabilistic analysis involves describing variables in terms of distributions to characterize their variability and/or uncertainty. In some cases, it uses distributions of food intake but with a fixed value for the concentration (Costa & Kristbergsson, 2008). In probabilistic risk assessment, input variables are entered as probability density functions (PDFs) instead of single values. For example, instead of using a single body weight of 70 kg in the risk calculation, a distribution of body weights would be entered that reflects the variability in body weight of the exposed population. These PDFs are then combined in such a way as to yield a risk distribution, representing the range and frequency of risks anticipated to exist in the exposed population. One of the most commonly used techniques to combine PDFs is Monte Carlo simulation. With Monte Carlo simulation, a computer program creates a simulated population designed to resemble the exposed population in every key aspect. For each risk calculation, it takes a value from each input PDF and calculates a numerical risk. This process is repeated several times (iterated) and the resulting range of risk values is tallied in the form of a distribution. This distribution represents the risk distribution for the population. From this distribution, the variability in risk among individuals can be visualized and the risk level at various percentiles of the population determined (Williams et al., 2000). This gives a more complete exposure assessment since the whole distribution of the consumption and concentration data is considered.

1.8.5 Risk characterization

In this final stage of the risk assessment process, information from the three previous steps are evaluated to produce a determination of the nature and magnitude of human risk. The

integration of information from other steps to develop a qualitative or quantitative estimate of the likelihood that any of the hazards associated with the chemical(s) of concern will be realized. The risk assessment process is completed with a summary of the risk information. The information developed in the risk assessment process will be utilized in the risk management processing which decisions are made as to the need for, the degree of and the steps to be taken to control exposures to the chemical of concern (Derelanko & Hollinger, 2001).

The characterization of risk must often encompass multiple populations having varying exposures and sensitivities. A descriptive characterization of the nature, severity and route dependency of any potential health effects, as well as variation within the population(s) of concern should be considered. Any uncertainties and limitations in the analysis are described in the risk characterization, so that the strengths, weaknesses and overall confidence in the risk estimates can be understood (Williams et al., 2000). Based on the risk characterization, the need for and the degree of risk management will be determined. When threshold levels are available, the risk can be estimated by direct comparison of the outcome of exposure assessment with the thresholds such as ADI or TDI or RfD. In absence of the threshold, the following approaches can be applied: extrapolation of higher dose data from rodent carcinogenicity assays in which 25% of the animals develop cancer (T25 value), application of the threshold of toxicological concern (TTC) approach as explained below and calculation of the margin of exposure (MOE) as explained further (O'Brien et al., 2006).

The threshold of toxicological concern (TTC) is a risk assessment tool for evaluating substances with few or no toxicity data. The TTC is a pragmatic risk assessment tool that is based on the principle of establishing a human exposure threshold value for all chemicals, below which there is a very low probability of an appreciable risk to human health (Cramer, Ford, & Hall, 1978; Kroes et al., 2004). It is to be considered as a screening tool for priority setting in an integrated testing strategy (Hennes, 2012). In absence of full toxicity database, the TTC concept uses the chemical structure of the chemical in question and the known toxicity of chemicals which share similar structural characteristics to set an exposure threshold value below which there is very low probability of a risk to human health (Kroes et al., 2004). The US FDA developed a so-called 'threshold of regulation' value, which is defined as 0.5 ppb in the diet corresponding to an exposure of 1.5 µg/person/day. Because EFAs are not known to be carcinogenic and they have unknown toxicity, this value can

tentatively be used for the EFAs in exposure assessment to characterize their risk to human consumers (Hennes, 2012). A decision tree provides a systematic structured approach for consistent application of the TTC principle to chemicals in food at low exposure. When chemicals are assessed using the TTC approach, a review of prior knowledge and use should always be performed preceding application of the decision tree (Kroes, Kleiner, & Renwick, 2005). Chemicals are classified into three classes according to Cramer decision tree and the TTC values for chemicals belonging to Cramer class I, II and III are, 1800, 540, 90 μg person d^{-1} respectively (Cramer et al., 1978).

Numerical estimates of the risk associated with the human exposure can be derived by extrapolation of the animal dose–response data or using the median toxic dose (TD50), or the dose giving a 25% incidence of cancer in an appropriately designed animal experiment (T25). However, this approach has a high level of uncertainty related to the mathematical models applied which do not always reflect the complexity of the biological phenomena involved and may not estimate the real risk for humans (Kroes et al., 2004). Validity of this approach depends on the quality of the data used to define the dose–response relationship, the exposure data and the relevance of the mathematical extrapolation model (O'Brien et al., 2006).

The MOE approach can be applied to both the exposure to one individual substance or the aggregate exposures to a group of substances with a similar toxicological profile (Benford et al., 2010). The MOE is defined as the ratio of the no-observed-adverse-effect level (NOAEL) or benchmark dose lower confidence limit (BMDL) for the critical effect to the theoretical, predicted, or estimated exposure dose (WHO, 2009). It is not scientifically valid to identify a NOAEL for substances that are genotoxic and carcinogenic. Therefore, the MOE is calculated from a point of departure (PoD) (also known as a reference point) on the dose–response relationship curve. The benchmark dose (BMD) approach offers the best tool for deriving a suitable PoD. The dose that causes a low but measurable response (benchmark response or BMR, typically 5% or 10%) is designated as the BMD, and its lower 95% confidence limit is the BMDL. The BMDL is considered as the most appropriate PoD since it accommodates uncertainty in the data. A benchmark response (BMR) of 10% is preferred to 5%, since the modelling of lower responses generally results in greater uncertainty. If the data is insufficient to derive a $BMDL_{10}$, then use of the T25 is the preferred option (Barlow et al., 2006).

Interpretation of the MOE for a genotoxic carcinogen would be difficult for risk managers, without some advice about the uncertainties, assumptions and limitations present in the data used to derive the ratio. Risk managers should be informed of the magnitude of an MOE that could be considered to represent a low priority for risk management actions, after considering uncertainties related to the precision of the dose– response relationship and the quality of the human exposure data, which in some cases may be quite poor (O'Brien et al., 2006). The magnitude of the MOE reflects but does not attempt to define the possible magnitude of the risk (O'Brien et al., 2006; Williams et al., 2000). The EFSA Scientific Committee considers that a MOE of 10,000 or more, based on genotoxicity or animal cancer bioassay data, ''would be of low concern from a public health point of view and might reasonably be considered as a low priority for risk management actions" (EFSA, 2005). Risk managers should be informed of the magnitude of an MOE that could be considered to represent a low priority for risk management actions, after considering uncertainties related to the precision of the dose–response relationship and the quality of the human exposure data, which in some cases may be quite poor (O'Brien et al., 2006).

1.9 TOXICITY AND TOXICOLOGY OF EFAS

There is a growing interest to EFAs as oxidation products due to their presumed high reactivity, high toxicity and possible absorption through diet (Goicoechea & Guillen, 2010). Toxicology studies performed on animals have shown that EFAs are toxic particularly *cis*-9,10-epoxystearic acid, *cis*-9,10-epoxyoctadec-12-enoic acid and *cis*-12,13-epoxyoctadec- 9-enoic acid (Chu et al., 1980; Le Quéré, Plée-Gautier, Potin, Madec, & Salaün, 2004). The last two compounds are commonly known as leukotoxin and isoleukotoxin respectively. They are called so because they produce their primary toxic effects against leukocytes (Hayakawa et al., 1986). These acids can be formed endogenously and can cause degeneration and necrosis of leukocytes and they are associated with multiple organ failure, breast cancer, cell proliferation in vitro and disruption of reproductive functions in rats (Goicoechea & Guillen, 2010). It has been previously demonstrated that the observed toxicity of leukotoxin and isoleukotoxin is due to the metabolism of the epoxides to their corresponding diols by soluble epoxy hydrolase (Moghaddam et al., 1997).

For a chemical to exert its potency, it must be absorbed into the blood stream. Although EFAs are absorbed in humans, their absorption has been reported to be poor which may lead to accumulation in the colon and this may be linked to colon cancer. Wilson et al, 2002, found

that dietary mono EFAs are absorbed intact and are not converted to vicinal dihydroxy fatty acids and that 17% was absorbed. However, mono EFAs were better absorbed than di-EFAs (Wilson et al., 2002). During in vitro studies, the toxicity of Leukotoxin and isoleukotoxin methyl esters to cells was further confirmed to be due to the enzyme soluble epoxide hydrolase (sEH) metabolizing the acids to more water soluble hydroxyl fatty acids (diols) (Greene, Newman, Williamson, & Hammock, 2000a; Greene, Williamson, Newman, Morisseau, & Hammock, 2000b; Halarnkar, Wixtrom, Silva, & Hammock, 1989) which are more toxic (Hayakawa et al., 1990; Moghaddam et al., 1997; Zheng, Plopper, Lakritz, Storms, & Hammock, 2001). Furthermore, it is believed that dietary EFAs could exert their toxic effects through their reactivity towards amines, SH-groups of proteins and DNA (Wilson et al., 2002).

In a study on mice, a lethal concentration (LC_{50} = 0.040 – 0.126 mM) for the leukotoxin and isoleukotoxin in presence of human soluble epoxy hydrolase (hsES) was reported (Greene et al., 2000a). Whereas cytotocity studies done on rabbit renal tubule models using linoleic acid and linolenic acid monoepoxides and their diols showed toxicity at 100-500 µM concentrations (Moran et al., 2000). Additionally, the leukotoxin dose resulting in 50% mortality was approximately 400 mg/kg, whereas that of the diols of leukotoxin was around 100 mg/kg (Zheng et al., 2001). A study on in vitro cytotoxicity performed in *Spodoptera frugiperda* (Sf-21) cells with human soluble epoxy hydrolase (hsEH) and β-galactosidase (Lac Z) as control enzymes also elucidated the toxicity of EFAs. This study showed that toxicity of EFAs to cells depends on several factors among which the hydrocarbon chain length was critical. Toxicity was observed to increase from C11-18 with exception of one isomer (Z)-6,7-epoxyoctadecanoic acid methyl ester and then it decreased with increase in chain length. Increasing ester length gradually decreased the toxicity of the compounds (Greene et al., 2000a). Cytochrome P450 is able to epoxidise linoleic acid to produce active epoxy metabolites which are associated with many pathological conditions which lead to renal failure (Le Quéré et al., 2004; Mitchell, Moran, & Grant, 2002; Moran et al., 2000; Moran, Mon, Hendrickson, Mitchell, & Grant, 2001). However, in a more recent study, cytotoxicity of epoxystearic acid has been investigated in human liver carcinoma cells (HepG2 cells). Results indicate that this could induce cytotoxicity, DNA damage, apoptosis, and oxidative stress (Liu, Cheng, Li, Wang, & Liu, 2018).

To judge toxicity hazards, acceptable daily intake (ADI) or tolerable daily intake (TDI) values are used, which are released by the Scientific Committee on Food and the European Food Safety Authority (EFSA). Toxicity data to set tolerable standards for EFAs has not been readily available. However, based on ESBO with a minimum 7% - maximum 8% oxirane oxygen content, the EFSA suggested a TDI (tolerable daily intake) of 1 mg/kg bw/day resulting in a maximum tolerated migration corresponding to the overall migration limit (60 mg/kg) for a 60 kg adult (Hammarling et al., 1998).

Two systems of classifying chemicals per their toxicity can be used to group EFAs**.** According to International Agency for Research on Cancer (IARC) studies, EFAs have not shown to be potent carcinogens. One EFA which is more abundant, *cis*-9,10-epoxystearic acid, is classified into class III a group for compounds that are not classifiable according to their carcinogenicity to humans (IARC, 1999; IARC/WHO, 1987). This classification however, does not consider toxicity of a chemical but only its probability to cause cancer. In addition, this approach does not allow deriving a health-based safety value.

A second approach is typically used for chemicals for which limited toxicological studies are available or for which no health-based safety values were derived yet. As explained before, this approach is based on the chemical structure of the molecule and proposes, a structure-dependent default tolerable daily intake, on basis of a decision tree (Toxtree version 2.5.4 available online). As such, EFAs are classified as Class III substances which are highly toxic with a tolerable daily intake of 1.5 µg kg^{-1} bw day^{-1} taking a body weight of 60 kg. The decision tree classifies every structurally defined organic or metallo-organic chemical based on structure and biochemistry. This classification is concerned with oral toxicity (Cramer et al., 1978). Accordingly, Class III substances contain structural features that permit no strong initial presumptions of safety, or that may even suggest significant toxicity (Cramer et al., 1978; Munro, Renwick, & Danielewska-Nikiel, 2008). These compounds have low reported threshold which implies that they deserve high priority for further investigation. This is particularly when per capita intake is high or a significant subsection of the population has a high intake. The implied hazard would then require the most extensive evidence for safety-in use (Cramer et al., 1978).

In the proceeding chapters, development and validation of new methods for the analysis of EFAs will be discussed. Furthermore, studies on exposure assessment and characterization of the potential risk in the Belgian population in relation to consumption of specific food

categories will be discussed. Finally, the formation of EFAs under various conditions and its reactivity towards other biomolecules is investigated.

CHAPTER

2 IMPROVED GAS CHROMATOGRAPHY-FLAME IONISATION DETECTOR ANALYTICAL METHOD FOR THE ANALYSIS OF EPOXY FATTY ACIDS

Redrafted from:

Edward Mubiru, Kshitij Shrestha, Antonios Papastergiadis and Bruno De Meulenaer (2013). "Improved gas chromatography-flame ionization detector analytical method for the analysis of epoxy fatty acids." Journal of Chromatography A 1318: 217-225.

Oral presentation:

May 4 - 7th, 2014: The 105th AOCS Annual Meeting & Expo, San Antonio, Texas, USA.

ABSTRACT

In this study an improved method for analysis of epoxy fatty acids is reported. Data obtained from analysis of polar fatty acids has previously been presented, but due to the high number of compounds that co-elute in the polar fraction, the resultant chromatograms are complex which may lead to compromising the accuracy of the data. A three steps separation of fatty acid methyl esters (FAMEs) by solid-phase extraction (SPE) on a silica gel column to remove hydroxy fatty acid interferences was proposed. This approach is opposed to a two-step separation procedure that has been often used to prevent analytical interferences caused by non-altered fatty acids. A gas chromatograph with a flame ionisation detector (GC-FID) equipped with a polar CP-Sil 88™ column was used. Quantification was based on the use of methyl nonadecanoate (C19:0), as an internal standard. Individual mono EFAs were well separated without co-eluting compounds. The optimised method was finally applied to screen EFAs in 37 fresh oil samples. Results obtained for the total EFAs were in the range 0.03 – 2 mg g^{-1} of oil with repeatability coefficient of variation (CV) ranging from 2.8 to 9.9% for duplicate analysis showing that the results obtained are repeatable.

Keywords: Lipid oxidation, epoxy fatty acids, oxygenated polar fatty acids, GC-FID, FAMEs

2.1 INTRODUCTION

Lipid oxidation is one of the most important chemical reactions in food which results in food deterioration. In addition, polar fatty acids with one or more oxygenated functional groups such as epoxy, keto and hydroxyl can be formed in at least one of the fatty acyl chains of the triacylglycerols. These compounds are believed to be stable final products that result from the decomposition of hydroperoxides (Berdeaux, Dutta, Dobarganes, & Sebeio, 2009). The major precursors of these oxygenated fatty acids are polyunsaturated fatty acids (PUFAs) (Cui et al., 2008b; Dobarganes, 2009). Because the drive today is to enrich oils with polyunsaturated fatty acids thus making them more prone to oxidation, the need for sensitive methods for analysis of these compounds is timely. The analysis of oxygenated fatty acids has received less attention and yet they are reported to form in high amounts especially in thermally oxidised foods (Marmesat et al., 2008; Velasco et al., 2002; Velasco et al., 2004). Generally these oxygenated fatty acids have been reported to have toxic effects (Christie, 2012; Greene et al., 2000a), particularly the long chain epoxy compounds have been reported to be protoxins (Greene et al., 2000a).

The analysis of oxygenated polar fatty acids is generally based on the pre-separation of polar and non-polar fatty acid methyl esters (FAMEs) in a silica column followed by the gas chromatographic separation coupled with a flame ionisation detector (GC-FID). However, this analysis is challenging as revealed by the complex gas chromatograms reported earlier (Fankhauser-Noti et al., 2006; Kalogeropoulos, Salta, Chiou, & Andrikopoulos, 2007; Marmesat et al., 2008; Velasco et al., 2002) due to coelution and interference from other compounds especially the hydroxy fatty acids.

The objective of the study was to improve the method of analysis of EFAs. Emphasis was put on the EFAs containing eighteen carbon atoms. To achieve this, a simple three steps SPE of the methyl esters on 10% moisture silica gel column has been proposed. The three steps SPE involved the use of three solvent systems of different polarity to partition the FAMEs.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and materials

Tert- butyl methyl ether (TBME) and 3-chloroperoxybenzoic acid (70-75%) were purchased from Acros Organics (Geel, Belgium). Sodium methoxide solution (25%), silica gel 60 for column chromatography (particle size = 0.063-0.100 mm), methyl 12-oxostearate and methyl 12-hydroxystearate were purchased from Sigma Aldrich (St. Louis, MO, USA). Methyl oleate (C18:1), methyl linoleate (C18:2), methyl linolenate (C18:3), methyl nonadecanoate (C19:0) and a mixed GLC 68D standard were obtained from Nu-Chek-Prep. Inc (USA). All other chemicals and reagents were of analytical grade and obtained from the local suppliers.

2.2.2 Oil samples

A total of 37 fresh oil samples were purchased from five different local markets in Belgium. They were brought to the laboratory and analysed immediately. No details of their previous history were retrieved.

2.2.3 Qualitative Standards

Standards that were used for qualitative identification of EFAs on GC-FID were prepared by thermoxidising methyl oleate, linoleate, α-linolenate and sunflower oil in a Muffle furnace (Heraeus Instruments, Germany) for 10 hr at 180 ± 2°C. About 1 g of the FAMEs and sunflower oil was placed in a Duran test tube (GL 14) without a cap and put in a beaker containing glycerol to aid uniform transfer of heat to the samples.

2.2.4 Synthesis of *cis*-9, 10-epoxystearate a quantitative standard

The epoxy analyte was synthesized based on a method described by Gunstone and Jacobsberg as reported by Christie (Christie, 2011a). The methyl oleate was reacted with *3*-chloroperoxybenzoic acid in chloroform at room temperature for 4 hr. The epoxy ester was purified on silica gel column (25 g) by eluting the unreacted fatty acids with 200 mL hexane-diethyl ether (98:2 v/v) as the mobile phase and the EFA with 300 mL of hexane-diethyl ether (90:10 v/v). Confirmation of the identity and purity of the synthesized compound was done by use of GC-MS.

2.2.5 Base-catalyzed- transmethylation with sodium methoxide at room temperature

Transmethylation followed Berdeaux *et al*. method (Berdeaux et al., 1999a) with modifications: Briefly 500 mg oil sample was accurately weighed into 25 mL glass centrifuge tube and a volume of 5.0 mL of TBME was added. Then, 2.5 mL volume of 0.2 M sodium methoxide solution in methanol was added and vortexed for 1 min, and allowed to stand at room temperature for 2 min. For neutralization purposes and prevent saponification, a 0.17 mL volume of 0.5 M sulphuric acid was added and the mixture was vortexed for a few seconds. Finally, 5.0 mL of water was added, vortexed for 30 s and centrifuged at 3600 ×*g* for 1 min. The organic layer was collected over a layer of sodium sulphate and the extraction was repeated two times with 5.0 mL of TBME. The organic layer was evaporated on a rotavap and finally dried under nitrogen. The resultant FAME was dissolved into 5.0 mL of n-hexane–diethyl ether (98:2, v/v) and accurately 2.0 mL was loaded onto the silica column.

2.2.6 SPE column preparation and packing

Silica gel was dried in a muffle furnace at 450°C for 12 hr and later cooled in a desiccator. Finally, the moisture content was adjusted to 10% and equilibrated on a shaker for 1 hr before use. An empty SPE cartridge column (6 mL, 6.5 x 1.3 cm) was filled with 2.0 mL of the elution solvent; n-hexane–diethyl ether (98:2, v/v). Silica gel slurry was prepared by mixing 1.0 g in 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.

2.2.7 Optimisation of the column chromatography-partitioning

An aliquot of 2.0 mL FAMEs was loaded onto the prepared silica column and the non-polar fraction which comprises of the non-altered FAMEs was eluted with 15.0 mL of n-hexane–diethyl ether (98:2, v/v). The remaining polar compounds in the silica were eluted in two steps: polar fraction 1 comprising mainly of epoxy compounds was eluted with 15.0 mL of n-hexane-diethyl ether (90:10, v/v) and 10 µg of methyl nonadecanoate (C19:0) prepared in iso-octane, was added as internal standard. The second fraction, polar fraction 2 mainly comprising of the hydroxy fatty acids was eluted with 25 mL of hexane-diethyl ether (70:30, v/v). Separation of the fractions was confirmed by thin-layer chromatography (TLC) as per Marmesat (Marmesat et

al., 2008), using a plate of silica gel 60 (5 cm×10 cm plates, 0.25 mm thickness). The plate was deliberately overloaded, developed with hexane–diethyl ether–acetic acid (80:20:1, v/v/v) and visualized with iodine vapour. Results from TLC showed that the non-polar, the polar fraction 1 and the polar fraction 2 were clearly separated.

2.2.8 SPE recoveries and silylation of the FAMEs

Recoveries of the analytes were determined on a model system consisting of a mixture of methyl *trans*-10-heptadecanoate, methyl *cis*-9,10-epoxystearate, methyl 12-oxostearate and methyl 12-hydroxystearate in iso-octane at the same concentration level of 100 µg mL^{-1}. The mixture (1.0 mL) was separated on the silica columns in triplicate and the recoveries were calculated. The quantification was based on methyl nonadecanoate as an internal standard. The recovery was calculated as the ratio of the concentration of analyte remaining after the separation divided by the analyte's initial concentration in the mixture before partitioning (Harvey, 2000). The fractions were silylated using 10% trimethylchlorosilane (TMCS) in N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA). The FAMEs to be silylated were dried under nitrogen using a nitrogen generator (Domnick Hunter, Parker, Cleveland, USA) and the silylating reagent was added in excess (100 µL). The reaction could proceed at room temperature for 20 min, the reagent was evaporated under nitrogen and then the analytes were dissolved in iso-octane prior to GC injection.

2.2.9 Analytical methods

2.2.9.1 Gas chromatography-FID conditions

The FAMEs were analyzed by GC-FID using an Agilent 6890N series gas chromatograph (Agilent, USA). The samples were dissolved in iso-octane, 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 x 0.25 mm I.D) capillary column coated with a 0.2 µm film. Deactivated fused silica pre-column 3 m x 0.25 mm i.d. (Agilent, Belgium) 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^{-1}, 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) was 40, 400 and 20 mL min^{-1} respectively. The column flow rate of helium as a carrier gas was 1 mL min^{-1}. Identification of individual FAMEs was carried out by comparison of retention times with those of authentic

standards (GLC 68D, Nu-Chek Prep, Inc, and USA). The epoxy FAMEs were confirmed by use of GC-MS and for quantification the response factor based on epoxystearate was used.

2.2.9.2 Gas chromatography-mass spectrometer conditions

A GC-MS was used for the qualitative identification of the polar FAMEs. The FAMEs were injected in an Agilent 7890A GC equipped with a 5975C Mass Spectrometer (Agilent Technologies, Palo Alto, CA). Chromatographic conditions and column were the same as those applied on GC-FID. The injection volume was 1 µL and the PTV injector was kept at 53°C for 0.2 min. then ramped to 200°C at a rate of 700°C min^{-1}. The MSD conditions were: capillary direct interface temperature, 250 ^{o}C; ionization energy, 70 eV; operating in a scan mode between *m/z* 30 and *m/z* 600; scan rate 3.64 cycles sec^{-1}.

2.2.9.3 Fatty acid composition and peroxide value (PV) analysis

Fatty acid composition was determined after preparation of FAMEs according to American Oil Chemists' Society (AOCS) Official Method (Ce 1b-89) (AOCS, 1990). The peroxide value was determined according to AOCS official method (Cd 8b-90) (AOCS, 2011).

2.2.10 Statistical analyses

Analytical determinations were carried out in duplicate unless otherwise indicated. Evaluation of the standard deviation from duplicate results for reliability testing was performed according to Synek (Synek, 2008).

2.3 RESULTS AND DISCUSSION

2.3.1 Optimisation of the SPE separation and spectral peak identification

FAMEs obtained after transesterification of the thermally oxidised sunflower oil were separated into polar and non-polar FAMEs using the extensively applied two steps SPE method (Berdeaux et al., 2009; Kalogeropoulos et al., 2007; Marmesat et al., 2008; Velasco et al., 2002; Velasco et al., 2004). The gas chromatograms of these fractions analysed on a CP-Sil 88™ GC column are shown in **Figure 2.1**. As the hydroxy compounds, do not elute easily from this column, resulting in the formation of broad peaks, the polar fraction was analysed only after silylation. Identification of the peaks was based on the results obtained from mass spectrometry data and

are shown in **Table 2.1.** Spectral data interpretation in this table was done by consulting study reports (Berdeaux et al., 2012; Christie, 2012; Esselman & Clagett, 1969; Kleiman & Spencer, 1973; Neff, Frankel, Scholfield, & Weisleder, 1978), reference to the NIST library and evaluating the fragmentation pattern of these compounds. Normally it is difficult to identify cis or trans isomers by mass spectrometry, but the CP-Sil 88™ GC column used has a known elution order of trans isomers first. Correct elution order and peak identification corresponded well with the previous studies (Kalogeropoulos et al., 2007; Marmesat et al., 2008; Velasco et al., 2002) except for Velasco et al, (2004) who had a different elution order on an Innowax fused-silica capillary column.

In **Table 2.1**, two peaks of saturated EFA isomers from oleic acid, namely methyl *trans*-9,10-epoxystearate (*trans*-9,10-ES, peak 1) and methyl *cis*-9,10-epoxystearate (*cis*-9,10-ES, peak 2) were confirmed. Four unsaturated EFAs peaks (3-6) from linoleic acid namely methyl *trans*-12,13-epoxyoleate (*trans*-12,13-EO, peak 3), methyl *cis*-12,13-epoxyoleate (*cis*-12,13-EO, peak 5), methyl *trans*-9,10-epoxyoleate (*trans*-9,10-EO, peak 4) and methyl *cis*-9,10-epoxyoleate (*cis*-9,10-EO, peak 6) were confirmed as previously reported (Berdeaux et al., 1999b; Marmesat et al., 2008; Velasco et al., 2002).

In **Figure 2.1,** a peak corresponding to *cis*-9,10-ES is visibly distorted, while there are interfering compounds eluting together with *trans*-9,10-ES. These interfering compounds were not observed when the polar fraction was injected without silylation, inferring that they were because of hydroxy compounds. The interference due to silylated hydroxy compounds was further confirmed in GC-MS by studying the characteristics ions (*m/z* 73 and 75) due to silylation. It is not recommended to inject polar FAMEs containing such hydroxy compounds without silylation in this column, as these hydroxy compounds either stay on the column and shorten the column life or emerge very late as broad tailing peaks which would increase the analysis time. Furthermore, silylating agents can also quickly foul and eventually plug the GC injector inserts. To prolong column life, Christie (Christie, 2003) recommends a cleanup step after silylation by liquid-liquid extraction with hexane and water and then drying the organic phase over sodium sulphate. This extra step increases the analysis time and may also affect recoveries. In this way, such chromatographic interferences due to hydroxy compounds could present both qualitative and quantitative analytical problems for epoxy compounds and reduce the reliability of the results obtained.

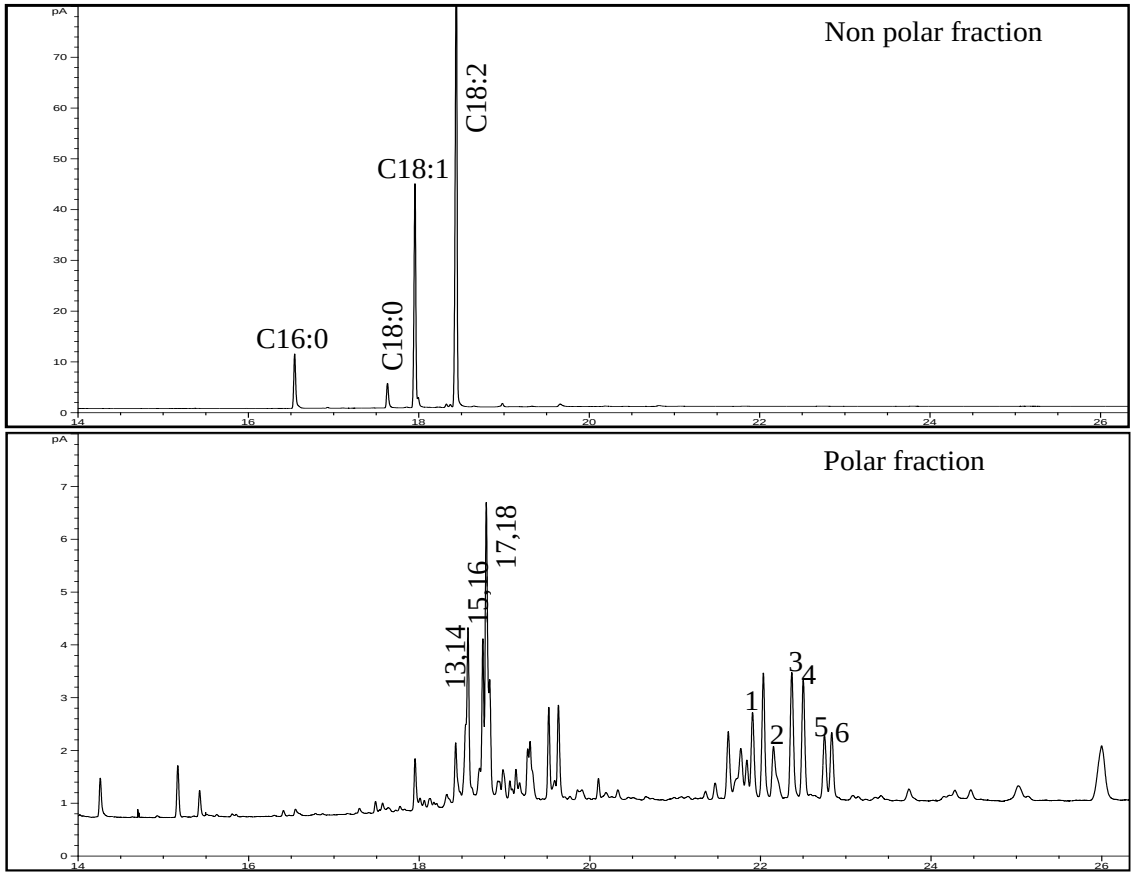


Figure 2.1: Chromatograms of the non polar and silylated polar fraction of sunflower oil FAMEs after two steps SPE showing distorted peaks on GC-FID coupled with a CP Sil 88 column. Peaks 1-18 see Table 2.1

To reduce the interferences, hydrogenation of the polar fatty acids to saturate them and reduce the number of peaks has been preferred by many researchers. Although it can partially solve the problem, quantification of the individual unsaturated polar fatty acids becomes impossible, as they would be converted into saturated ones. During this study, when the polar FAMEs from the thermoxidised methyl linoleate were hydrogenated, methyl *trans*-9,10-ES and methyl *trans*-12,13-ES co-eluted as a single peak, while methyl *cis*-9,10-ES and methyl *cis*-12,13-ES were separated individually. In this way, hydrogenation was not a solution for a better analytical method of EFAs as we would lose information not only about unsaturation, but also on the positional isomer distribution.

To remove the interferences due to hydroxy and oxo fatty acids, a three steps separation of FAMEs in a silica SPE column so as to collect the non-polar fatty acids, EFAs and hydroxy fatty acids in three different fractions was developed and has been described in the method. The developed method is based on the differences in polarity of the oxygenated fatty acids as

described previously by different researchers (Berdeaux et al., 1999a; Berdeaux et al., 1999b; Marmesat et al., 2008; Neff, Frankel, & Weisleder, 1981; Velasco et al., 2002; Velasco et al., 2004). The method was optimized using the mixture of thermally oxidised methyl oleate, methyl linoleate and methyl linolenate. Results of the GC-FID chromatograms obtained after a three steps SPE separation on silica gel column are shown in **Figure 2.2**. The GLC 68D standard has also been presented on the same **Figure 2.2A** to evaluate the interference that could be expected from the neutral fatty acids.

Table 2.1: Mass spectral ions of some of the polar fatty acid methyl esters identified in the different fractions after SPE separation

Name of compound	Peak	Spectral ions; *m/z* (relative abundance%)
Methyl 9,10-epoxyoctadecanoate	1 (*trans*) 2 (*cis*)	*312 (ND), 281 (1),* **155 (70), 199 (10),** 294 (7), 171 (11), 109 (29), 97 (41), 83 (44), 55 (100)
Methyl 12,13-epoxy-octadec-9-enoate	3 (*trans*) 5 (cis)	*310 (1), 279 (3),* **164 (20), 207 (4),** 167 (18), 149 (11), 136 (21), 123 (23), 99 (45), 95 (57), 81 (89), 74 (25), 55 (100)
Methyl 9,10-epoxy-octadec-12-enoate	4 (*trans*) 6 (*cis*)	*310 (1), 279 (2), 200 (4),* **155 (26), 185 (12),** 168 (9), 135 (10), 109 (25), 95 (44), 81 (70), 67 (70), 55 (100)
Methyl 12,13-epoxy-9,15-octadecadienoate	7 (*trans*) 9 (*cis*)	*308 (1), 277 (2), 189 (5), 171 (6), 151 (6), 239 (2),***207 (11), 111 (38),** 161 (9),189 (5) ,211 (1), 147 (18),123 (17), 108 (10), 95 (47), 83 (61), 81 (77), 67 (100)
Methyl 15,16-epoxy-9,12-octadecadienoate	8 (*trans*) 11 (*cis*)	*308 (1), 279 (1), 189 (2), 250 (1),* **236 (10), 247 (1),** 207 (2), 189 (2), 121 (20), 107 (37), 79 (100), 67 (49)
Methyl 9,10-epoxy-12,15-octadecadienoate	10 (*trans*) 12 (cis)	*308 (ND),* **185 (4), 155 (17),108 (52),** 167 (1), 93 (42), 79 (100), 67 (23)
Methyl 9-hydroxyoctadecanoate trimethylsilylether	13	*386 (ND), 371 (1), 355 (2), 339 (4),* **229 (89), 259 (100),**155 (16), 129 (28), 103 (14), 75 (56), 73 (85)
Methyl 10-hydroxyoctadecanoate trimethylsilylether	14	*386 (ND), 372 (1), 355 (3), 339 (7),* **215 (100), 273 (95),**129 (3), 103 (13), 75 (33), 73 (55)
Methyl 12-hydroxy-octadec-9-enoate trimethylsilylether	15	*384 (ND), 369 (1), 353 (1), 337 (2),* **187 (100), 299 (3),** 270 (11), 129 (4), 103 (15), 97 (7), 81 (6), 75 (14), 73 (52)
Methyl 10-hydroxy-octadec-12-enoate trimethylsilylether	16	*384 (ND), 369 (1), 353 (1), 337 (2),* **213 (5), 273 (100),** 185 (23), 173 (9), 169 (17), 129 (18), 103 (8), 97 (5), 81 (25), 75 (28), 73 (82)
Methyl 9-hydroxy-octadec-12-enoate trimethylsilylether	17	*384 (ND), 369 (3), 353 (1), 337 (5),* **227 (45), 259 (42),** 294 (19), 155 (27), 130 (32), 103 (11), 97 (12), 81 (56), 75 (57), 73 (100)
Methyl 13-hydroxy-octadec-9-enoate trimethylsilylether	18	*384 (ND), 369 (2), 353 (1), 337 (6),* **173 (75), 313 (17),** 130 (28), 103 (22), 97 (10),81 (45), 75 (61), 73 (100)

ND, not detected.

Italics: the common fragment M; M-15, M-31, M-43, M-47 for all silylated methyl esters; M-31 for EFAs.

Bold: The characteristic or identifier ions and the other most abundant fragments are presented in normal font.

Six mono epoxy FAMEs (peaks 1-6) from methyl oleate and methyl linoleate were observed in polar fraction 1 as shown in **Figure 2.2B**. The identification of these peaks using GC-MS has already been discussed. Additionally, six mono epoxy FAMEs (peak 7-12, **Table 2.1**) from

methyl linolenate were also observed in the same fraction, out of which coelution of peak 9 and 10 was confirmed by GC-MS.

These epoxy FAMEs eluted according to their polarity with the less polar one first (Cui et al., 2008b) in the order 12,13- epoxide, 15,16-epoxide and 9,10-epoxide. It is already reported that it is not easy to explain the fragmentation pattern of the methyl epoxy octadienoates on positive EI-mode (Kleiman & Spencer, 1973; Orellana-Coca et al., 2005), however, some characteristic ions were present to confirm the identity of the different EFAs. Methyl *trans*-12,13-epoxy-9,15-octadecadienoate (peak 7) and methyl *cis*-12,13-epoxy-9,15-octadecadienoate (peak 9) were confirmed by use of the molecular ion (m/z = 308) and the characteristic ions with m/z values 207 after loss of mass m/z =69 and methanol (m/z =32) on one side, and 111 which forms after loss of mass m/z =197 on the other side of the epoxy group. Methyl *trans*-15,16-epoxy-9,12-octadecadienoate (peak 8) and methyl *cis*-15,16-epoxy-9,12-octadecadienoate (peak 11) were confirmed by use of the molecular ion (m/z = 308) and the characteristic ions with m/z values 247 after loss of methanol and 236 which form after the cleavage on both sides of the epoxy group. Methyl *trans*-9,10-epoxy-12,15-octadecadienoate (peak 10) and methyl *cis*-9,10-epoxy-12,15-octadecadienoate (peak 12) were confirmed by use of the molecular ion (m/z = 308) and the characteristic ions with m/z values 108 and 155 which form after the cleavage on both sides of the epoxy group. Also ion m/z = 185 due to cleavage inside the epoxy ring was characteristic to these isomers (Orellana-Coca et al., 2005). When the polar fraction 1 was injected in GC-FID after silylation, the interfering hydroxy FAMEs were not detected in the chromatogram, confirming their removal from this fraction. The polar fraction 2 was injected in GC-FID after silylation to reveal the hydroxy FAMEs and has been shown in **Figure 2.2C**. In **Table 2.1,** two hydroxy trimethylsilyl ether derivatives which were partially separated on GC were identified as monohydroxyoctadecanoates (peak 13&14). Two isomers, 9-OH (peak 13) and 10-OH (peak 14) were confirmed by the base peak at *m/z* = 259. Four peaks, (15-18) eluting very closely were found to be the positional isomers of hydroxyoctadecenoates. These were confirmed to be 12-hydroxyoactadecenoate (peak 15), 10-hydroxyoctadecenoate (peak 16), 9-hydroxyoctadecenoate (peak 17) and 13-hydroxyoctadecenoate (peak 18) as previously reported (Neff et al., 1978).

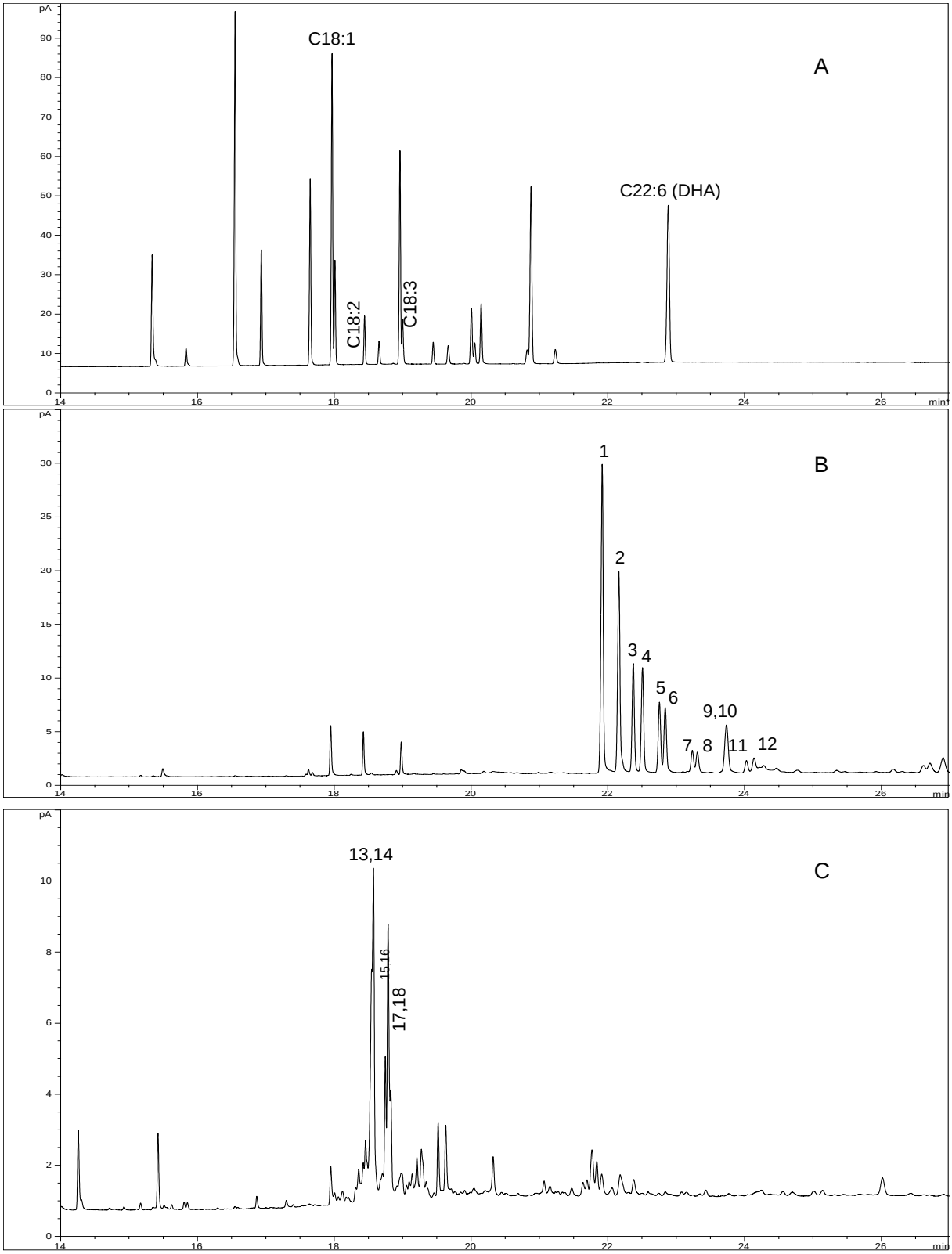


Figure 2.2: Chromatograms of a GLC 68D standard and a mixture of C18:1, C18:2 and C18:3 thermoxidised FAMEs after three steps SPE showing well resolved peaks on GC-FID coupled with a CP Sil 88 column. (A) GLC 68D standard, (B) polar fraction 1 and (C) the silylated polar fraction 2. For peak identity, see Table 2.1

The results displayed in **Figure 2.2** demonstrate that the interferences observed after the two steps SPE separation (**Figure 2.1**) in the epoxy region can be removed by application of the proposed separation method. Use of three steps separation on silica column separated the hydroxy and other polar FAMEs, which led to obtaining high resolution chromatograms with well separated peaks of epoxy compounds. The interferences caused by some of the unmodified FAMEs such as C22:0, C24:0 and C24:1 as reported by different authors (Berdeaux, Velasco, Márquez-Ruiz, & Dobarganes, 2002; Kalogeropoulos et al., 2007; Velasco et al., 2002) were not observed because these unmodified FAMEs eluted earlier than the epoxy FAMEs. The only possible interference would come from methyl ester of docosahexaenoic acid (DHA) 22:6 a common fatty acid on fish oil which was not present in vegetable oils used in this study**.** Another advantage of the three steps SPE separation is that minor quantities of the unmodified FAMEs (C18:0, C18:1 and C18:2; **Figure 2.2B**) remaining in the silica column after elution of non-polar FAME fraction, would be completely removed during elution of polar fraction 1. Those FAMEs could otherwise interfere with quantification of hydroxy FAMEs if all the polar compounds would have been collected together in one fraction as reported previously in a two steps SPE separation.

The use of a three steps SPE separation and a polar FAME column CP-Sil 88™ enabled individual separation of each of the six EFA isomers obtained from C18:1 and C18:2 fatty acids that are common in most oils. Such well resolved peaks can be used in studies to determine isomeric distribution of EFAs in oxidised fats. It was found that at temperatures >180°C (data not shown), the trans isomers were dominating and in methyl oleate their percentage was 65%. The two trans isomers in methyl linoleate were both present at 30% each. Methyl linolenate although had two peaks co-eluting, their percentages were: 21% for *trans*-12,13-epoxide and 22% for *trans*-15,16-epoxide. The two peaks that co-eluted namely *trans*-9,10-epoxide and *cis*-12,13-epoxide were present at 3%. The two cis peaks namely *cis*-15,16-epoxide and *cis*-9,10-epoxide were present at 13% each.

2.3.2 Calibration curves, linearity, LOD, LOQ and recoveries

As commercial epoxy standards are not readily available, the analyte used in the recovery study was synthesized. The response factor used to quantify all the isomers was based on C19:0 as an internal standard and the synthesized *cis*-9,10-epoxystearate because similarity of the analytical response of saturated and unsaturated EFAs on GC-FID has been reported (Velasco et al., 2002).

The working range was 0, 20, 40, 60, 80 and 100 µg mL^{-1} and the internal standard was kept at 50 µg mL^{-1} in all cases. The response factor was calculated by linear regression of area ratio of the analyte to the internal standard (y axis) versus the concentration ratio of the analyte to the internal standard in iso-octane. The linearity obtained was satisfactory at this range with R^2 = 0.9992. The response factor obtained was 1.04 and this is what was used in all the calculations. The LODs and LOQs were determined by a method based on two variable regression of the calibration curve, LOD = 3 (SE / Slope) and LOQ = 2 x LOD; where SE is the standard error of the intercept. The LOD and LOQ were found to be 1.45 and 2.9 µg g^{-1} of oil respectively.

To assess the efficiency of the separation step, and to ascertain that there were no losses of the analytes of interest during separation, the developed method was evaluated for recoveries as this is critical in quantification. Good recoveries for all the analytes were obtained and with each compound being recovered in the fraction as expected (not shown). The percentage recoveries were in the range of 98% and above in all the three analytes (epoxy, oxo and hydroxy FAMEs) showing that the SPE conditions used were optimal. Silylation was done to confirm that there were no hydroxy compounds remaining in the epoxy fraction and the chromatograms obtained did not show any new peaks (data not shown).

2.3.3 Application of the developed method on fresh oil samples

To evaluate the developed method, 37 different fresh oil samples bought from the Belgian market were analysed EFA content. The results of the analyses are summarized in **Table 2.2**, where also the unsaturated fatty acids relevant in the production of EFAs are shown. To measure the extent of oxidation in the samples, the peroxide value (PV) was determined. Results showed that all the oils analysed were fresh as indicated by the very low peroxide values ranging from 0 to 12.55 meq O_2 kg^{-1}. However, it should be noted that some oils had a low percentage of fatty acids < 80% which is normally attributed to a high content of unsaponifiable matter or due to being oxidized. The latter is because during oxidation fatty acids undergo polymerisation and oxygenation reactions resulting in compounds which are not eluting from the column despite the use cool on-column injector. Repeatability was checked for the duplicate analysis and the CV was found to be 9.9% for EFAs in the range 0 -10, 6.9% for 11- 49, 4.8% for 50 - 249 and 2.8% for oils with EFA content of > 250 µg g^{-1} of oil.

Table 2.2: Epoxy fatty acids (µg g^{-1} of oil), PV (meq O2 kg^{-1} of oil) and fatty acid composition (g 100 g^{-1} of oil) in fresh oils obtained from Belgian markets

		Epoxy fatty acid composition (µg g^{-1} of oil) [a]						Fatty acid composition (g 100 g^{-1} of oil)			
Type of oil	PV[a]	*trans*-9,10-ES	*cis*-9,10-ES	*trans*-12,13-EO	*trans*-9,10-EO	*cis*-12,13-EO	*cis* – 9,10-EO	C18:1	C18:2	C18:3	Others
Arachid	1.5	22.8	685.0	10.3	6.9	65.7	350.1	43.7	7.5	0.0	10.4
Arachid	2.4	13.1	172.1	7.2	4.6	32.0	125.9	44.9	16.0	0.2	14.0
Colza	4.4	15.1	25.7	9.5	0.8	7.3	6.5	43.9	14.8	7.7	6.6
Colza	2.5	8.7	19.5	6.5	0.4	4.5	6.9	39.1	13.2	6.4	5.5
Colza	2.3	21.9	64.7	0.9	0.9	24.8	67.2	45.6	14.6	7.2	6.0
Colza	4.0	7.7	19.0	2.6	2.1	7.5	7.7	54.2	16.9	8.9	7.7
Colza	3.6	6.7	12.2	6.1	0.4	3.5	2.5	59.1	19.1	9.4	8.8
Corn	1.8	12.1	59.8	19.3	9.1	48.6	79.7	28.1	46.8	0.9	12.4
Corn	0.9	7.4	75.6	13.1	3.8	59.1	107.7	28.9	54.0	0.9	13.0
Corn	0.0	9.1	61.0	14.0	3.9	46.8	92.3	24.5	46.1	0.8	11.2
Corn	3.1	9.4	118.1	15.8	16.6	98.1	161.9	18.7	32.5	0.6	8.3
Corn	1.8	12.9	92.0	18.6	10.3	67.9	123.1	29.7	49.9	1.2	13.2
Frying	3.7	9.2	179.4	7.5	7.2	44.5	570.1	36.0	41.0	0.9	15.4
Frying	3.3	7.4	78.2	6.7	5.3	24.1	173.8	37.9	38.9	2.5	14.4
Frying	5.3	8.9	60.4	6.6	3.4	23.6	161.4	33.7	48.0	2.7	11.8
Frying	2.2	14.8	315.8	2.2	7.8	66.4	485.8	33.0	34.1	1.4	10.7
Frying	3.7	9.6	177.3	4.0	7.9	54.7	576.1	24.5	38.4	0.8	11.9
Frying	5.2	6.9	34.1	1.7	1.9	9.5	52.6	50.6	22.0	3.7	12.8
Frying	5.2	6.5	125.3	1.6	3.8	31.2	391.1	27.3	43.7	0.1	8.5
Frying	3.3	11.2	60.7	8.8	2.8	24.0	138.6	42.7	37.6	3.8	9.8
Mixture	4.5	13.7	95.3	9.3	2.9	24.5	109.6	43.5	23.3	3.8	7.8
Mixture	2.6	9.8	56.5	5.1	2.0	16.0	109.3	42.7	19.9	3.6	6.7
V.Olive	5.2	9.5	218.3	5.3	0.0	7.6	10.7	72.8	4.3	0.6	15.2
V.Olive	6.9	9.9	221.5	4.5	0.0	8.6	16.7	75.8	4.5	0.7	15.5
V.Olive	8.8	9.0	165.3	7.5	0.8	13.5	20.3	51.4	7.4	0.5	13.4
V.Olive	6.0	6.8	218.3	0.0	0.3	9.3	12.0	55.9	3.4	0.5	12.1
R.Olive	12.4	6.8	214.6	0.0	0.4	13.1	18.2	52.7	5.5	0.5	10.8
V.Olive	12.6	7.9	134.7	6.8	0.7	11.4	16.1	47.3	6.3	0.5	12.6
V.Olive	5.8	4.3	76.5	4.1	0.0	3.3	6.4	75.1	8.1	0.8	12.5
Plant oil	8.2	5.6	7.5	4.3	3.4	4.7	4.8	47.8	14.4	7.5	8.5
Salad	1.7	8.0	38.5	5.2	5.3	16.7	99.8	33.6	33.5	3.5	8.0
Soya	5.9	7.6	3.7	12.5	3.1	3.3	19.5	15.1	31.3	3.2	10.2
Sunflower	2.1	10.9	231.0	8.4	8.5	54.7	747.5	34.4	50.7	0.1	10.7
Sunflower	2.2	33.2	414.3	15.7	10.2	102.2	1434.0	23.8	60.8	0.1	10.7
Sunflower	1.8	12.8	397.7	5.7	13.4	110.4	1393.3	20.3	51.7	0.1	9.1
Sunflower	4.8	8.2	223.9	4.2	9.6	68.8	759.3	18.9	47.7	0.1	8.4
Sunflower	3.1	8.8	239.3	9.5	10.1	69.6	826.0	22.6	58.8	0.1	10.6

Abbreviations: ES, epoxystearate; EO, epoxyoleate; PV, peroxide value; Mixture, (Rape seed, high oleic sunflower, grape seed and corriander oil); Plant oil, (ω-3 enriched); Soypro, (liquid cooking margarine made from soy); V.Olive, virgin olive oil; R.Olive, refined olive oil; [a]Values are means of duplicate analyses.

Fatty acid composition results showed that most of the oils were rich in C18:1 and C18:2, except for colza and the plant oil which had significant amount of C18:3 fatty acids. Generally, all the oils had higher amounts of unsaturated EFAs corresponding to the original high amounts of unsaturated fatty acids and in all cases the *cis* isomers dominated. However, more *trans* fatty acids have been reported to form in thermally oxidized samples mainly due to the conversion of the *cis* isomers to more stable *trans* isomers at elevated temperatures that are normally used during deodorization of oils.

On average, sunflower oil had the highest amount of EFAs while colza oil had the lowest, despite the comparable PV values. Results of higher amounts of unsaturated EFAs in fresh oil samples are in a similar range as those obtained by Fankhauser-Noti *et al.* (2006). It was interesting to note that sunflower oil had comparable amounts of epoxy stearate to olive oil despite the low levels of C18:1 fatty acids in sunflower oil. This seems to suggest that in oils rich in PUFAs, the formation of epoxy stearates from oleic acid is facilitated.

To explain the observed trend of EFA content, correlations were performed and results are presented in **Figure 2.3** and **2.4**. There was a strong correlation ($p < 0.001$; r= 0.97; n = 37) between the fatty acids ratio (C18:1/C18:2) and the ratio of the amount of EFAs (ES/EO) formed**.** Also, a strong correlation ($p < 0.001$; r = 0.99; n = 28) exists between the total saturated and unsaturated EFAs of colza, corn, frying, mixture, plant, salad, soya and sunflower oils which had comparable amounts of C18:1 and C18:2. The strong correlation observed indicate that the rate of formation of the EFAs may be related to the amount of unsaturated fatty acids present in oil. However, there was no correlation ($p> 0.05$; r = -0.310; n = 37) between the EFAs formed with the peroxide value. The occurrence of high amounts of EFAs in fresh oil samples and the lack of correlation between PV and the EFAs may be because of multiple reasons: 1) PV is an unspecific method and it does not give any information about the concentration of the different peroxides present. 2) Probably be an indication that there is another more specific route of formation for EFAs apart from the breakdown of the hydroperoxides (Cahoon et al., 2002; Earle, 1970; Orellana-Coca et al., 2005). However, the known processes of formation of EFAs are through direct oxygen addition either at the site of the double bond or nearby it (Neff & Byrdwell, 1998). Also during thermal oxidation at 80°C it is reported that 9,10-epoxyoctadecanote can be formed by the reaction between oleate and oleate hydroperoxide (Frankel, 2005; Neff et al., 1978).

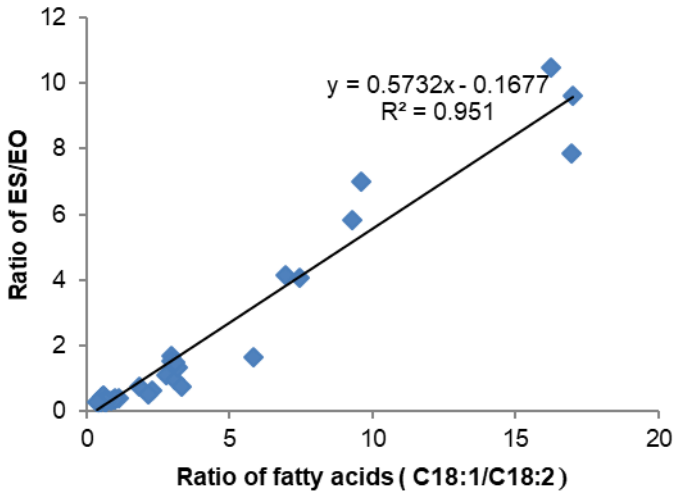


Figure 2.3: Regression of the ratio of EFAS and the ratio of major 18C fatty acids of fresh oils, (n=37). (ES) epoxystearate; (EO), epoxyoleate.

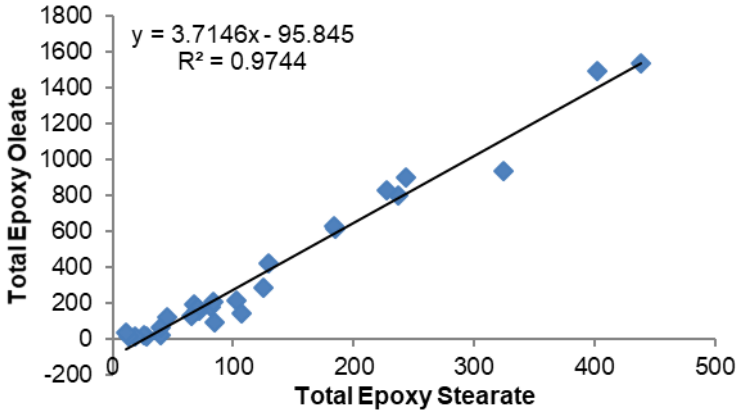


Figure 2.4: Regression of total saturated and unsaturated EFAs for oils (colza, corn, frying, mixture, plant, salad, soya and sunflower) with comparable amounts of C18:1 and C18:2 fatty acids (n =28)

Epoxidation can as well occur when a peracid reacts with hydroperoxides but in all these routes of formation, epoxidation happens by replacing the point of unsaturation in the fatty acid (Christie, 2012; Dobarganes, 2009). The total amounts of EFAs present in fresh oils varied between 0.03 – 2 mg g^{-1} of oil. Based on these amounts detected in the fresh oils, the safety issue of EFAs is being underestimated. However, due to lack of toxicity and intake data, the risk posed by these fatty acids cannot be inferred. Although it has not been possible to determine from this study how these fatty acids form in the oils that have been analysed,

the study has been able to separate the co-eluting hydroxy fatty acids thus accurately quantify the EFAs in oils.

2.4 CONCLUSIONS

The innovation of this method is the three steps SPE separation technique of the different fatty acids on silica gel which has improved the analysis of the EFAs. This purification step separates the co-eluting hydroxy fatty acids and thus better peaks are obtained on a polar FAME CP Sil 88™ GC column. After this step, it is possible to determine polar fatty acids in less oxidised samples because of increased sensitivity. The need to hydrogenate the FAMEs to avoid the coelution of hydroxy and fatty acids has been overcome thus determine each compound in its original form. Strong correlations were illustrated, between the ratios of fatty acids to the ratio of EFA formed and between saturated and unsaturated EFAs. The new approach exhibits excellent chromatographic performance with well resolved peaks and low detection limits.

CHAPTER

3

DEVELOPMENT AND VALIDATION OF A GAS CHROMATOGRAPHY-FLAME IONISATION DETECTOR METHOD FOR DETERMINATION OF EPOXY FATTY ACIDS IN FOOD MATRICES

Redrafted from:

Edward Mubiru, Kshitij Shrestha, Antonios Papastergiadis and Bruno De Meulenaer (2014). "Development and Validation of a Gas Chromatography–Flame Ionization Detection Method for the Determination of Epoxy Fatty Acids in Food Matrices." Journal of Agricultural and Food Chemistry 62(13): 2982-2988.

Oral presentation:

May 4 - 7th, 2014: The 105thAOCS Annual Meeting & Expo, San Antonio, Texas, USA.

ABSTRACT

A reliable and suitable method for the determination of epoxy fatty acids in various food matrices based on the Bligh and Dyer lipid extraction procedure was developed and validated. The method involves the use of a methylated EFA as internal standard (IS), extraction of the analytes from the matrices followed by room temperature methylation, a three-step solid phase extraction (SPE) separation of the fatty acid methyl esters (FAMEs) and detection with gas chromatography flame ionisation detection (GC-FID). The method was validated in four different food matrices chosen as model systems namely, vegetable oils, unprocessed pork, fried potato crisps and infant formula. The extraction technique allows the method to be applied for routine analysis of many samples. Intra-day repeatability ranged from 1 to 19% and inter-day reproducibility ranged from 2 to 9%. The limit of quantification (LOQ) ranged from 3.32 to 20.47 µg $g^{-1}$of sample with recoveries ranging from 94 to 115%. The results verify the accuracy and reproducibility of the analytical technique and its ability to provide reliable quantification of EFAs. Finally, levels of EFAs in several food products on the Belgian market were screened and presented.

Keywords: Lipid oxidation, epoxy fatty acids, fatty acid methyl esters, transesterification, Bligh and Dyer

3.1 INTRODUCTION

In a bid to provide convenient foods and to prolong their shelf life, food processing and packaging have been used more frequently. However, during food processing and storage, exposure to high temperatures, light, metal ions and oxygen makes foods susceptible to lipid oxidation and formation of its associated products (Cardenia, Waraho, Rodriguez-Estrada, McClements, & Decker, 2011; Choe & Min, 2006). Usually precautions such as active packaging, modified atmosphere packaging, vacuum packaging and many other techniques have been taken to prevent lipid oxidation (Tian, Decker, & Goddard, 2013). When lipid oxidation occurs in a food, it impacts on nutritional and safety of a food through, flavour deterioration and sometimes production of toxic compounds (Frankel, 2005).

To improve the analytics of EFAs in oils, a sensitive and accurate method used two internal standards and this required two methylation steps (Velasco et al., 2002; Velasco et al., 2004). Fankhauser-Noti et al., (2006) used a direct methylation method developed by Suter et al., (1997) to analyse EFAs without SPE column separation. This direct transesterification method in food matrices has a drawback that sometimes it involves use of pre-treatment steps which differ per food matrix and this limits its universal applicability to all food matrices (Suter et al., 1997).

To analyse EFAs from food samples, a method which can reliably extract oil from the food matrix is required. A good method for extraction of lipids from various food matrices should be able to overcome the interaction between the lipid and tissue matrix (Christie, 2003). In this study a universal method for extraction of EFAs from different fat containing food matrices based on a modified Bligh and Dyer method was validated and implemented. This method uses a synthesized epoxy FAME IS at the beginning of the analysis which reduces analytical variation within and between samples and has proved to be a robust and successful approach.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and reagents

Methyl *cis*-10-heptadecenoate (C17:1), and a mixed FAME GLC 68D standard were obtained from Nu-Chek-Prep. Inc. USA. Dichloromethane (99% purity) was bought from Chem-Lab NV (Zedelgem, Belgium) and methanol was obtained from Fischer Scientific (Tournai, Belgium). Other reagents are described in **Chapter 2 section 2.2.1.**

3.2.2 Standards

3.2.2.1 Synthesis of methyl cis-10,11-epoxyheptadecanoate (IS) and cis-9,10-epoxystearate

Both epoxy FAMEs of the IS and the *cis*-9,10-epoxystearate **(**analyte) were synthesized based on a stereospecific method described by Gunstone and Jacobsberg (1972). Briefly, the corresponding FAMEs of the IS and the analyte were reacted with *3*-chloroperoxybenzoic acid in chloroform at room temperature for 4 h with periodic agitation. The formed epoxy esters were purified on dry activated silica gel column (25 g) by eluting the unreacted fatty acids with 200 mL hexane-diethyl ether (98:2 v/v) as the mobile phase and the epoxy FAMEs were collected with 300 mL of hexane-diethyl ether (90:10 v/v). Confirmation of the identity and purity of the synthesized compounds was done by use of Gas chromatography–mass spectrometry (GC-MS).

3.2.2.2 Standard preparations

The synthesized IS and analyte at a concentration of 20 mg mL^{-1} each was prepared in isooctane. Stock solutions at a concentration of 10 mg mL^{-1} each were also prepared in isooctane, and all stored at -20°C. From the individual stock standard solutions, working solutions were prepared by making dilutions in isooctane to obtain a calibration range of 40, 80, 120, 160, 200 and 200, 400, 600, 800, 1000 µg mL^{-1} for the lower and upper range respectively. The upper range was diluted ten times before injection on GC-FID. All necessary precautions were taken to avoid photo-degradation of the light sensitive standards, such as wrapping the standard solutions and the extracts with aluminium foil and by storing them in the dark.

3.2.3 Samples

Validation was based on the following samples namely, coconut oil, crisps, milk powder (infant formula) and pork samples which were purchased from a supermarket in Belgium. Normally the samples would be brought to the laboratory on the day of analysis and analysed immediately. Several different food samples here referred to as market samples were screened for EFAs namely, mayonnaise, crisps, biscuits, peanuts, minced beef, cooked ham, butter-margarine and a special biscuit known as speculoos were bought from supermarkets in Belgium and analysed.

3.2.3.1 Sample preparation

Validation samples: Ground and homogenized sample (except oil) of 500 ± 0.50 mg was weighed into a 250-mL glass centrifuge tube and spiked with epoxy analyte at different concentrations (0- 200 µg g^{-1} of sample). A fixed concentration (50 µg) of the IS was always added. Water was adjusted to have 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 homogenised using an Ultra-Turrax blender (Janke & Kunkel, IKA-Werk, Staufeb, Germany) for 1 min at 14000 rpm. Another 15 mL of dichloromethane was added and then homogenised for 30 s and finally 15 mL of water (pH ≤ 2) was added and homogenised for 30 s. The tubes were centrifuged at 3600 x *g* for 10 min using a centrifuge (Rotina 380R Heltich Zentrifugen, Germany), the pH of the aqueous layer was checked to be less than two, 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 sulphate over a 150 mm filter paper. The solvents were removed using a rotary evaporator and finally dried on nitrogen.

Market samples: When applying the extraction procedure to screening market samples, a weight (depending on the amount of lipid in the sample) that does not exceed 200 mg of oil was taken. The IS amount always added was 10 µg and the same extraction procedure above was followed.

3.2.4 Transmethylation and SPE separation

3.2.4.1 Transmethylation

The base catalysed transmethylation procedure followed is described in **chapter 2 section 2.2.5.**

3.2.4.2 SPE separation and column chromatography partitioning

The SPE separation procedure is described in **chapter 2 section 2.2.6.**

3.2.5 Instrumentation

3.2.5.1 Gas chromatography-FID and GC-MS conditions

The epoxy FAMEs were analyzed by GC-FID using an Agilent 6890N series gas Chromatograph (Agilent, USA) as described in **chapter 2 section 2.2.9.1 and 2.2.9.2.**

3.2.6 Method validation

The analytical method here presented was optimised using four different food matrices covering a wide category of common foods as indicated under the samples section. Although foods were analysed to obtain blank samples, it was not possible to obtain one without a signal of the analyte, however the IS did not have any interfering signal. In-house validation procedures were followed as recommended by the Commission Decision 2002/657/EC (European Commission, 2002; Ricard, Alicia, Jordi, & Rius, 2002). Performance criteria assessed included: linearity, matrix effect, limit of detection (LOD), limit of quantification (LOQ), repeatability (intra-day; RSDr) and reproducibility (inter-day; RSDR) and stability. Because there are no certified reference materials available for EFAs, trueness of the obtained results was assessed using spiked samples (Ricard et al., 2002). The concentration levels used to spike the different food matrices were decided on after considering the epoxy levels obtained in fresh oil samples (Mubiru et al., 2013).

3.2.6.1 Intra-day repeatability, inter-day reproducibility and stability

Precision of the method was assessed by repeatability and the intermediate precision called inter-day reproducibility. The repeatability was assessed by spiking the epoxy standard solutions to the different food matrices at five different concentration levels with three replicates at each level (n=3) and analysing on the same day on GC-FID. Inter-day reproducibility was determined by repeating the experiment on two different days for all the matrices. Stability was evaluated by storing the extracts at -20°C and analysing them after four months of storage.

3.2.6.2 Calibration curves, LOD, LOQ and recoveries

Linearity and working ranges were evaluated by the correlation coefficient of standard calibration curve and by plotting the residuals for the curve**.** Because EFAs are occurring at a wide concentration range, two calibration curves with uniformly spaced ranges were used in the beginning, 0-200 and 200-1000 µg g^{-1} of sample. The calibration curve was obtained by directly injecting to the GC the mixture of IS and analyte. The calibration and standard addition curves were constructed by plotting area ratios of analyte to IS (y) versus the concentration ratios of the analyte to that of the IS (x). During standard addition, the same concentrations as those in the calibration curve were used and known amounts of the epoxy analyte were added to a fixed mass

of each matrix separately. Regression of the calibration curve using the general linear regression model was performed to obtain the calibration curve equation (R^2, slope and intercept). LOD and LOQ were determined from the standard addition curves using the linest function of Microsoft Office Excel 2007 program. LOD was defined as the concentration equal to 3 (SE / Slope) and LOQ = 2 x LOD; where SE is the Standard error of the intercept (Ricard et al., 2002). Because certified reference materials are not available, it is acceptable that trueness of measurements is assessed through apparent recovery of additions of known amounts of the element to the unknown samples (European Commission, 2002). The same addition curves (spiked samples) used to calculate the LOD were further used to obtain the apparent recoveries of the method. Apparent recovery is defined as the quantity observed value/reference value, obtained using an analytical procedure that involves a calibration graph (Burns, Danzer, & Townshend, 2002). Quantification of the validation samples was based on external calibration curve and for real samples it was based on the response factor of epoxystearate.

3.2.6.3 Matrix effect evaluation

The matrix effect was assessed by comparing the slopes of the calibration curve with that of the standard addition curves (Papastergiadis, Mubiru, Van Langenhove, & Meulenaer, 2013). Where there was no matrix effect, the same slope was obtained.

3.2.7 Statistical analyses

Analytical determinations were conducted in triplicate and where duplicate analysis was performed, evaluation of precision was done according to Synek (2008). Comparison of the calibration and addition curve slopes was done by performing a one-way ANCOVA test carried out using GraphPad Prism 6 2012 program.

3.3 RESULTS AND DISCUSSION

3.3.1 Method development

During method development and validation both the Folch method and Bligh and Dyer method were applied and the recovery results (not shown) for both methods were not different. Because Bligh and Dyer method has an advantage of using less solvent (Iverson, Lang, & Cooper, 2001),

it was chosen to reduce on the use of expensive and hazardous solvents (Cequier-Sánchez, Rodríguez, Ravelo, & Zárate, 2008).

An important factor during method development was to optimise the solvent ratios of the liquid-liquid extraction procedure. The possible extraction agents are dichloromethane and chloroform. Because dichloromethane is less hazardous it was preferred in the extraction. As a quality note, high quality solvents should be used and where such a quality is not achievable, the solvents should be distilled and use of glassware is highly recommended.

Since the Bligh and Dyer method was initially developed using chloroform, it was required to optimise the use of dichloromethane. The ternary diagram of water + methanol + dichloromethane (Bligh & Dyer, 1959; Merzougui, Hasseine, Kabouche, & Korichi, 2011) was used to optimise both sample and the solvent ratios required during extraction. The water content of dry samples had to be adjusted as indicated below because this had a bearing on the phase separation which is the basis of this method. Addition of solvent mixtures in the following ratio and in the following order was optimal; water 29 mL (considering water in the sample), dichloromethane (22 mL) and methanol (52 mL). The system should be monophasic, there should not be phase separation if the solvent ratio is correct and if sufficient homogenization is done. Addition of 30 mL of dichloromethane to the mixture, followed by addition of 30 mL of acidic water (pH $\leq$ 2 acidified with 6 M hydrochloric acid) and homogenizing again with an Ultra-Turrax blender for 30 s allowed phase separation. Methanol is a poor solvent for triacylglycerols but is fully miscible with water in all proportions. It can thus serve to strip away the hydrating water and allow dichloromethane, to dissolve the polar fatty acids. The water in the system causes the dichloromethane to separate cleanly, with the methanol component retaining the water and suppressing emulsion formation (Christie, 2003).

Because epoxy standards are not available from commercial sources (Mubiru et al., 2013), the IS and epoxy analyte used during this study were both synthesized by use of a solvent based epoxidation procedure described by Gunstone and Jacobsberg (1972). Although it is possible to synthesize epoxides using hydrogen peroxide based catalytic epoxidation, the ‘‘Tetrakis’’ phosphotungstate catalyst and by lipase-mediated reactions (Orellana-Coca et al., 2005; Poli, Clacens, Barrault, & Pouilloux, 2009), the internal standard used was prepared by using the method reported by Gunstone and Jacobsberg (1972). The method had a yield of >90% which is a sign of purity. The synthesized compounds were confirmed by mass spectral data after GC-MS

analysis. It is well known that during analysis of EFAs, the method of sample preparation must consider the fact that the highly reactive epoxide ring can be opened by heat or chemicals (Piazza, Nunez, & Foglia, 2003b). Thus, the methylation procedure adopted for formation of FAMEs during the study was the room temperature methylation. However, this has a disadvantage that a free fatty acid cannot be used as an IS during the analysis of EFAs. As observed by Suter et al. (1997), that methylation and saponification reactions both compete in the presence of water, care had to be taken that the reaction mixture was not kept for unnecessarily long for the formed methyl esters to be saponified. Use of acidified water also helped to stop the possibility of saponification. The FAMEs were extracted twice in MTBE to increase the efficiency of the extraction procedure. During this study, the use of a methylated IS was evaluated by calculating the area ratios of both the analyte and the methylated IS. It was found that there was no change in the ratios; furthermore, during validation, the analyte and the IS were both epoxy FAMEs which reduced variability during the analysis. However, it is important to note that care should be taken during the evaporation step not to lose the IS, so finally after the rotary evaporation step, extracts were always dried under a gentle stream of nitrogen. The use of an epoxy FAME IS has made this method more sensitive and accurate because analytical variations can be corrected by the IS added at the beginning of the extraction as recommended by the Commission Decision 2002/657/EC (European Commission, 2002). In analytical methods where the IS may not be added at the start of the analysis, control of analytical variations is not possible.

3.3.2 Method validation

3.3.2.1 Linearity, LOD, LOQ and recovery

Because the EFAs occur over a wide range, in the beginning of method development, two calibration ranges were chosen, the low and the high range. Six concentration levels (0, 40, 80, 120, 160, 200 µg g^{-1}) plus the blank were used during method development and validation. In both ranges the linearity was evaluated by plotting the residuals and linear regression (R^2) values. It must be emphasized that concentrations of the high range calibration curve, could not be injected directly onto the GC–FID as they would overload the column, so they were diluted ten times. In all the two ranges, there was no sign of detector saturation which would cause loss of linearity, so a good linear range was observed in both calibration curves. When the two concentration range data were pooled, and plotted into one calibration curve, still good linearity

with a regression coefficient of 0.9994 and a slope of 1.23 was obtained. The calibration curves showed a measure of goodness-of-fit of linear regression (R^2) ranging from 0.9979-0.9999. Whereas the standard addition curves showed coefficients (R^2) ranging from 0.9959-0.9973 in oil, 0.9885-0.9927 in crisps, 0.9990-0.9994 in milk powder and 0.9915-0.9995 in pork. The curves with $R^2 \geq 0.995$ are considered linear (Van Loco, Elskens, Croux, & Beernaert, 2002) and these results show how well the developed method can estimate EFAs in the different matrices.

Mean percent recoveries based on spiked samples for the EFAs in the different matrices were in the range 94 to 115 (**Table 3.1**). The values are the mean of three replications of analysis performed on two different days.

Table 3.1: Intra-day precision (RSDr), inter-day precision (RSDR) expressed as relative standard deviation (%) and apparent recovery obtained for oil, crisps, pork and milk powder at five concentration levels of *cis*-9,10-epoxystearate analyte

Matrix	Concentration (µg g^{-1})	RSDr (%)	RSDR (%)	Apparent recovery (%)
Oil	40	3	9	100
	80	4	4	100
	120	6	5	101
	160	8	5	101
	200	2	3	101
Crisps	40	19	3	115
	80	1	5	99
	120	3	2	95
	160	4	6	97
	200	5	6	97
Pork	40	10	7	96
	80	1	8	94
	120	2	3	96
	160	7	9	99
	200	5	6	96
Milk powder	40	3	4	98
	80	2	5	98
	120	2	6	99
	180	3	3	101
	200	2	5	102

The LOD was determined according to the definition given by IUPAC and the regression of the calibration equation using the linest method was used. Accordingly, the LOD values (µg g^{-1} of sample) determined for the different matrices validated were 2.75 in oil, 10.23 in crisps, 1.66 in milk powder and 5.16 in pork. The lowest LOD was obtained in milk (**Table 3.2**).

Table 3.2: Limit of detection (LOD) and Limit of quantification (LOQ) obtained for the different matrices in µg g^{-1} of sample

	Oil	Crisps	Pork	Milk powder
LOD	2.8	10.2	5.2	1.7
LOQ	5.5	20.5	10.3	3.3

The matrix effect on the recovery of the analytes was evaluated by comparing the slopes of the calibration curves with those of the addition curves. The comparison was done by using a statistical tool in GraphPad Prism to obtain a *p* value. The results of the statistical evaluation of the slopes of the calibration and addition curves are shown in **Table 3.3**. Only crisps samples appear to have a statistically significant matrix effect (p=0.0002). The observed matrix effect in crisps could be attributed to the high signal of EFAs that is detected in the blank samples. Normally a good evaluation of matrix effect is obtained if a sample without the analyte of interest (blank) is obtained, but in all cases, it was not possible to get one. However, in other matrices for which the signal was low, it was possible to objectively evaluate whether it is the matrix interfering with the analysis or not.

Table 3.3: Matrix effect evaluation by statistical comparison of the calibration curve with the standard addition curve

	Calibration curve		Addition curve		
	Slope	R^2	Slope	R^2	*p* value
Oil	1.209	0.9999	1.222	0.9973	0.6019
Crisps	1.180	0.9987	0.986	0.9885	0.0002*
Pork	1.174	0.9998	1.185	0.9998	0.2615
Milk powder	1.180	0.9987	1.200	0.9994	0.3498

An asterisk indicates a statistical difference within rows between the two slopes ($p < 0.05$).

3.3.2.2 Intra-day precision (repeatability) and inter-day precision (reproducibility) and stability

Results for intra-day and inter-day precision measures are presented in **Table 3.1**. These results are presented as percent relative standard deviations in both cases as a measure of precision. The repeatability values presented are the highest that were obtained during the validation study. Although it was not possible to vary all factors that may affect precision as recommended in method validation studies (European Commission, 2002; Ricard et al., 2002), the relative standard deviations obtained, revealed that accurate estimation of precision was obtained. In the lower concentration ranges, higher variations of up to 19% were observed in crisps. The stability of the extracts was evaluated by reinjection of the same extracts after storage. Results after reinjection of the same extracts for all the three matrices used did not show any degradation after four months of storage. This was true with the calibration standards which when re-injected after four months, the slopes obtained did not differ significantly. This is a clear indication that EFA extracts can be stored at -20°C for some good time without any degradation.

3.3.3 Application of the method on market samples

After validation and optimization of the analytical method, it was applied on commercially available samples collected from Belgian supermarkets. The fatty acid composition was not analysed and their history was not known. For each sample, the analytical determination was performed in duplicate. The results of the analysis are summarised in **Table 3.4**. The food samples analysed were chosen to cover a wide range of samples of fat origin with differing physical and chemical composition. It was important to note that for samples which had a high dry matter content, their weight during analysis was reduced significantly as to avoid the solid matter covering the organic layer. Furthermore, depending on the fat content of the sample, the sample weight and the solvent ratio were increased to be able to recover enough amount of fat. The results obtained indicate that the EFAs are occurring in high amounts in most samples although beef samples had less. The coefficient of variation for duplicate analysis ranged from 13.9 to 14.1%. It was generally observed that on fat basis, biscuits had the highest total epoxy content. There is no clear relationship between the sample type and the total EFAs determined. However, the levels of EFAs are expected to depend on the amount of unsaturated fatty acids present which are the precursors of EFAs (**chapter 2, section 2.3.3**), sensitivity to oxidation and the level of oxidation.

Table 3.4: Epoxy fatty acid levels analysed in different food samples from Belgium supermarkets

Sample Name	Epoxy fatty acid content (µg g^{-1} of sample)								
	***Fat content (%)**	***trans*-9,10-ES**	***cis*-9,10-ES**	***trans*-12,13-EO**	***trans*-9,10-EO**	***cis*-12,13-EO**	***cis*-9,10-EO**	**Total epoxy (µg g^{-1} of sample)**	**Total epoxy (µg g^{-1} of fat)**
Biscuit	12	14.90	13.92	3.89	1.03	27.40	12.67	73.81	615.08
Speculoos A	19	3.58	19.16	1.06	0.76	39.13	10.80	74.49	392.05
Speculoos B	19	2.91	17.55	1.11	0.97	34.63	9.41	66.58	350.42
Speculoos C	19	3.49	12.28	1.11	1.15	22.12	6.80	46.95	251.07
Speculoos D	17	4.04	13.96	1.25	1.94	24.58	7.99	53.76	308.97
Mayonnaise A	80	10.92	9.50	4.15	7.00	7.27	5.25	44.09	54.91
Mayonnaise B	81	17.33	7.87	4.49	6.54	6.96	5.97	49.16	60.69
Mayonnaise C	81	10.15	5.14	3.74	4.65	4.31	4.61	32.6	40.05
Peanuts A	51	9.42	129.41	4.39	4.34	4.69	18.53	170.78	334.86
Peanuts B	NI	4.70	47.10	2.29	3.37	2.76	14.09	74.31	ND
Butter-Margarine	82	53.66	16.79	18.30	0.00	9.38	17.21	115.34	140.66
Cooked ham	3	0.75	0.66	0.47	0.00	0.48	0.88	3.24	115.71
Minced beef	30	1.48	1.09	1.72	0.00	0.66	0.63	5.58	18.60
Walnuts	65	4.27	6.42	3.68	1.84	25.60	7.07	48.88	75.20

Abbreviations: ES, epoxystearate; EO, epoxyoleate; NI, not indicated; ND, not determined.

* Fat content as reported on the package label.

3.4 CONCLUSIONS

The method presented and validated is accurate and reproducible and can be applied in the determination of EFAs in a wide range of food samples of fat origin. The well resolved and clean chromatograms obtained on real sample matrices indicate the reliability of the method for confirmatory purposes. The method developed is robust and can be applied to such a wide coverage. High recoveries for the EFAs and the low LOD and LOQ levels indicate that the method is sensitive and can be used in risk assessment studies.

CHAPTER

4

EXPOSURE ASSESSMENT OF EPOXY FATTY ACIDS THROUGH CONSUMPTION OF SPECIFIC FOODS AVAILABLE IN BELGIUM

Redrafted from:

Edward Mubiru, Liesbeth Jacxsens, Antonios Papastergiadis, Carl Lachat, Kshitij Shrestha, N. H. M. Rubel Mozumder & Bruno De Meulenaer (2017): Exposure assessment of epoxy fatty acids through consumption of specific foods available in Belgium, Food Additives & Contaminants: Part A, 34:6, 1000-1011, DOI: 10.1080/19440049.2017.1310399

ABSTRACT

Epoxy fatty acids (EFAs) are secondary oxidation products formed from unsaturated fatty acid hydroperoxides. Seventeen food categories were analyzed for C18 mono-EFAs of food products available on the Belgian market. A quantitative exposure assessment was performed based on deterministic and probabilistic approaches combining these concentration data with consumption data obtained from the Belgian National Food Consumption Survey of 2004. A preliminary evaluation of any potential risk related to the intake of the studied EFAs through the studied foods was performed by applying the Threshold of Toxicological Concern (TTC) concept. Three food categories out of seventeen foods, mayonnaise, butter-margarine and ready to eat meals were found to contribute most to the intake of EFAs. According to probabilistic determination, these foods had a P50 intake of 0.41, 0.33 and 0.30 mg kg^{-1} bw day^{-1} respectively. They had a P99.5 intake of 3.72, 2.79 and 38.61 mg kg^{-1} bw day^{-1} respectively. The intake below the TTC value was from consumption of cooked meat, smoked salmon and raw cured ham, with a P50 intake of 0.0006, 0.0007 and 0.0011 mg kg^{-1} bw day^{-1} respectively and the other foods were above the TTC value. Based on the TTC concept a risk to human health could be identified related to the consumption of cheese, snacks foods, plant oils, French fries, dry nuts, chips, cured minced raw meat, cookies, fresh and frozen salmon and bacon.

Keywords: Lipid oxidation, epoxy fatty acids, fatty acid methyl esters, threshold of toxicological concern, risk assessment, exposure assessment

4.1 INTRODUCTION

Long chain EFAs have gained a lot of interest mainly because they are more abundant in nature. They can be manufactured in large amounts by epoxidation of appropriate alkene esters for industrial purposes (Gunstone & Jacobsberg, 1972; Gunstone & Schuler, 1975). EFAs are potential toxic compounds to humans which is attributed to their high reactivity because of the presence of the oxirane ring in their structure. This under conducive conditions can react with proteins and DNA, consequently leading to structural damage and alteration of their functionality (Greene et al., 2000b; Gunstone et al., 2007). Specifically, the 9,10-epoxyoctadec-12-enoic acid and its isomer 12,13-epoxyoctadec- 9-enoic acid are leukotoxic and isoleukotoxic respectively. The EFAs have been reported to have low absorptivity in the body a property which suggests that high levels may be detected in the colon. Although there has not been strong link of EFAs to health risks, some studies have suggested that they may cause cancers (Wilson et al., 2002). To date there is no available robust toxicological data of EFAs and there is a limited knowledge concerning the dietary exposure of humans to these compounds except for an experimental study on women (Wilson et al., 2002). In absence of this specific toxicological data, a risk assessment can be done based on the Threshold of Toxicological Concern (TTC) concept.

Exposure assessment studies on secondary lipid oxidation products which are potentially toxic to humans are missing except a recent study done on malondialdehyde (MDA), 4-hydroxy-2-hexenal (HHE) and 4-hydroxy-2-nonenal (HNE) (Papastergiadis et al., 2014). During this study, it was reported that consumption of the specific foods did not pose a risk to most Belgian consumers based on the TTC concept. However, exposure to MDA through consumption of especially dry nuts was significant and 3.8% of the population consuming cured and minced raw meat products was found to be at a risk of HNE exposure (Papastergiadis et al., 2014). The objective of this study therefore was to determine EFAs in specific foods available on the Belgian market and then perform a quantitative exposure assessment of dietary intake of the EFAs for the Belgian population both consumers and total population using the food consumption data of the Belgian National Food Consumption Survey (BNFCS) of 2004. Finally, evaluation of the potential risk due to total EFAs consumption was performed by comparing the estimated intakes with the corresponding TTC

value of the EFAs (class III) and discussed against the earlier reported findings of other secondary lipid oxidation products (MDA, HHE and HNE) by Papastergiadis et al. (2014).

4.2 MATERIALS AND METHODS

4.2.1 Supplies and reagents

The reagents used are described in **chapter 3 section 3.2.1.**

4.2.2 Sampling plan for EFA determination in foods

The choice of study foods (**Table 4.1**) was adapted from a recent study done by Papastergiadis et al (2014), this is a directed or risk-based sampling where specific foods were sampled per their potential risk to be contaminated with secondary oxidation products (Kroes et al., 2002). Fat content and the degree of unsaturation of the fatty acids were considered. Foods were divided into three groups including foods of plant origin, foods of animal origin and chilled cooked meals (containing multiple ingredients) respectively (Papastergiadis et al., 2014). Two other food groups, mayonnaise, butter-margarine were included based on their importance in the normal diet of most Belgian consumers. A total of 390 samples were purchased from supermarkets in the region of Ghent, Belgium, excluding French fries and fried snacks that were purchased from local fast food restaurants. Foods were purchased and always analyzed immediately upon delivery to the laboratory. For purposes of obtaining a representative sample, all the components of the foods which were homogenised using a waring stick blender prior to taking the required weights before analysis.

4.2.3 Determination of EFAs in foods

The samples were extracted using the validated method described in **chapter 3 section 3.2.3.1**. Base catalysed transmethylation procedures and the SPE separation steps followed have previously been described in **chapter 2 sections 2.2.5** and **2.2.6.** The LODs of the method considered were determined previously in **Table 3.2**.

4.2.4 Consumption data

Food consumption data were obtained from the Belgian National Food Consumption Survey (BNFCS) of 2004 the aims and the methodology are described by De Vriese et al., (2005).

Dietary intake was collected from 3245 individuals of fifteen years old and above, residing in Belgium (Vandevijvere et al., 2009). The survey was based on two non-consecutive 24 h recalls combined with a self- administered food frequency questionnaire. Consumption information of the specific foods were extracted from the BNFCS database based on their description (food category and food name data) as included in **Table 4.1**. The usual food intake was determined from the total data set by correcting for intra person variability using the Multiple Source Method (MSM) program (German Institute of Human Nutrition Potsdam-Rehbrücke (DIfE), 2013; Harttig, Haubrock, Knueppel, Boeing, & Consortium, 2011; Haubrock et al., 2011). All subjects were considered habitual consumers of the foods and food intake data were expressed in kg of food as kg^{-1} bw day^{-1} using the body weight (bw) data collected in the survey. The food consumption database used does not contain information about cooked chilled meals (bought as ready-to-eat products). As such a discrete function was used to model their intake which was obtained from a consumer survey data conducted by Daelman et al. (2013) where a total of 679 individuals of fifteen years and above were interviewed on their consumption behavior of ready-to-eat meals during the spring of 2011.

Table 4.1: Description of the foods analysed

Food group	Food category	Food description
Foods of plant origin	Chips	Chips with different flavours salted and non-salted
	Cookies	Biscuits, waffles, cakes, speculoos
	Dry nuts	Roasted, peeled and non-salted peanuts, walnuts, hazelnuts, almond, pistachios, cashew
	French fries	Fries with different sauces (mayonnaise, tartar, andalouse)
	Plant oils	Refine (corn, sunflower, arachis, colza, soya, salad, frying, a mixture) and extra virgin oils
Foods of animal origin	Bacon	Salted and smoked and non-smoked
	Butter-Margarine	Salted and non-salted
	Cheese	Gouda and cheddar
	Cooked meat	Grilled or boiled Paris sausage, frankfurter
	Mayonnaise	Normal, light and similar sauces
	Milk	Full fat pasteurized and sterilized milk
	Raw Ham	Salted and ripened
	Cured minced raw meat	Dry sausage, salami
	Smoked salmon	Filleted and stored at 4°C
	Fresh and frozen salmon	Stored at -20°C
	Snacks foods	Boulet, chicken finger, hamburger, fish sticks
Cooked chilled food	Ready to eat meals	Lasagna, spaghetti, chicken fillets, fried rice, stewed beef, pasta, pork meat balls, burgers

This survey was based on frequency of consumption where respondents had to answer the question 'how often do you eat a ready-to-eat meal?' and seven answers were possible: 5-7

times a week, 2-4 times a week, once a week, 3-5 times a month, once a month, once a year and never. These responses were first converted to a daily consumption by a conversion factor (i.e. 5-7 times a week corresponded to 1/day; once a week corresponded to 1/7 days; 3-5 times a month corresponded to 4/30 days, once a month corresponded to 1/30 and once per year corresponded to 1/365), followed by a multiplication of the average weight of all analyzed products. The outcome was divided with the average body weight of 60 kg (Kroes et al., 2005). At this point it should be mentioned that the total weight of each packaging was considered as a whole single personal portion (Daelman et al., 2013; Papastergiadis et al., 2014).

For those data in **Table 4.2**, a risk discrete function has been attributed, with the first argument in the discrete function expressing the consumption of ready-to-eat meals is the set of possible values (relative respondents), and the second is the set of corresponding probabilities. By application of this function, the consumption dataset of kg ready-to-eat meals kg^{-1} bw day^{-1} was obtained. To obtain information about the total intake of the population including consumers and non-consumers, the IF function was applied to the consumers distributions, because the percentage of consumers was known, random intake values could be returned that were used to infer for the population intake.

4.2.5 Exposure assessment

During EFA analysis no concentrations were below LODs (non detects), so no data censoring was considered during exposure assessment (Kroes et al., 2002; Picot & Roudot, 2012; WHO/FAO, 1985). For the exposure assessment, foods were divided into seventeen categories: plant oils, dry nuts, potato crisps, French fries, cookies, fried snacks, frozen and fresh salmon, smoked salmon, full fat milk, cheese, cured and cooked meat products, bacon, cured raw ham, cured minced raw meat products, mayonnaise, butter-margarine and ready to eat meals (**Table 4.1**).

4.2.6 Deterministic exposure assessment

Dietary exposure of the consumers and total Belgian population to the EFAs was initially performed using the deterministic approach, this refers to using a model with no uncertainty and variability consideration which may not give a full estimate of the intakes. Estimated

intakes were calculated by multiplication of a fixed mean of EFA contamination data with the mean, maximum or P99.5 percentile of the consumption data of each food category. This approach was deemed necessary to minimise the risk to the consumers (Vromman et al., 2010). Deterministic analysis could not be applied to the cooked chilled meals because of the nature of the consumption data.

4.2.7 Probabilistic exposure assessment

Best fit distributions for both consumption and contamination data of each food category were determined. The best fit distributions were defined based on chi square statistics, probability-probability plots (P-P) and the quantile-quantile plots (Q-Q). Distribution fitting was feasible when at least five positive data were available (Medeiros Vinci et al., 2012; Vose, 2008). Monte Carlo simulations were performed for each food category to develop the exposure model considering uncertainty and variability. The probability of existence of EFAs in the different foods, its level in those foods and the probability of human exposure were all the outputs of the mathematical model. The estimated daily intake (mean, standard deviation, maximum and percentiles) was expressed in mg kg^{-1} bw day^{-1} of EFA. Distribution fitting and Monte Carlo simulations were performed with the @Risk for Microsoft Excel software version 5.7.1 (Palisade Corporation, USA), with 50 000 iterations and three simulations.

4.2.8 Threshold of Toxicological Concern

Chemicals are classified into three classes according to Cramer decision tree and the TTC values for chemicals belonging to Cramer class I, II and III are 1800, 540, 90 μg person day^{-1} respectively taking the normal body weight to be 60 kg (Cramer et al., 1978). The decision tree comprises a sequence of questions such that compounds with structures indicative of a high potential for toxicity are assigned to structural class III (Kroes et al., 2004). Classification of the compounds based on the Cramer decision tree was carried out with Toxtree (Version 2.5.4) software available online by the German Institute of Human Nutrition, Potsdam-Rehbrücke. EFAs were confirmed to be grouped under class III.

Table 4.2: Best fit distribution functions used, the minimum, mean and maximum concentration of EFAs (mg kg^{-1}) and food intakes of of Belgian consumers (mg kg^{-1} bw day^{-1})

Food categories	Variable	Function	Min	Mean	Max	% Consumers
Chips	Food intake	RiskExpon(0.0000429428;RiskShift(-0.0000000132662))	0	0.00004	+∞	10
	EFA content	RiskTriang(55.347;55.347;934.61)	55.35	348.43	+∞	
Cookies	Food intake	RiskExpon(0.0000392046;RiskShift(-0.0000000121414))	0	0.00004	+∞	12
	EFA content	RiskLoglogistic(53.82;85.797;3.7601)	53.82	150.48	+∞	
Dry nuts	Food intake	RiskExpon(0.0000301116;RiskShift(-0.0000000094246))	0	0.00003	+∞	5
	EFA content	RiskGamma(1.3566;393.79;RiskShift(152.76)	152.76	686.98	+∞	
French fries	Food intake	RiskExpon(0.00032186;RiskShift(-0.0000000993093))	0	0.00032	+∞	25
	EFA content	RiskExtvalue(98.855;50.892)	−∞	128.23	+∞	
Plant oils	Food intake	RiskExpon(0.00029172;RiskShift(-0.0000000900938))	0	0.00029	+∞	36
	EFA content	RiskExpon(380.31;RiskShift(19.814)	19.81	400.12	+∞	
Bacon	Food intake	RiskExpon(0.0000630029;RiskShift(-0.0000000195116))	0	0.0001	+∞	9
	EFA content	RiskTriang(7.2532;7.2532;210.85)	7.25	75.12	+∞	
Butter-Margarine	Food intake	RiskInvgauss(0.0012506;0.0038708;RiskShift(-0.00011301))	-0.0001	0.00114	+∞	91
	EFA content	RiskInvgauss(331.75;577.04)	77.18	408.93	+∞	
Cheese	Food intake	RiskExpon(0.00045229;RiskShift(-0.000000139467))	0	0.00045	+∞	45
	EFA content	RiskExtvalue(248.63;105.24)	−∞	309.38	+∞	
Cooked meat	Food intake	RiskExpon(0.0000890779;RiskShift(-0.0000000275017))	0	0.00010	+∞	10
	EFA content	RiskExtvalue(9.0545;5.4844)	−∞	12.220	+∞	
Mayonnaise	Food intake	RiskGamma(2.4914;0.00063544;RiskShift(0.00031812))	0.0003	0.00190	+∞	44
	EFA content	RiskLognorm(159.21;240.26)	135.84	295.05	+∞	
Milk	Food intake	RiskExpon(0.0078497;RiskShift(-0.00000242049))	0	0.00785	+∞	64
	EFA content	RiskLogistic(36.963;11.249)	−∞	36.96	+∞	
Raw ham	Food intake	RiskExpon(0.0000362156;RiskShift(-0.0000000112192))	0	0.00004	+∞	11
	EFA content	RiskExpon(50.159;RiskShift(13.407)	13.41	63.57	+∞	
Cured minced raw meat	Food intake	RiskExpon(0.0000536806;RiskShift(-0.0000000166194))	0	0.00005	+∞	14
	EFA content	RiskLoglogistic(-23.764;211.55;3.7606)	-23.7600	214.55	+∞	
Smoked salmon	Food intake	RiskExpon(0.0000188984;RiskShift(-0.00000000591313))	0	0.00002	+∞	4
	EFA content	RiskInvgauss(66.628;139.396;RiskShift(2.4924)	2.4900	69.12	+∞	
Fresh & frozen salmon	Food intake	RiskExpon(0.0000838218;RiskShift(-0.0000000263756))	0	0.00008	+∞	4
	EFA content	RiskExtvalue(54.337;47.07)	−∞	81.5100	+∞	
Snack foods	Food intake	RiskGamma(0.94776;0.0016524)	0	0.00160	+∞	3
	EFA content	RiskLogistic(190.92;38.031)	−∞	190.9200	+∞	
Ready to eat meals	Food intake	RiskDiscrete(0.003.0.022.0.138.0.415.0.239;0.855.0.427.0.142.0.066.0.033.0.003)	0.0030	0.03800	0.4153	78
	EFA content	RiskLoglogistic(17.1;29.533;2.0843)	17.1000	61.7000	+∞	

EFA epoxy fatty acids methyl esters

4.2.9 Statistical analysis

One-way analysis of variance (ANOVA) was applied to detect differences in the mean of the total concentrations of the EFAs between different food categories. In case of lack of homogeneity of data, a logarithmic transformation was applied prior to statistical analysis to achieve homoscedasticity. For significant differences, a Student-Newman-Keuls test of mean comparisons was applied at a p value of 0.05. Calculations were performed with SPSS 22 statistical package (IBM, SPSS, Inc).

4.3 RESULTS AND DISCUSSION

4.3.1 Occurrence of EFAs in foods on Belgian market

Results of the concentrations of EFAs for a total of 390 food samples analysed are presented in **Table 4.3**. In the table, the total EFA content is shown, but in most cases the total comprises of 12 mono EFA isomers especially *cis* isomers. The method used to analyse EFAs in food matrices and oils were previously in-house validated (**chapters 2 and 3**) and it should be noted that, free EFAs cannot be analysed by these methods. Only EFAs that are still attached to triglyceride backbone are analysed. EFAs were detected in high amounts in most of the food samples especially those in the plant foods category. There were no non-detects (below LODs) in all the samples.

According to published data (Fankhauser-Noti et al., 2006; Mubiru et al., 2013, 2014), EFAs can be found in food samples up to mg levels and twelve isomers of C18:1, C18:2 and C18:3 fatty acids are known to occur of which the *cis*-9,10-epoxystearate is the most abundant isomer. All the foods could be categorised into three groups according to the amount of EFAs determined, low EFA (< 100), medium EFA (100-200) and high EFA (> 200) mg kg^{-1} of sample. Generally, animal based foods had low levels of EFAs compared to plant foods ranging from 12 to 82 mg kg^{-1} of sample and specifically these were found in cooked meat, chilled cooked meals, raw ham, smoked salmon, fresh and frozen salmon, milk and bacon. These low levels seem to be related to the types of fatty acids that are common with animal based foods as they have low content of C18:1 and C18:2 fatty acids. Foods which had > 200 mg kg^{-1} of EFAs were, plant oils, butter-margarine, cheese, mayonnaise, chips, cured minced raw meat (salami), cookies. Cured minced raw meat, a category of sausages, had higher

incidence of EFAs up to 209 mg kg^{-1} of sample compared to the raw meat which had 66.9 mg kg^{-1}. This may be linked to the further ripening and drying treatment which is given to the meat during processing.

Table 4.3: Total C18 epoxy fatty acid concentrations (mg kg^{-1}) in the different foods analysed

Food categories	N	Mean	SD	Max
Chips	24	297.71^{def}	218.46	872.27
Cookies	29	148.22^{cd}	43.65	263.16
Dry nuts	24	686.98^{g}	513.19	2,117.43
French fries	19	128.50^{c}	92.42	360.32
Plant oils	36	410.68^{def}	427.67	1,933.26
Bacon	25	73.49^{b}	53.52	187.74
Butter-Margarine	27	408.93^{f}	229.30	1,028.96
Cheese	24	309.59^{ef}	138.96	668.54
Cooked meat	22	12.54^{a}	8.17	34.72
Mayonnaise	25	309.61^{def}	318.47	1,754.00
Milk	17	40.22^{b}	24.37	118.75
Raw ham	22	65.85^{b}	68.41	225.95
Cured minced raw meat	26	208.98^{cde}	106.48	488.59
Smoked salmon	21	69.12^{b}	40.33	146.24
Fresh & frozen salmon	9	82.25^{b}	62.23	178.73
Snack foods	20	186.06^{cde}	65.96	325.27
Ready to eat meals	20	59.37^{b}	38.56	150.85
Total	**390**			

Values with different superscripts along the column are significantly different $p < 0.05$

Among the plant category foods, dry nuts had the highest EFA content of 687 mg kg^{-1}. This high incidence may not be surprising because some oil seeds have been reported to have naturally occurring EFAs to a level of 60 to 80% (Gunstone, 1954). This observation could partly be attributed to the high content of oleic and linoleic fatty acids found in most of plant based foods based on fat and fatty acid content. Coupled to this observation, some of the seeds could further be processed by roasting and salting which may affect the EFA content. However, a correlation between roasting and its effect on the production of EFAs is not known but may probably be due to further treatments of nuts which can increase the incidence of EFAs since high temperatures are involved which may accelerate the rate of lipid oxidation.

Although butter is obtained from milk which had a low EFAs content, the butter-margarine category had a high content of EFA as high as 409 mg kg^{-1} on average. This is probably because margarine which is in the same category as butter is made by fractionation or

hydrogenation of plant oils that may already have a high epoxy content with fats. The higher level of EFAs in butter could be also due to the greater interfacial area created due to emulsification. However, when compared separately margarine had a higher EFA content than butter. However, the relationship of EFA content between cheese and milk vis-a-vis the fat or fatty acid content is not clear. But what is clear is that irrespective of the fatty acid content, foods that are further processed had higher EFAs when compared to those that remain in the 'raw' form.

Most of the vegetable oils used in this study were fresh and refined with very low initial peroxide values (data not shown), possibly because of the refining process. It was observed that big standard deviations in some data sets could be obtained; this may be an indicator of the heterogeneity of the distribution of EFAs in the different food matrices. This problem can be overcome by increasing the number of samples which can help to reduce this cause of variation and increase the validity of the results (Kroes et al., 2002).

4.3.2 Consumption data

The total sample of 3245 individuals included in the BNFCS provided useful information on the consumption of the analyzed samples from the Belgian population. The highest number of consumers was obtained from the butter-margarine food group with 91% consumers. This high percentage may be attributed to the wide range of foods that fall under this category especially the margarine which all contribute to the daily intake. The rest of the foods with the percentage number of consumers are shown in **Table 4.2**, foods consumed by > 25% of the Belgian respondents were, milk (64%), mayonnaise (44%), cheese (45%) and plant oils (36%). Foods that were consumed by between 10% to 25% of consumers included, French fries (25%), cured processed meat products (14%), cookies (12%), cured unprocessed meat products (11%), cooked and processed meat products (10%) and chips (10%). Foods that were found to be consumed by < 10% of the individuals included bacon (9%), dry nuts (5%), frozen and fresh salmon (4%), smoked salmon (4%) and fried snacks (4%). Ready to eat meals were consumed by 78% of the participants as reported (Daelman et al., 2013).

4.3.3 Deterministic exposure assessment

In **Table 4.4** the estimated intakes (mg kg^{-1} bw day^{-1}) by the Belgian adult consumers for the different foods are presented. Initial estimation of the exposure to EFAs through consumption

of each individual food category was based on a deterministic approach. Deterministic analysis for the chilled cooked meals could not be applied because of the nature of the available consumption data.

Table 4.4: Deterministic analysis of C18 epoxy fatty acid estimated intake (Mean, P99.5, Max) in mg kg^{-1} bw day^{-1}

Food categories	Mean	P99.5	Max
Chips	0.0128	0.3434	2.6425
Cookies	0.0058	0.1478	0.8570
Dry nuts	0.0204	0.6198	9.9099
French fries	0.0413	0.8774	1.8511
Plant oils	0.1196	1.6808	2.6300
Bacon	0.0046	0.1716	0.4262
Butter-Margarine	0.4652	1.6243	2.9110
Cheese	0.1400	2.1054	5.5076
Cooked meat	0.0011	0.1716	0.4262
Mayonnaise	0.5887	1.7289	2.0754
Milk	0.3156	3.5879	8.8297
Raw ham	0.0024	0.0584	0.4254
Cured minced raw meat	0.0112	0.2711	0.8130
Smoked salmon	0.0013	0.0579	0.3388
Fresh and frozen salmon	0.0068	0.4164	1.1062
Snack foods	0.0089	0.2744	3.0166
Ready to eat meals	N/A	N/A	N/A

N/A, not applicable

Food categories that had the lowest contribution to the mean intake of EFAs were cooked meat products followed by smoked salmon, raw cured ham, bacon, cookies and fresh and frozen salmon with levels of 0.0011, 0.0013, 0.0024, 0.0046, 0.0058 and 0.0068 mg kg^{-1} bw day^{-1} respectively. Since no toxicological reference values exist for EFAs, the TTC value of 0.0015 mg kg^{-1} bw day^{-1} was used to estimate the risk towards human health. This is the US FDA 'threshold of regulation' set value, which is defined as 0.5 ppb in the diet corresponding to 1.5 μg/person/day (FDA, 1995). The lowest exposure to EFA was through consumption of cooked meat, this can be attributed to the lowest mean contamination levels (12.54 mg kg^{-1}) combined with the low average daily meat intake (1.0 x 10^{-4} kg of food kg^{-1} bw day^{-1}). Exposure due to the consumption of milk appears higher although EFA content is low (mean concentration 40 mg kg^{-1}), which is mainly because higher amounts are consumed daily by the individuals (mean consumption 8 x 10^{-3} kg of food kg^{-1} bw day^{-1}). The highest exposure for the consumers was found to originate from the consumption of mayonnaise, butter-margarine,

fried snacks, cheese and plant oils. The highest exposure to the EFAs is specifically attributed to mayonnaise and butter-margarine as the most significant sources of mean EFA intake estimated at the levels of 0.589 and 0.465 mg kg^{-1} bw day^{-1} respectively. Although dry nuts are not consumed in large amounts (3 x 10^{-5} mg kg^{-1} bw day^{-1}), a high mean daily intake of 2 x10^{-2} mg food kg^{-1} bw day^{-1} was observed and this is due to the high contamination levels (687 mg kg^{-1} of nuts). Concerning chips and cookies which are important snacks among the consumed foods in Belgium even with preschool children (Huybrechts et al., 2008), the exposure was approximately 9 and 4 times higher than the TTC value. Considering P99.5 and max intake EFAs in all the foods using a deterministic approach, high levels of exposure were registered and confirmed that the portion of the consumers who are consuming highly contaminated specific foods in large amounts are more exposed to high amounts of EFAs than the average consumer. This maximum intake of EFAs in all foods is of concern and because there was exceedance of the intake by the consuming population, use of probabilistic approach was deemed necessary to calculate further the exposure and the risk to consumers.

4.3.4 Probabilistic exposure assessment

More accurate determinations of exposure are obtained during probabilistic analysis because each possible value that each variable can take and the possible probabilities of its occurrence are considered (Vose, 2008). Consumption and contamination data for the different food categories were fitted to the best distributions which were defined based on the lowest chi square statistics and P-P plots attributed in @Risk software (**Table 4.2**). Before quantitative analysis using the fitted distributions, stability and reproducibility were tested, whereby three simulations with 50000 iterations were made and the results were found to be consistent and reproducible. In **Table 4.2** the best fit distribution functions are shown and the parameters describing them. The accompanying statistics that are required for the calculation (min, mean and max) are shown. These are random values chosen by @Risk after considering the uncertainty in the two data inputs (concentration and consumption) to represent the minimum, mean and maximum values the concentration of EFAs and the consumption of the foods in question can have within the defined distributions. Results from the probabilistic estimates of the intake (mean, standard deviation, maximum, percentiles) resulting from the consumption by both the consumers and the total population of each food category are presented in **Table 4.5**.

Food ranking according to the percentage exceeding the TTC show that butter-margarine, mayonnaise and ready to eat meals had 100% of the consumers above the TTC value which corresponded to 50% of the total population in butter-margarine and mayonnaise. Other foods in decreasing order of exceedance were ranked as follows: cheese, snacks foods, plant oils, French fries, dry nuts, chips, cured minced raw meat, cookies, fresh and frozen salmon, bacon, raw cured ham, smoked salmon, and cooked meat. At P50, it can be concluded that the highest contribution to the intake of EFAs comes from mayonnaise, butter-margarine and the ready to eat meals which is 0.41, 0.33 and 0.30 mg kg^{-1} bw day^{-1} respectively. The probabilistic approach had a higher mean intake via ready to eat meals (2.52 mg kg^{-1} bw day^{-1}) by a magnitude of eight times compared to the median (P50) (0.3 mg kg^{-1} bw day^{-1}) because of the high positive skewness in the distributions.

Both the deterministic and probabilistic approach had nearly the same mean estimation of the intake except in the snack foods where the deterministic approach overestimated the intake by 3%. The P99.5 estimate of the deterministic approach had higher exposure values by a magnitude of 2-7 times in other foods, however, cooked meat estimate was 21 times higher (**Table 4.4** and **Table 4.5**). Similar observations were made by Papastergiadis et al. (2014) when they studied intake of aldehydes in similar food categories, where the deterministic approach had similar mean estimation and it tended to overestimate the intake at both P99.5 and at the maximum intake. This observation mainly depends on the type of distribution that are fitted to the data, highly skewed distributions tend to have higher means and P99.5 estimates (Papastergiadis et al., 2014).

Table 4.5: Estimated intakes (mg kg^{-1} bw day^{-1}) of C18 epoxy fatty acids from different foods using a probabilistic approach

Food categories		Mean	StDev	P50	P75	P90	P97.5	P99.5	Max	% >TTC
Chips	Consumers	0.0150	0.0196	0.0081	0.0191	0.0377	0.0705	0.1113	0.3330	86
	Total population	0.0075	0.0155	0.0000	0.0081	0.0232	0.0528	0.0907	0.3095	43
Cookies	Consumers	0.0059	0.0065	0.0038	0.0079	0.0139	0.0233	0.0362	0.1002	76
	Total population	0.0029	0.0055	0.0000	0.0038	0.0092	0.0185	0.0304	0.1002	38
Dry nuts	Consumers	0.0207	0.0286	0.0112	0.0256	0.0499	0.0981	0.1686	0.5925	90
	Total population	0.0103	0.0228	0.0000	0.0113	0.0309	0.0717	0.1350	0.5925	45
French fries	Consumers	0.0413	0.0511	0.0243	0.0538	0.0989	0.1809	0.2966	0.8278	95
	Total population	0.0208	0.0415	0.0000	0.0246	0.0652	0.1384	0.2452	0.7068	48
Plant oils	Consumers	0.1165	0.1929	0.0483	0.1343	0.2989	0.6488	1.1498	4.9023	97
	Total population	0.0583	0.1500	0.0001	0.0481	0.1700	0.4553	0.9220	4.9023	48
Bacon	Consumers	0.0047	0.0064	0.0024	0.0060	0.0120	0.0227	0.0370	0.1117	62
	Total population	0.0024	0.0052	0.0000	0.0024	0.0074	0.0173	0.0313	0.1117	31
Butter-Margarine	Consumers	0.4656	0.4460	0.3328	0.5751	0.9489	1.6584	2.7921	8.6917	100
	Total population	0.2337	0.3944	0.0370	0.3336	0.6588	1.2757	2.2471	8.6917	50
Cheese	Consumers	0.1395	0.1642	0.0866	0.1844	0.3292	0.5826	0.9543	2.6107	99
	Total population	0.0702	0.1367	0.0002	0.0871	0.2176	0.4498	0.7973	2.4784	50
Cooked meat	Consumers	0.0011	0.0014	0.0006	0.0014	0.0027	0.0050	0.0083	0.0263	23
	Total population	0.0005	0.0011	0.0000	0.0006	0.0017	0.0038	0.0069	0.0263	12
Mayonnaise	Consumers	0.5616	0.6026	0.4085	0.6527	1.0491	1.9647	3.7183	22.1083	100
	Total population	0.2821	0.5122	0.0750	0.4076	0.7404	1.4665	2.9125	18.3956	50
Milk	Consumers	0.2899	0.3663	0.1712	0.3917	0.7187	1.3009	2.0624	5.8169	96
	Total population	0.1438	0.2929	0.0000	0.1724	0.4670	0.9877	1.6630	4.2087	48
Raw ham	Consumers	0.0023	0.0034	0.0011	0.0028	0.0057	0.0115	0.0211	0.0534	42
	Total population	0.0011	0.0027	0.0000	0.0012	0.0033	0.0084	0.0168	0.0534	21
Cured minced raw meat	Consumers	0.0115	0.0151	0.0067	0.0146	0.0272	0.0513	0.0872	0.4979	84
	Total population	0.0057	0.0121	0.0000	0.0067	0.0176	0.0382	0.0710	0.2834	42
Smoked salmon	Consumers	0.0013	0.0018	0.0007	0.0016	0.0031	0.0060	0.0108	0.0306	27
	Total population	0.0007	0.0014	0.0000	0.0007	0.0020	0.0045	0.0085	0.0306	14
Fresh and frozen salmon	Consumers	0.0068	0.0098	0.0035	0.0087	0.0174	0.0338	0.0563	0.1811	69
	Total population	0.0034	0.0078	0.0000	0.0034	0.0106	0.0251	0.0471	0.1811	34
Snack foods	Consumers	0.2982	0.3411	0.1869	0.4032	0.7171	1.2255	1.8712	6.5076	99
	Total population	0.1478	0.2794	0.0000	0.1854	0.4713	0.9679	1.5586	4.0098	49
Ready to eat meals[a]	Consumers	2.5223	10.3656	0.2997	1.3978	7.0671	17.4940	38.6068	961.2216	100

[a], The population was obtained from the survey conducted by (Daelman et al., 2003); TTC, Threshold of Toxicological Concern

4.3.5 Risk characterization

Due to the lack of toxicological data on EFAs the TTC principle was applied. Based on the Cramer decision tree, EFAs were grouped in class III and therefore, a TTC level of exposure of 0.0015 mg kg^{-1} bw day^{-1} was considered in evaluating the intake take data that was obtained from the probabilistic analysis. The average body weight used was 60 kg (Kroes et al., 2005). Based on the probabilistic analysis of the consumption and the contamination data it can be suggested that, the consumers of the studied food categories may be at risk, because exposure was far above the defined TTC. The exception was the cooked meat, smoked salmon and raw cured ham which were below the TTC value with a P50 intake of 0.0006, 0.0007 and 0.0011 mg kg^{-1} bw day^{-1} respectively. This corresponded to 77, 73 and 58% of the consumers of these specific food groups exposed to EFAs at levels below the TTC value 1.5 mg kg^{-1} bw day^{-1} respectively. The rest of the consumers of the other foods were exposed to high levels which were up to 200 times far above the TTC value with 100% of the consumers of mayonnaise, butter-margarine and ready to eat meals at a risk. Thus, a potential risk may occur for this portion of consumers frequently consuming these specific food categories.

4.3.6 Uncertainty evaluation of the exposure assessment

There are always uncertainties related to exposure assessments that should be considered for the interpretation of the results. The BNFCS used in this study was conducted in 2004, and it is known that eating habits might change over time, therefore it is not clear if the dietary intake of the Belgian population has currently changed, this might lead to some uncertainties during result interpretation. However, a new evaluation study was conducted in 2014 by the Public Health Authority and the results are not yet out. A possible under or overestimation of the consumption of the studied food groups resulting from misreporting during consumption data collection (e.g. inaccuracies on consumed quantities reported, foods reported in wrong food groups) could be some of the limiting factors in the precision of the estimated intakes for the EFAs. Despite this, the 24 h recall is usually the best recommended method used to estimate dietary food intake in big consumption surveys in Europe (de Boer et al., 2011). For the chilled cooked meals, the consumption data does not represent the total population residing in Belgium, thus deviations could be expected from the national consumption of these products. The current calculations and interpretations are performed for the individual

food categories, no inference can be made for the total exposure resulting from consumption of other foods of the Belgian consumers.

Uncertainty from the concentration data is that the risk from the exposure to EFAs is evaluated as total but not as individual isomers. In view of their structure differences in terms of unsaturation, this may lead to reactivity differences for each isomer which may imply that a combined estimation of all the EFAs could be an underestimation of the possible risk that they can cause depending on the individual isomer behaviour in the body. However, on the other hand it is also possible that a combined exposure of all the twelve isomers would lead to an overestimation of the risk that may be posed and lead to wrong conclusions that many consumers are at risk as predicted from such a study that does not consider the individual isomer exposure. In case of salmon which is rich in omega-3 fatty acids such as eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6), there could be an underestimation since the method of analysis only determined C18 EFAs. The best estimation can be provided if the concentration data is obtained after hydrogenation to obtain all the EFAs present, but then again potential reactivity differences between the different isomers are neglected. This study also considered only mono-epoxy fatty acids, while it is probable that in foods rich in polyunsaturared fatty acids, also di- or triepoxy fatty acids may be present.

4.4 CONCLUSIONS

This is the first study concerning exposure assessment of a population to EFAs through consumption of specific foods. The exposure related to the consumption of the specific food categories and the resulting potential risk has been reported. Results indicate that consumers of the studied food categories are exposed to high levels of EFAs. Based on the TTC principle as a preliminary risk characterization approach, results suggest that a risk could exist due to the consumption of these foods for the vast majority of the consumers. Since the TTC is a preliminary approach, it is recommended that extensive toxicity testing and safety evaluation of EFAs be done to fully characterise the risk involved especially in plant-based foods.

An aggregate exposure approach for lipid oxidation compounds to assess the potential risk should be done. In such a study, EFAs should be combined with those oxidation products

(MDA, HNE and HHE) presented by Papastergiadis et al. (2014), to get a total picture of the toxic oxidation compounds which consumers may be exposed to. It is also known that EFAs once formed cannot be removed and highly nutritious foods like nuts have been seen to contribute significantly to the intake of EFAs. Therefore, it can be advised that reduction of intake of EFAs through prevention of lipid oxidation is needed. This can be achieved by taking precautions to prevent lipid oxidation in foods especially during processing and storage, which may lower consumption of such oxidised foods.

CHAPTER 5

INSIGHTS INTO THE FORMATION OF EPOXY FATTY ACIDS DURING AUTOXIDATION AND PHOTOOXIDATION OF VEGETABLE OIL MODELS

ABSTRACT

Formation of epoxy fatty acids (EFAs) from oleic, linoleic and linolenic acid was investigated in different oil blends during photosensitized oxidation (at 6°C in the presence of chlorophyll and its degradation products), photoxidation (at 6°C) and autoxidation (at 70°C). Progressive lipid oxidation was observed in all experimental conditions on basis of the peroxide value and the conjugated diene content. In most of the samples, the occurrence of these oxidation markers was followed by the occurrence of EFAs. The EFA content of the samples were comparable with earlier reports on the presence of EFAs in fresh oils and increased upon progressive oxidation. Formation of EFA proved to occur in a stereospecific manner and in addition only specific place isomers of EFA were formed. On basis of these observations, it was suggested that EFA are most likely produced as a result of a 1,3 cyclisation of the hydroperoxide radical or alkoxy radical to the double bond of the oxidizing fatty acid, resulting initially in the formation of *cis* stereoisomers. Upon progressive oxidation a radical supported *cis-trans* isomerization is suggested resulting into an increasing *trans/cis* ratio.

Keywords: Lipid oxidation, epoxy fatty acids, fatty acid methyl esters, transesterification, photooxidation

5.1 INTRODUCTION

In the previous chapters, analytical methods were developed to quantitatively analyse the C18 mono EFAs in oils and fats and more complex food matrices. These methods were applied to determine the concentration of these understudied lipid oxidation products in a variety of oils and foods. Surprisingly, in several blends relatively high concentrations were found despite the classical lipid oxidation parameters indicated a low oxidation status. In addition, no information is currently available with respect to the formation of EFAs as a result of photooxidation.

Photosensitized oxidation involves the oxidation in presence of a photosensitizer which is a molecule able to capture the energy from visible photons in order to become sensitized. The molecule is then able to transfer this energy to the lipid directly enabling it to form a radical which will react with triplet atmospheric oxygen leading to the formation of hydroperoxides as in the autoxidation reaction (*type I photooxidation*). Alternatively, the sensitized molecule can transfer its energy to triplet oxygen, due to which the significantly more reactive singlet oxygen is generated which will directly react with unsaturated fatty acids to form hydroperoxides (*type II photooxidation*). Naturally present pigments such as chlorophyll, pheophytin, myoglobin, hemoglobin, protoporphyrins and riboflavin act as sensitizers as do dyes, including erythrosine, xanthenes, anthraquinone, rose Bengal and methylene blue (Gunstone et al., 2007; Shahidi, 2005). The only fat soluble photosensitizers in foods are chlorophyll and its degradation products pheophytin, pyropheophytins and pheophorbide (Daun, 2012; Usuki, Endo, & Kaneda, 1984). Chlorophyll as a photosensitizer produces both free radicals and singlet oxygen with the dominant reactions depending on substrate and reaction conditions (Yam, 2009). Chlorophyll pigments are extracted into the oil where they undergo several transformations resulting in crude oil containing mostly pheophytins and pyropheophytins (Daun, 2012). It has been reported that pheophytins and pheophorbides have a higher prooxidant activity than chlorophyll (Usuki et al., 1984). Apart from photosensitized oxidation, photooxidation can be induced by UV-light and proceeds in a similar way as the autoxidation reaction.

As outlined before, upon decomposition of the lipid hydroperoxides, the formed alkoxy radicals can lead to the formation of EFAs by replacing a site of unsaturation or adding nearby it (Neff & Byrdwell, 1998). Till now the formation of EFAs has mainly been

documented and related to oxidation at high temperatures, typically during frying (Berdeaux et al., 1999b; Kalogeropoulos et al., 2007; Marmesat et al., 2008; Velasco et al., 2002; Velasco et al., 2004). Yet it is well known that thermal oxidation (frying temperatures) due to the amount of energy involved leads to the production of high amounts of secondary oxidation products including EFAs. Data presented in **Chapter 2 and 3** however suggest that also at moderate (elevated) temperatures EFAs can be generated via autoxidation. Previous studies have also reported high amounts of EFAs in fresh and oxidised foods (Fankhauser-Noti et al., 2006).

Therefore, the objective of this study was to investigate the extent to which EFAs form during the common lipid oxidation mechanisms, i.e. photosensitized induced oxidation, photooxidation and autoxidation using bulk oil blends that are rich in the three unsaturated fatty acids C18:1 (predominately present in olive oil), C18:2 (predominately present in sunflower oil) and C18:3 (predominately present in lineseed oil). It was hypothesized that each of the lipid oxidation mechanisms leads to EFA formation however the amounts may differ in each case. As in each case of oxidation specific geometrical hydroperoxides are formed, it was expected that this would be reflected in the final isomeric distribution (ID) of the formed EFAs as well.

Photosensitized oxidation was carried out using a fat soluble fotosensitizer (chlorophyll and its degradation products as present in virgin olive oil) at refrigerated temperatures as before we have showed that in riboflavin containing emulsions, oxidation proceeded readily at 6°C (Mestdagh et al. 2011). Virgin olive oil was blended with three different types of stripped oil. Stripped oils, void of antioxidants was used, to ensure oxidation occurred during storage, because virgin olive oil does not only contain chlorophyll and its degradation products as a pro-oxidant, but also a number of potent anti-oxidants. Control experiments were included respectively considering blends void of the photosensitizer (and the antioxidants present in the virgin olive oil) and all the blends stored without illumination. These additional blends were obtained by stripping olive oil in order to remove the chlorophyll and its photosensitizing degradation products.

Autoxidation studies at moderately elevated temperatures, relevant for pasteurized foods or foods stored for a particular time at a higher temperature were carried out at 70°C using the similar oil blends as used during the photooxidation studies for consistency. Non-stripped

blends were included as well in these experiments, as it was expected that the oxidation in the stripped blends would proceed too fast to be really representative.

5.2 MATERIALS AND METHODS

5.2.1 Reagents and chemicals

Aluminum oxide activated, basic, Brockmann I (150 mesh) was purchased from Sigma-Aldrich (Bornem, Belgium) and the other reagents and chemicals used are fully described under **chapter 2 and 3 sections 2.2 and 3.2** respectively**.**

5.2.2 Stripping of oils and oil blend preparation

The triacylglycerols (TAGs) were isolated from olive, sunflower and linseed oil according to a modified method (Mariod, Matthäus, & Hussein, 2011). Briefly, 25 g of oil were dissolved in 30 mL of petroleum ether and loaded onto a glass column containing 25 g of activated silica. The oil was eluted with 50 x 3 mL of petroleum ether and then recovered by evaporating the solvent off under a vacuum in a rotating evaporator (Heidolph Instruments GmbH & Co, Schwabach, Germany) operating at 30 ^{o}C. Furthermore, a total of 100 g of activated aluminum oxide was packed in another glass column. The oil obtained from the previous step was then dissolved in 60 mL of petroleum ether, loaded onto this column and eluted with 2 x 50 mL of hexane. The solvent was later evaporated on a rotating evaporator as above and the oils were further used for the preparation of the oil blends. More residual solvents were removed by flushing the oils with nitrogen for 12 hr. Further oil oxidation during and after stripping was prevented by limiting direct exposure to light, flushing with nitrogen and cold storage at -20°C.

5.2.3 Experimental setup

5.2.3.1 Blending of the oils

Oils were blended together using an Ultra-Turrax blender (Janke & Kunkel, IKA-Werk, Staufeb, Germany) for 1 min at 14000 rpm and later sonicated to ensure homogeneity. The oils were always protected from light by wrapping in an aluminum foil. The specific composition of the blends studied is outlined in **Table 5.1** and **Table 8.1** in **Appendix**.

5.2.3.2 Oxidation studies at 6°C under illumination and in the dark

Six oil blends were considered for this study, identified as blends A - C and D - F (**Table 8.1 in Appendix**). The first blends (A - C) were prepared by mixing the stripped oil (respectively refined olive, sunflower and linseed oil) to a fixed amount of non-stripped virgin olive oil in a ratio 2:1 to obtain a chlorophyll level of about 11 mg kg^{-1} oil. The second group of blends (D - F) were prepared by mixing the same stripped oils (refined olive, sunflower and linseed oil) to a fixed amount of stripped refined olive oil (by stripping the refined olive oil used to prepare blends A and C) in a ratio 2:1. Consequently these blends (D-F) did not contain any chlorophyll (or its degradation products).

Four mL of each blend was introduced in 20 ml transparent SPME vials. Samples were placed in a cold room equipped with two 40 W cool white fluorescent lights (Philips TL-D 36W/840 fluorescent tubes) which were suspended at 0.5 m above the surface of the oil containers. The illumination intensity measured at the level of the bottlenecks using a Lux meter (PAR-cell 532; 400 − 700 nm, Skye Instruments, Llandrindod Wells, UK) was 2630 Lux and the temperature inside the cold room was at 6 ± 1°C. In parallel, samples in similar amber SPME vials were stored in a cardboard box to prevent any possible exposure to light. Sampling was done at 0, 12, 19, 27, 43 and 54 days, except that for the samples stored in the dark, no sample was taken at 12 days of storage.

5.2.3.3 Oxidation studies at 70°C

For this study, twelve different oil blends were considered. The first six blends were the same as those used in the oxidation studies at 6°C, albeit originating from a different batch. In addition to these blends, three additional blends (A'-C') were prepared by mixing the original non-stripped oil (refined olive, sunflower and linseed oil) to a fixed amount of virgin olive oil in a ratio 2:1. Blends E'-F' were of the same composition but only differed by replacing the non-stripped virgin olive oil with non-stripped refined olive oil (**Table 8.1** in **Appendix**). Sampling for elevated temperature was done at 0, 1, 3 and 5 days since the reaction was proceeding very fast.

5.2.4 Analytical methods

5.2.4.1 Peroxide value determination

The PV in meq O_2 kg^{-1} oil was determined using an iron based spectrophotometric method as described previously (Shantha & Decker, 1994). A calibration curve was constructed just before measurement of the peroxide value of the sample was done. The calibration curve was constructed using a standard iron (III) chloride solution as described in the same method. A six-point calibration in the range of 5 to 30 μg of iron (III) was done in duplicate. A R-squared value of more than 0.99 for the calibration was always obtained.

5.2.4.2 Conjugated dienes and conjugated trienes measurement

Spectrophotometric determination of conjugated dienes (CD) and trienes (CT) by UV absorption at 232 nm and 268 nm was carried out according to AOCS standard method 2.501 (AOCS, 1998). Briefly accurately 0.01 to 0.05 g oil sample was weighed into a 10-mL volumetric flask and dissolved in isooctane, mixed thoroughly and brought to volume. An Ultra-Turrax was used to aid in sample dissolution. To measure the CDs, absorbance was set at a wavelength of 233 nm. Measurements of absorbance of the dissolved oil sample was done using a quartz cuvette. An increase in absorbance suggests that the sample is oxidizing, where the absorbance of the sample was greater than 1, the sample was further diluted in the same solvent and measured again. This procedure was repeated at an absorbance of 268 nm for CT determination. To facilitate comparison between CD and CT, their content was calculated based on molecular extinction coefficient of 29000 $M^{-1}cm^{-1}$ (Frankel, 2005).

5.2.4.3 Chlorophyll pigments and fatty acid composition measurement

The determination of the total chlorophyll pigments content was based on absorption spectrophotometry of the oil according to the IUPAC commission standard method (Pokorny, Kalinova, & Dysseler, 1995). In fact, chlorophylls can absorb light in the UV/visible region because of the delocalization of the π electrons throughout the porphyrin system which is the chromophore for these pigments. The absorbance reading for chlorophylls was made at 630, 670 and 710 nm. The total chlorophyll content was expressed in mg of pheophytin kg^{-1} of oil.

Fatty acid composition was determined after preparation of fatty acid methyl esters (FAMEs) as described in **chapter 2** (**section 2.2.9.3**).

5.2.4.4 Transmethylation and SPE separation for EFAs

Base catalysed transmethylation procedures and the solid-phase extraction (SPE) separation steps followed, have previously been described in **chapter 2 section 2.2.5 and 2.2.6.**

5.2.4.5 Gas chromatography-FID conditions for fatty acids and EFAs

GC analysis for both EFAs (EFAs) and fatty acid methyl esters (FAMEs) was performed on a GC-FID Agilent 6890N series gas chromatograph (Agilent, USA) as described in **chapter 2 section 2.2.9.1.**

5.2.5 Statistical analysis

Comparison of the means of EFAs, PV, CD and CT of different experimental studies was done using one-way ANOVA test applying a significance level of $p < 0.05$. Normality was checked with a Shapiro-Wilk test and the Levene test was applied to confirm the homogeneity of variances. Logarithmic transformations of the different data sets which violated normal distribution were carried out before statistical analysis to have homoscedasticity in some cases. The statistics package used was SPSS 22.

5.3 RESULTS AND DISCUSSION

5.3.1 Fatty acid composition of the various blends

The oils blends used in this study (**Table 8.1** in **Appendix**) were composed in such a way that they contained varying amounts of the three fatty acid precursors of the EFAs considered in this study (**Table 5.1**). Therefore olive, sunflower and linseed oil were selected, known to be major sources of C18:1, C18:2 and C18:3 fatty acids, respectively. Since different feedstocks were used for respectively the photo- and autoxidation study, the fatty acid composition was separately determined. There were small variations between the different stocks used.

Stripped oils were used in order to ensure that oxidation actually would occur, especially during the photosensitized oxidation study. Virgin olive oil was added as a source of chlorophyll (and its degradation products), as photosensitizer. Virgin olive oil however also contains a number of potent antioxidants. By stripping, non-glyceride minor components like

tocopherols, chlorophylls, phytosterols, squalene, waxes, metals, pigments, free fatty acids and lipid oxidation products are removed (Khan & Shahidi, 2002; Mariod et al., 2011).

Considering the photooxidation study at 6°C, blends D-F were considered as chlorophyll free 'control' samples of the homologous blends A-C. For consistency, the same blends (A-F) were considered for the autoxidation study at 70°C, but non-stripped homologous blends (A'-F') were included as well because it was expected that the oxidation would proceed unrealistically fast in the stripped blends. The fatty acid composition of blends A'-F' were not significantly different from the stripped blends A-F (not shown) and were therefore not included in **Table 5.1** for clarity.

Table 5.1: Fatty acid composition (g 100 g^{-1} fatty acid) of the stripped oil blends used for the study

	Fatty acids for samples at 6°C				Fatty acids for samples at 70°C			
Oil blend[1]	**C18:1**	**C18:2**	**C18:3**	**others**	**C18:1**	**C18:2**	**C18:3**	**others**
A	64.0	10.6	0.7	18.2	71.0	9.6	0.6	18.9
B	34.7	29.5	0.4	9.2	46.4	39.7	0.4	13.5
C	30.4	9.0	26.4	10.2	39.9	12.3	34.3	13.5
D	44.2	9.6	0.5	14.8	68.4	11.0	0.6	19.9
E	31.3	31.4	0.4	10.3	43.4	41.5	0.5	14.6
F	25.2	10.2	24.2	10.1	37.3	13.7	34.2	14.8

[1]Blend: A = stripped refined olive + virgin olive oil; B = stripped sunflower + virgin olive oil; C = stripped linseed + virgin olive oil; D = stripped refined olive oil; E = stripped sunflower + stripped refined olive oil; F = stripped linseed + stripped refined olive oil

5.3.2 Oxidation studies at 6°C under continuous illumination

As mentioned before, the photooxidation study at 6°C included two sets of control experiments. A first 'control' consisted of a set of homologous blends void of chlorophyll (and its degradation products) as fat soluble photosensitizer (blends D-F). All blends were exposed to light and in parallel were stored in the dark, which consisted of the second group of control experiments, identified as 'dark' experiment further on.

Virgin olive oil as a source of the photosensitizer was chosen as chlorophyll in its pure state is expensive. It should be noted that the use of virgin olive oil not only added chlorophyll

(and its degradation products), but also added other minor oil components, especially the antioxidants present with it. By stripping, these are also removed. This could have changed the dynamics of the oxidation process**.** Therefore, it is clear that blends D-F cannot be considered strictly as true controls of blends A-C. The only way in which a true control can be obtained is by the addition of pure chlorophyll, which was not feasible. Chlorophyll isolates of a lower purity cannot be considered either because they are known to contain a number of carotenoids. In addition, it should be noted that in blends D-F the stripped olive oil used was obtained from a refined olive oil and not from the virgin olive oil used as a chlorophyll source in blends A-C. This is reflected in the small differences in fatty acid composition of the blends as reported in **Table 5.1**.

Table 5.2: Changes in chlorophyll content (mg pheophytin kg^{-1} oil) in stripped oil blends during oxidation under illumination and in the dark at 6 ± 1°C

Time (days)	Oil blend[1]											
	Dark[a]						Illuminated[b]					
	A	B	C	D	E	F	A	B	C	D	E	F
0	11.1	11.2	10.5	<LOD	<LOD	<LOD	11.2	11.3	10.5	<LOD	<LOD	<LOD
12	ND	ND	ND	ND	ND	ND	6.41	4.79	<LOD	<LOD	<LOD	<LOD
19	11.2	11.4	11.3	<LOD	<LOD	<LOD	4.69	3.33	<LOD	<LOD	<LOD	<LOD
27	11.2	11.3	11.3	<LOD	<LOD	<LOD	2.72	2.16	<LOD	<LOD	<LOD	<LOD
43	11.2	11.3	11.3	<LOD	<LOD	<LOD	1.26	0.83	<LOD	<LOD	<LOD	<LOD
54	11.2	11.3	11.3	<LOD	<LOD	<LOD	1.27	0.85	<LOD	<LOD	<LOD	<LOD

ND, not determined; LOD, limit of detection (1 mg/kg of oil as reported by Pokorny et al., 1995)

[a]Samples stored in the dark at 6 ± 1°C; [b]Samples stored under continous illumination (2630 lux) at 6 ± 1°C

[1]Blend: A = stripped refined olive + virgin olive oil; B = stripped sunflower + virgin olive oil; C = stripped linseed + virgin olive oil; D = stripped refined olive oil; E = stripped sunflower + stripped refined olive oil; F = stripped linseed + stripped refined olive oil.

The impact of this particular element on the oxidation process is however expected to be minor compared to the impact that in the 'control' blends D-F, apart from chlorophyll (and its degradation products) also the antioxidants present in virgin olive oil are absent. Results for total chlorophyll pigments and their evolution throughout the storage experiment, are presented in **Table 5.2**. The blends D-F did not contain chlorophyll in detectable amounts (LOD 1 mg/kg) as expected.

The chlorophyll content of the samples was monitored throughout the storage experiments at 6°C. For samples stored in the dark, it did not change significantly and thus chlorophyll and its degradation products could not act as sensitizer to promote lipid oxidation. In contrast, it could have acted as an antioxidant (Gutiérrez-Rosales, Garrido-Fernández, Gallardo-Guerrero, Gandul-Rojas, & Minguez-Mosquera, 1992). For samples, which were continuously illuminated, a time dependent degradation (exponential decay for blends A and B) was observed which moreover was more intensive in the more unsaturated oil blends.

The course of the oxidation reaction was monitored using the peroxide value, the conjugated diene and triene content in parallel to the EFA content of the respective blends as a function of incubation time (**Table 5.3**). Although it is known that photooxidation is faster than autoxidation, the kinetics of EFA formation under both mechanisms is not detailed in this study. The set up in such a study would require an EFA free sample that cannot be obtained even after stripping. EFA results were expressed as total EFAs to enable meaningful comparison of the amount formed during the storage time. Considering first the control experiments during which the samples were stored in the dark, Blend F was the only one which showed a clear trend of progressive oxidation for all parameters considered. For Blend C, characterized with a similar fatty acid composition, but containing virgin olive oil, the peroxide and CD content varied significantly as function of time, but no clear trend could be observed. On basis of these results it can be concluded that only in Blend F, significant autoxidation occurred, despite that the samples were stored in the dark and at refrigerated temperatures. The fact that this particular sample, containing only stripped oils, proved to be very prone to oxidation can be attributed to the combination of its high content of polyunsaturated fatty acids, in particular alpha-linolenic acid, and the absence of any antioxidants, which were actually present in its homologue, Blend C, as this one contained virgin olive oil.

For the samples exposed to light, a consistent progressive oxidation occurred in all samples, considering the PV, CD and CT, although the changes in CT were quite limited except in sample F. The PVs for Blends B, D and E, showed some remarkable jumps, but in view of the consistent increase in CDs, which normally are well correlated to the PV's (Shrestha et al., 2013), it seems plausible to assume indeed that also for these samples progressive oxidation occurred. For the blends containing initially chlorophyll (and its degradation products) (A-C), the increase in PV and CD levelled off near the end of the storage

experiment, while in the homologous blends, void of chlorophyll (and its degradation products) (D-F), a progressive increase was observed. Although it is known that the PV of oils normally increase and later give a plateau (Shrestha et al., 2013), it should be noted that chlorophyll (and its degradation products) decreased throughout the storage experiment (**Table 5.2**) which implies that the photosensitized induced oxidation was tempered. Due to the progressive increase in CD of the chlorophyll-free blends (D-F), this indicator became in fact higher compared to their respective homologues A-C throughout the storage experiment. This was not expected but can be explained by the phenomenon of chlorophyll degradation, as already described, and the fact that blends D-F did not contain the olive oil antioxidants present in blends A-C. So, blends D-F were in fact subjected to a photooxidation process, while blends A-C were initially subjected to a photosensitized oxidation process, which due to degradation of chlorophyll, transformed into a photooxidation process.

Comparing the EFA concentrations of the A-C vs the homologous D-F blends (**Table 5.3**) it is obvious that by stripping the oils, a major part of the original EFA present in the oils was removed. Given the fact that all blends stored in the dark (except blend F) did not show progressive oxidation, it was not surprising that no significant trends in their EFA content was observed either (**Table 5.3**). Contrary, for blend F, a parallel increase in its EFA content was observed.

With respect to the EFA content for the blends stored under continuous illumination, a progressive and significant increase was observed as function of storage time. Only for Blend C, a remarkable drop after 27 days of storage was observed, which could not be explained. As on the further days of sampling, a further increase in EFA was observed, an artefact on day 27 is not excluded. For Blend A a restricted drop in EFA content was observed after 43 days of storage, followed by a further limited decrease at the end of the experiment. No clear explanation can be found for this observation, although it was shown to be repeatable (1446 and 1651 µmol g^{-1} oil). As the main EFA present in this blend is ES and thus does not contain any additional double bound, a further oxidation of this particular EFA seemed unlikely.

Table 5.3: EFAs (EFA; µmol kg^{-1}), peroxide value (PV; meq O_2 kg^{-1} oil), conjugated dienes and trienes (CD and CT; µmol g^{-1} oil) content of stripped oil blends during oxidation in the dark and under illumination at 6 ± 1°C

Blend[1]	Storage time (days)	Dark[b]				Illuminated[c]			
		PV	CD	CT	Total EFA	PV	CD	CT	Total EFA
A	0	8.3	3.3	0.8	776.9	8.3	3.3	0.8	776.9
	12	ND	ND	ND	ND	13.8	11.3	1.1	1073.9
	19	10.8	5.7	0.9	624.6	14.6	12.9	1.0	1278.2
	27	9.1	6.0	1.0	470.3	17.0	15.5	1.2	1758.3
	43	9.6	6.4	1.0	676.7	25.8	18.2	1.4	1651.4
	54	8.8	5.8	1.0	658.1	23.0	18.2	1.4	1549.0
	p value[a]	0.003	0.000	0.001	0.008	0.002	0.000	0.000	0.116
B	0	11.4	4.6	2.5	834.5	11.4	4.6	2.5	834.5
	12	ND	ND	ND	ND	12.6	20.6	3.2	1281.4
	19	10.6	8.0	3.1	728.6	20.0	27.9	3.2	1452.4
	27	8.4	7.2	3.2	541.1	17.8	32.5	3.2	1461.4
	43	9.7	8.8	3.1	768.5	23.1	45.7	3.2	1677.3
	54	8.4	7.3	3.1	834.2	30.4	47.3	3.3	1813.7
	p value[a]	0.373	0.000	0.000	0.076	0.020	0.000	0.001	0.001
C	0	11.7	3.2	0.4	748.1	11.7	3.2	0.4	748.1
	12	ND	ND	ND	ND	9.4	18.9	0.6	1563.8
	19	14.1	6.3	0.3	820.5	22.5	25.4	0.5	1558.6
	27	13.1	5.7	0.3	510.8	11.9	30.0	0.7	1375.3
	43	15.5	7.8	0.4	942.7	15.6	37.1	0.9	1621.9
	54	16.1	7.3	0.4	843.1	19.1	44.3	1.2	1967.0
	p value[a]	0.004	0.000	0.195	0.195	0.043	0.000	0.001	0.000
D	0	0.9	2.7	1.0	105.1	0.9	2.7	1.0	105.1
	12	ND	ND	ND	ND	11.8	8.8	1.2	412.3
	19	1.8	5.9	1.3	107.5	6.3	15.1	1.0	476.9
	27	1.6	4.8	1.2	80.9	6.0	19.1	1.1	652.9
	43	1.5	6.2	1.2	80.1	7.1	32.3	1.3	1167.6
	54	1.7	5.3	1.3	84.1	16.6	45.0	1.3	1347.7
	p value[a]	0.004	0.001	0.002	0.926	0.000	0.000	0.019	0.001
E	0	1.3	4.2	2.7	187.5	1.3	4.2	2.7	187.5
	12	ND	ND	ND	ND	10.2	10.2	3.3	400.3
	19	2.1	7.3	3.4	141.3	6.2	21.0	3.0	459.5
	27	2.2	6.4	3.3	117.0	7.4	34.7	2.6	807.6
	43	2.9	7.8	3.3	115.5	20.9	65.4	2.2	1325.5
	54	3.0	7.7	3.4	163.8	22.7	91.8	2.1	1560.1
	p value[a]	0.000	0.001	0.001	0.366	0.000	0.000	0.002	0.000
F	0	5.4	2.9	0.6	296.4	5.4	2.9	0.6	296.4
	12	ND	ND	ND	ND	7.5	23.1	0.9	971.1
	19	9.2	12.5	0.7	507.6	12.8	40.6	1.7	947.7
	27	8.5	16.4	0.8	644.4	17.7	59.0	3.2	1449.0
	43	16.3	36.8	1.6	950.0	31.7	104.9	9.3	2325.7
	54	17.4	56.1	2.7	1517.7	35.9	126.8	13.7	2926.9
	p value[a]	0.088	0.001	0.011	0.011	0.000	0.000	0.000	0.000

All the data are expressed as the mean of two independent replicates. ND, not determined.

[a] *p* value <0.05 is significant according Tukey test in one-way ANOVA in every blend over time.

[b] Samples stored in the dark at 6 ± 1°C, [c] Samples stored under continous illumination (2630 lux) at 6 ± 1°C,

[1]Blend: A = stripped refined olive + virgin olive oil; B = stripped sunflower + virgin olive oil; C = stripped linseed + virgin olive oil; D = stripped refined olive oil; E = stripped sunflower + stripped refined olive oil; F = stripped linseed + stripped refined olive oil.

Similar as observed with the classical oxidation parameters, PV and CD, for the blends A-C originally containing chlorophyll (and its degradation products) as photosensitizers, it is observed that the increase of EFA as function of time occurred especially in the first 12 days of the experiment, while in the chlorophyll void homologues (D-F) a more equilibrated increase as function of time was observed. As explained before, this can be explained by the fact that for blends A-C the photosensitized oxidation diminished as a result of the chlorophyll degradation and moreover olive oil antioxidants were present. This explains also the increase in the EFA content of the stripped oil blends D-F, void of the photosensitizers and antioxidants, showed to be stronger and more consistent.

In **Table 8.2** and **Table 8.3** (**in Appendix**), the data for each individual EFA present in the studied samples are given. Not surprisingly, a good correspondence between the main fatty acids present in the original blends and the main EFAs produced throughout the incubation experiments (both in the dark and in the light) were observed.

Although care should be taken in comparing the homologous blends among each other (for instance blend A vs D), as the feedstocks used to prepare them are different and also because the initial amount of EFAs was considerably different, some interesting observations can be made considering illuminated blends. If the net amount of ES (including both isomers) produced throughout the storage period was compared between blend A vs blend D, it was observed that considerably more ES was produced in the stripped oil (blend D). This was also the case for the other homologous pairs (B vs E and C vs F). For EO, again considering all isomers, however, the net produced amounts in blend A vs C were comparable, while again for blend B vs E and blend C vs F, overall more EO was produced in the stripped oils. Finally, with respect to EOL, only considering blends C and F (as the alfa-linolenic acid concentration in the other blends was smaller than 1%), a similar observation was made. These observations again suggest that the stripped blends were more prone to oxidation then their virgin olive oil containing homologues, despite the fact that the latter ones were subjected to chlorophyll induced photosensitization.

As outlined before, EFAs exhibit stereoisomerism. In an attempt to get more insight in the mechanistics of EFA formation, the isomeric distribution of each individual type of EFA (ES, EO and EOL) was computed on a molar basis. Results are presented in **Table 5.4**. The isomeric distribution for each respective EFA was expressed as a percentage of the total

stearate, oleate or linoleate EFA isomer content. Two EFA isomers originating from linolenic fatty acid, *cis*-12,13 and *trans*-9,10-epoxylinoleate co-eluted thus their isomeric distribution could not accurately be determined. As the EFA formation in the dark was restricted, these were not considered in this analysis.

In general, *cis* isomers dominated in all oil blends before storage. In the blends E and F, only containing stripped oils, a somewhat higher portion of *trans*-EFA were present, especially for ES, but due to the stripping the overall EFA content of these blends prior to incubation was quite low (**Table 5.3**). During incubation of the blends containing virgin olive oil (A-C), the *cis*/*trans* ratio hardly changed, for all EFAs considered. Also, for *cis*-12,13 and *trans*-9,10-epoxylinoleate, it seems reasonable to suppose that the *cis* stereoisomer was and remained dominant, in view of the observations for the other epoxylinoleate isomers. So, it can be concluded that especially the *cis* stereoisomers were formed. In the blends containing only stripped oils (D-F), which were proven to be considerably more susceptible to oxidation (**Table 5.3**), it was obvious that the *trans* stereoisomers of all considered EFA were predominately formed. At the end of the incubation period considered, the ratio of both stereoisomers was almost 1:1 (for ES and EO, and probably for EOL as well, although this could not be concluded with certainty).

Thus, it seems that in absence of antioxidants and chlorophyll (and its degradation products) as photosensitizer, photoinduced oxidation favored the formation of trans stereoisomers. Despite the fact that in the first series of experiments, the oxidation is both due to photosensitized photooxidation (as long as chlorophyll is present) and photooxidation, the formation of cis stereoisomers is preferred. The formation of trans EFAs in blends D-F is more explained in section 5.3.4, in which we hypothesize that trans EFAs are formed from the initially formed cis EFA upon progressive oxidation via a radical mechanism. This hypothesis is in line with the observation in samples D-F, where trans isomers become dominant at longer incubation times (i.e. more progressed oxidation). This hypothesis also concurred with the observation that samples A-C containing virgin olive oil with antioxidants contained less trans isomers as the oxidation reaction was somewhat modulated.

Table 5.4: Percentages isomeric distribution (ID) of ES, EO and EOL on mole basis of the different stripped oil blends during oxidation under continuous illumination at 6°C

		Isomeric distribution										
		Oleic acid[a]		Linoleic acid[a]				Linolenic acid[a]				
Blend[1]	Storage Time (days)	*trans*-9,10-ES	*cis*-9,10-ES	*trans*-12,13-EO	*cis*-12,13-EO	*trans*-9,10-EO	*cis*-9,10-EO	*trans*-12,13-EOL	*cis*-12,13 & *trans*-9,10-EOL	*cis*-9,10-EOL	*trans*-15,16-EOL	*cis*-15,16 –EOL
A	0	8	92	<LOD	39	<LOD	61	<LOD	77	11	<LOD	13
	12	6	94	<LOD	36	13	51	3	44	36	<LOD	17
	19	4	96	<LOD	31	14	55	8	24	60	<LOD	8
	27	7	93	2	32	25	41	5	25	28	29	14
	43	10	90	<LOD	36	20	44	<LOD	23	49	28	<LOD
	54	11	89	<LOD	31	28	41	<LOD	30	48	23	<LOD
B	0	5	95	<LOD	43	<LOD	57	<LOD	100	<LOD	<LOD	<LOD
	12	6	94	1	36	4	59	14	36	32	<LOD	18
	19	8	92	1	31	9	59	16	33	19	<LOD	32
	27	12	88	3	31	16	50	20	26	24	30	<LOD
	43	7	93	4	34	9	54	<LOD	20	51	28	<LOD
	54	8	92	3	33	10	53	<LOD	40	33	27	<LOD
C	0	8	92	<LOD	42	<LOD	58	<LOD	33	34	<LOD	33
	12	8	92	3	31	4	62	4	26	41	2	28
	19	9	91	2	31	14	54	3	26	41	2	28
	27	12	88	2	33	12	53	3	28	39	4	27
	43	11	89	9	26	13	52	5	28	35	4	27
	54	10	90	3	30	9	58	4	29	37	4	26
D	0	9	91	<LOD	36	<LOD	64	<LOD	<LOD	<LOD	<LOD	<LOD
	12	17	83	8	33	9	50	<LOD	29	35	<LOD	35
	19	29	71	15	24	19	41	<LOD	4	70	<LOD	25
	27	36	64	19	26	19	36	4	9	70	4	13
	43	45	55	22	24	22	32	<LOD	7	80	<LOD	13
	54	49	51	28	17	27	28	<LOD	<LOD	94	<LOD	6
E	0	22	78	<LOD	39	<LOD	61	<LOD	<LOD	<LOD	<LOD	<LOD
	12	6	94	3	31	7	58	<LOD	20	13	<LOD	67
	19	24	76	11	28	11	50	<LOD	<LOD	<LOD	<LOD	<LOD
	27	29	71	15	26	17	41	<LOD	<LOD	100	<LOD	<LOD
	43	36	64	18	25	20	36	<LOD	<LOD	<LOD	<LOD	<LOD
	54	41	59	21	22	22	35	<LOD	<LOD	<LOD	<LOD	<LOD
F	0	18	82	<LOD	39	<LOD	61	6	27	33	4	30
	12	29	71	16	27	12	45	7	28	30	8	27
	19	34	66	21	22	16	41	10	31	24	13	22
	27	40	60	20	24	22	34	14	29	23	15	20
	43	50	50	26	23	25	26	18	31	16	20	15
	54	48	52	25	23	25	29	17	29	19	19	16

ES, epoxystearate; EO, epoxyoleate; EOL, epoxylinoleate; LOD, limit of detection; [1]Blend: A = stripped refined olive + virgin olive oil; B = stripped sunflower + virgin olive oil;

C = stripped linseed + virgin olive oil; D = stripped refined olive oil; E = stripped sunflower + stripped refined olive oil; F = stripped linseed + stripped refined olive oil;

[a] As% total of the originating fatty acid for the EFA.

5.3.3 Oxidation study at 70°C for stripped and non-stripped samples

For the autoxidation study at 70°C, similar oil blends were used as in the photoxidation experiment, but oils from a different batch were used. As it was expected that the oxidation process would proceed very fast because stripped oils were used, the set of blends was extended with a similar set of blends, but with non-stripped oils. This increased the total number of studied blends to 12

Table 8.1 in Appendix). The data with respect to the evolution of the PV, CD, CT and total EFA content are shown in **Table 5.5**

From these data it could be observed that especially in the blends solely composed of stripped oils (D-F), excessive oxidation occurred. Typically, the PV reached its maximum before the end of the incubation study and attained extremely high levels (> 100 meq kg^{-1} oil). A similar observation was made for the CD content, which is as mentioned before, normally strongly correlated to the PV. By substituting the stripped refined olive oil for virgin olive oil in these blends (blends A-C), only for the linseed oil containing blend C, such high PV were observed as well, although they were considerably smaller compared to blend F. Again, also in blends A-C the maximum PV was reached before the end of the incubation period. It can be concluded that the addition of virgin olive oil attenuated the autoxidation process to some extent, as was also observed in the photoxidation studies.

For the non-stripped blends (A'-F'), the PV's of all blends showed a significant and consistent increase as function of time, as was the case for the CD content. For these blends substituting virgin olive oil by refined olive oil did not impact the dynamics of the considered parameters between the homologous blends. PV's obtained were comparable as for blends A and B and were comparable with those obtained in the photooxidation study. On basis of all these data, it can be concluded that in all samples studied, autoxidation occurred and that in absence of antioxidants in the blends due to stripping of the oil, the oxidation process was quite excessive.

As for the evolution of the total EFA content in the blends solely containing stripped oils (D-F) it was obvious that also excessive amounts were produced, exceeding 10 mmol per kg of oil in blends D and F. In contrast to the drop in PV and CD content during prolonged storage, the EFA content still increased significantly for all these blends. For the blends containing stripped oils and virgin olive oil (A-C), also a significant increase of the EFA content as function of time was observed and levels obtained were comparable as those observed in the photooxidation studies. The increase after 1 day of incubation was still restricted, but was obvious after 3 days, while for the PV, an increase was already observed after 1 day of incubation.

Table 5.5: EFAs (EFA, µmol kg^{-1}), peroxide value (PV; meq O_2 kg^{-1} oil), conjugated dienes and trienes (CD and CT; µmol g^{-1} oil) content of stripped and non-stripped oils blends during autoxidation at 70 ± 1°C

		Stripped oil					Non- stripped oil			
Storage time (days)	Blend[1]	PV	CD	CT	Total EFA	Blend[2]	PV	CD	CT	Total EFA
0		6.6	3.7	0.7	318.6		7.0	6.3	1.5	906.2
1		8.0	4.4	0.9	304.1		6.7	6.4	1.7	1050.2
3	A	51.0	7.2	1.1	484.8	A'	8.2	7.1	1.5	899.8
5		34.1	4.0	0.5	494.2		10.2	7.6	1.6	1060.7
p value[a]		0.000	0.000	0.000	0.000		0.000	0.001	0.285	0.421
0		7.5	5.4	0.5	379.3		4.5	10.9	1.7	5637.2
1		12.9	5.9	0.5	412.8		5.6	11.1	1.9	5826.8
3	B	80.8	15.5	0.9	947.1	B'	19.2	18.1	1.8	5688.8
5		45.3	10.6	0.5	1032.3		40.0	26.2	1.9	5929.0
p value[a]		0.000	0.000	0.000	0.271		0.000	0.000	0.151	0.072
0		7.8	2.5	0.3	433.7		4.4	5.9	0.7	1996.5
1		21.5	7.0	0.4	556.5		5.5	6.6	0.5	2147.6
3	C	142.4	18.8	1.7	1844.6	C'	27.3	14.6	0.9	2257.7
5		203.6	17.2	1.9	3265.0		53.8	25.6	2.0	3038.0
p value[a]		0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.104
0		0.5	3.4	1.0	26.6		4.0	6.3	2.0	971.9
1		20.4	9.7	0.7	649.1		4.0	6.4	2.0	1052.0
3	D	329.3	46.6	1.8	6973.9	D'	5.5	7.5	2.0	923.5
5		274.3	31.7	1.6	12134.7		9.3	8.1	2.2	1009.8
p value[a]		0.000	0.000	0.001	0.000		0.000	0.000	0.302	0.404
0		2.4	4.6	0.7	79.1		2.4	11.4	2.4	5869.7
1		25.3	24.5	0.7	1044.3		3.1	11.6	2.2	5764.4
3	E	537.9	120.4	3.6	6769.1	E'	14.8	17.4	2.3	6082.1
5		498.1	70.8	3.9	9050.3		37.2	24.8	2.5	6269.8
p value[a]		0.000	0.000	0.000	0.007		0.000	0.000	0.014	0.319
0		3.1	3.0	0.5	79.8		1.8	6.1	1.3	2156.5
1		21.9	21.1	1.4	1549.5		2.6	6.9	1.1	2319.5
3	F	656.6	69.9	12.0	8904.6	F'	26.0	15.5	1.4	2482.7
5		280.9	44.6	13.0	16273.6		54.6	23.1	2.5	3222.9
p value[a]		0.000	0.000	0.000	0.011		0.000	0.000	0.000	0.000

All the data are expressed as the mean of three independent replicates.

[a]*p* value <0.05 is significant according Tukey test in one-way ANOVA in every blend over time.

[1]Blend: A = stripped refined olive + virgin olive oil; B = stripped sunflower + virgin olive oil; C = stripped linseed + virgin olive oil; D = stripped refined olive oil; E = stripped sunflower + stripped refined olive oil; F = stripped linseed + stripped refined olive oil.

[2]Blend: A' = refined olive oil + virgin olive oil; B' = sunflower oil + virgin olive oil; C' = linseed oil + virgin olive oil; D' = refined olive oil; E' = sunflower oil + refined olive oil; F' = linseed oil + refined olive oil

This suggests that hydroperoxides should be formed first before EFA can be produced. This is in line with the formation mechanisms suggested earlier.

Considering the non-stripped oils (A'-F'), it was obvious that the initial EFA content for some blends was really high. Although the PV content was not < 1 meq O_2 kg^{-1} oil as could be expected for a fresh oil, the PV's were not exceptionally high either (all < 10 meq O_2 kg^{-1} oil) These results are in line with the observations made in **Chapter 3**. As for the EFA

evolution as function of time no significant changes in any of the samples, except in sample F, were observed, which was on basis of its composition the most vulnerable for oxidation. It should be noted however that for this sample, it was only because at the last day of incubation that such a significant increase was observed. In fact, for the homologous blend C', such an increase at the last incubation day was observed, but because of the variability of the data it was for this blend not statistically significant. These results contrast with the fact that on basis of the PV and CD data, clearly oxidation did occur in these samples in a time consistent way. In view of the observations made for blends A-C, it can be concluded that although hydroperoxides were building up in blends A'-F', the subsequent formation of EFAs was delayed due to the presence of a variety of anti-oxidants present in the non-stripped blends.

Comparing the stripped blends containing virgin olive oil amongst each other (blends A-C) with respect to the net amount ES formed considering the content of oleic acid in the respective blends (**Table 8.4 in Appendix**), it could be concluded that on a relative basis more ES was formed in the more unsaturated blends. This was also the case for EO, considering the linoleic acid content of the respective blends. This indicates that due to the higher vulnerability of oils rich in alfa-linoleic acid for oxidation, also linoleic and oleic acid are more prone to oxidation and conversion to their respective EFAs. A similar observation was made in chapter 2 table 2.2.

Finally, also the percentage isomeric distribution of the considered EFA for the blends A-F was considered (**Table 5.6**). For the sake of completeness, the data with respect to the non-stripped blends (A'-F'), are included in **Appendix** (**Table 8.6)**, but as only in the most unsaturated blends a significant formation of EFAs was observed at the end of the storage period, these data will not be discussed in detail.

Before the start of the incubation, again the *cis* stereoisomer for each EFA was predominant in all samples (also in Blends A'-F'). Upon progressive oxidation the *trans* stereoisomers' contribution to the total EFA content became more important, similarly as observed during the photoxidation study for blends D-F. In the photoxidation study it was observed that in the samples containing photosensitizers and antioxidants, the *cis* stereoisomer remained dominant, but this was not the case in the autoxidation study

Table 5.6: Percentage isomeric distribution (ID) of ES, EO and EOL on mole basis of the different stripped oil blends during autoxidation at 70 ± 1°C

Isomeric distribution												
		Oleic acid[a]		Linoleic acid[a]				Linolenic acid[a]				
Blend[1]	Storage time (days)	*trans*-9,10-ES	*cis*-9,10-ES	*trans*-12,13-EO	*cis*-12,13-EO	*trans*-9,10-EO	*cis*-9,10-EO	*trans*-12,13-EOL	*cis*-12,13 & *trans*-9,10-EOL	*cis*-9,10-EOL	*trans*-15,16-EOL	*cis*-15,16-EOL
A	0	4	96	<LOD	39	<LOD	61	<LOD	<LOD	<LOD	<LOD	<LOD
	1	6	94	<LOD	40	<LOD	60	<LOD	<LOD	<LOD	<LOD	<LOD
	3	10	90	6	30	16	48	<LOD	100	<LOD	<LOD	<LOD
	5	15	85	9	38	<LOD	53	<LOD	100	<LOD	<LOD	<LOD
B	0	6	94	<LOD	42	<LOD	58	<LOD	<LOD	<LOD	<LOD	<LOD
	1	13	87	<LOD	43	<LOD	57	<LOD	<LOD	<LOD	<LOD	<LOD
	3	29	71	12	32	13	44	<LOD	100	<LOD	<LOD	<LOD
	5	28	72	16	30	17	37	<LOD	100	<LOD	<LOD	<LOD
C	0	6	94	<LOD	40	<LOD	60	<LOD	34	36	<LOD	30
	1	27	73	16	27	17	40	11	37	21	12	19
	3	39	61	20	24	24	32	15	33	20	16	17
	5	42	58	25	24	27	24	18	32	16	19	15
D	0	<LOD	100	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	1	67	33	35	13	35	17	<LOD	<LOD	<LOD	<LOD	<LOD
	3	72	28	35	13	39	13	16	39	30	6	9
	5	70	30	35	16	36	12	14	43	29	5	8
E	0	<LOD	100	<LOD	38	<LOD	62	<LOD	<LOD	<LOD	<LOD	<LOD
	1	61	39	32	15	32	20	<LOD	<LOD	<LOD	<LOD	<LOD
	3	60	40	33	16	34	17	11	43	35	5	6
	5	55	45	31	19	32	18	<LOD	100	<LOD	<LOD	<LOD
F	0	<LOD	100	<LOD	<LOD	<LOD	100	<LOD	30	42	<LOD	28
	1	63	37	34	14	35	17	23	33	11	24	9
	3	48	52	28	23	27	22	20	33	14	20	14
	5	40	60	24	27	24	25	17	31	18	17	17

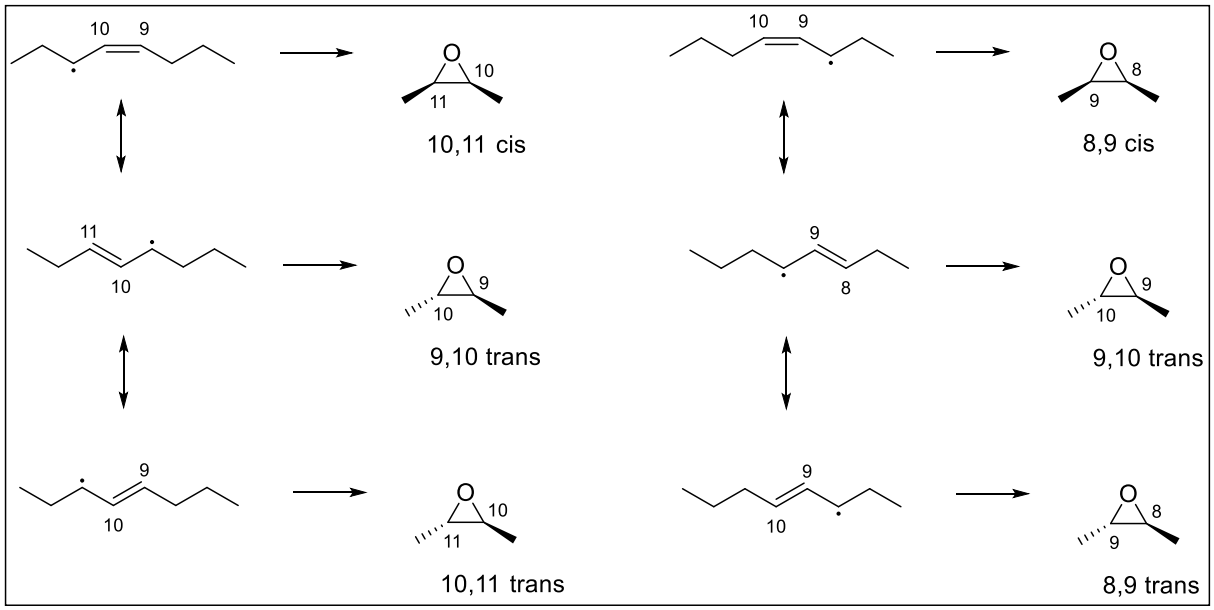
LOD, level of detection; ES, epoxystearate; EO, epoxyoleate; EOL, epoxylinoleate.

[1]Blend: A = stripped refined olive + virgin olive oil; B = stripped sunflower + virgin olive oil; C = stripped linseed + virgin olive oil; D = stripped refined olive oil; E = stripped sunflower + stripped refined olive oil; F = stripped linseed + stripped refined olive oil.

[a] As% total of the originating fatty acid for the EFA.

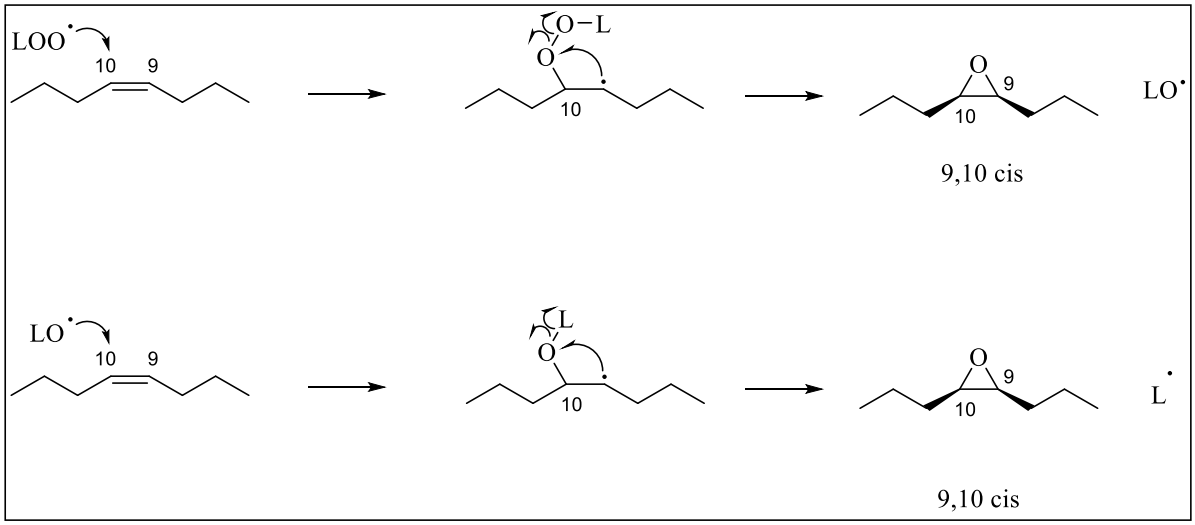
5.3.4 Potential implications with respect to the formation mechanism of epoxy fatty acids

Considering the suggested most probably reaction pathway in which EFA are formed, outlined in **Scheme 1-5** (1,2 addition to the adjacent double bond of LO• radical), the formation of a mixture of various EFAs from oleic acid can be rationalized as shown in **Scheme 5-1.**

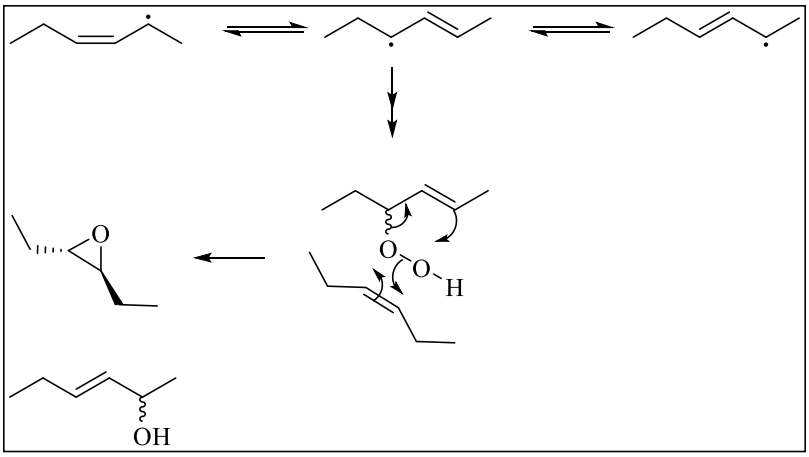


Scheme 5-1. Different ES isomers potentially formed from oleic acid after 1,2 addition to the adjacent double bond of LO• radical

From the data obtained throughout the experiments in this chapter and also from the observational data reported in the preceding experimental chapters, it is obvious that the plethora of isomers suggested to be potentially formed, is not supported by experimental evidence. Indeed, only the 9,10-ES could be detected in all samples analysed. This implies that the suggested reaction scheme should be questioned. The experimental data support more the reaction pathways shown in **Scheme 5-2**. The problem however with this pathway is the fact that it only leads to the production of *cis* stereoisomers, while throughout several oxidation studies carried out in this chapter, it was shown that *trans* stereoisomers are preferentially formed.



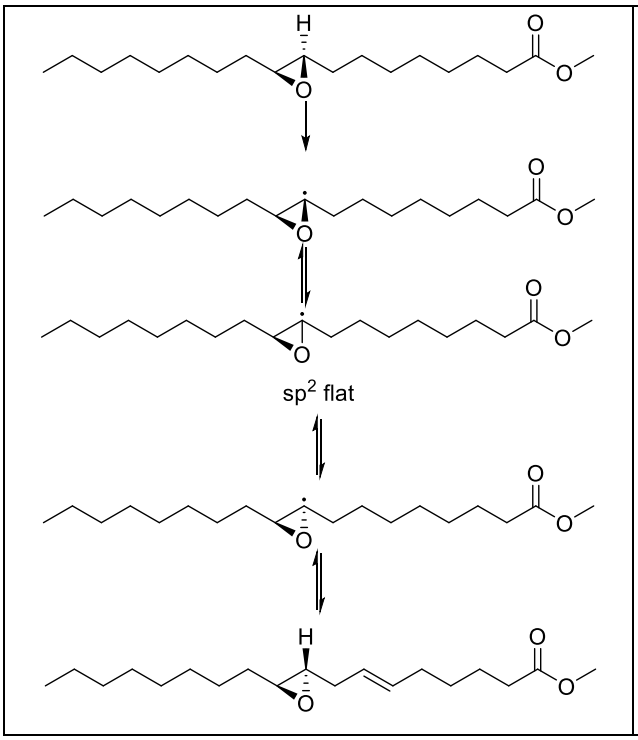
Scheme 5-2. 1,3 cyclisation of the LOO• or LO• radical to the double bond



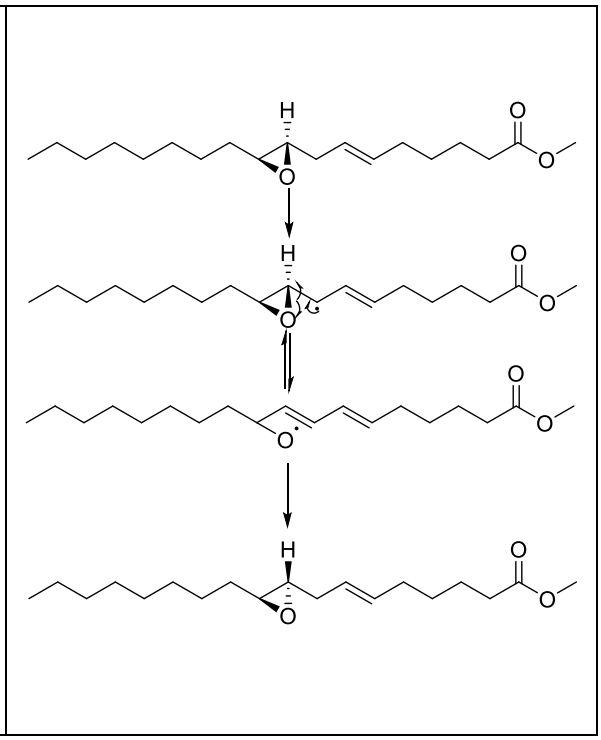
Scheme 5-3. Alternative formation pathway of *trans* stereoisomers of EFA (which on basis of experimental data seems not probable)

The possibility of the occurrence of another pathway via a hydroxyl transfer from the hydroperoxide to an unsaturated fatty acid, leading to the *trans* stereoisomer as shown in **Scheme 5-3**, could not be supported by experimental evidence as tertiary-butyl hydroperoxide added to soybean oil in various amounts and incubated respectively at room temperature and at 60°C did not result in a significant increase of the EFA content of the oil. This reaction pathway is however described in literature, but requires a strong catalyst (eg molybdenum-based metal catalyst) which is not present in oils or foods (Iwahama et al., 2000). Given the fact that the 1,3 cyclisation of the LOO• or LO• radical to the double bond is retained as the most likely reaction pathway, it can be rationalized that initially the *cis*-stereoisomer should be formed, which further on can isomerise to the *trans*-stereoisomer.

This isomerization can be accomplished via a radical reaction scheme as outlined in **Scheme 5-4** for ES and in **Scheme 5-5** for EO. These mechanisms would require more intensive oxidation conditions, which is actually in line with the observations made in this chapter.



Scheme 5-4. Suggested *cis* - *trans* isomerization scheme of epoxystearate



Scheme 5-5. Suggested *cis* - *trans* isomerization scheme of epoxy oleate

5.4 CONCLUSIONS

It can be concluded that EFAs are formed via photosensitized oxidation in oils containing chlorophyll and its degradation products at cold temperatures. In absence of antioxidants and a photosensitizer, oils were however even more sensitive to EFA formation if exposed to light. During storage at elevated temperatures (70°C, during 5 days), EFA formation was only apparent if the oil blends contained stripped oils, i.e. had a lower content of anti-oxidants or if the non-stripped oil blends contained a high (>30%) content of alfa-linolenic acid. A stereospecific formation of EFAs was observed, suggesting a radical supported isomerization of the initially formed *cis* to the *trans* stereosoisomers in more challenging oxidation conditions.

CHAPTER

6 INSIGHTS INTO THE INTERACTION OF EPOXY FATTY ACIDS WITH CASEIN AND WHEY PROTEINS IN OIL-IN-WATER EMULSIONS

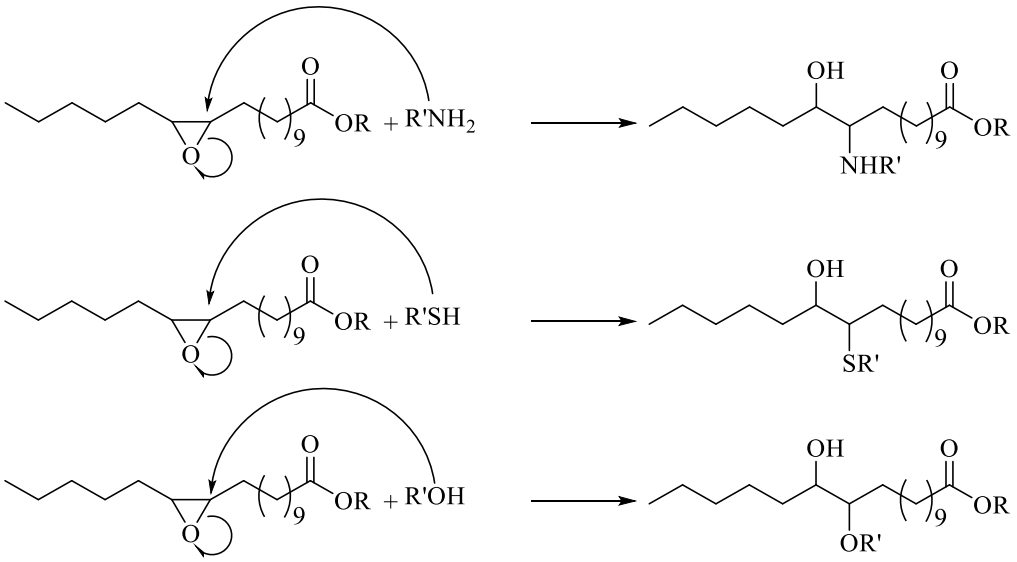
ABSTRACT

In foods, lipids will oxidize in the presence of other food components. Due to this, co-oxidation with the present proteins may occur. Reactive carbonyl species originating from lipid oxidation are known to react readily with proteins leading to substantial changes in the proteins. In view of their reactive oxirane ring, it is expected that also EFAs are prone to interact with proteins. To verify this hypothesis, oxidized soybean oils containing a varying amount of EFAs were emulsified in the presence of respectively casein and whey proteins. Remarkably, an overall increase in the EFA content of the lipid phase was observed. Therefore, an additional experiment was performed in which *cis*-9,10-epoxystearate dissolved in triheptanoin was emulsified excluding any additional EFA formation due to progressive oxidation, as a fully saturated oil was used. Emulsions were prepared in the presence of dairy proteins, and in presence and absence of Tween 20. In none of the emulsions however, a significant decrease in the *cis*-9,10-epoxystearate concentration could be noted after incubation. This preliminary study suggests an unexpected absence of reactivity of EFAs towards the studied proteins.

Keywords: Lipid oxidation, epoxy fatty acids, fatty acid methyl esters, co-oxidation

6.1 INTRODUCTION

In food systems, lipid oxidation generally occurs simultaneously with "co-oxidation" phenomena, which affects other molecules. Especially the interaction with proteins and oxidizing lipids is of interest in view of earlier observed strong protein aggregation in the presence of oxidizing lipids (Cucu, Devreese, Mestdagh, Kerkaert, & De Meulenaer, 2011) or during the co-oxidation of lipids and proteins (Mestdagh, Kerkaert, Cucu, & De Meulenaer, 2011). This interaction may lead for instance to a decreased protein digestibility (Obando, Papastergiadis, Li, & De Meulenaer, 2015). The strong interactions between lipids and proteins in foods can cause the oxidation reactions to be transferred easily from lipids to proteins (Viljanen, Kivikari, & Heinonen, 2004). Proteins and lipids are prone to oxidation but it is not known which one occurs first (Karel, 1973; Schaich, 2008). Amino acids such as methionine, histidine, tryptophan, tyrosine and cysteine are known to be more susceptible to oxidation compared to the other amino acids (Andersen et al., 2006; Cucu et al., 2011; Gardner, 1979). As described before, during the oxidation of unsaturated fatty acids, primary and secondary oxidation products are produced. Both can interact with proteins, together with a variety of fatty acid radicals which will be present. Thus, it was recently shown that the use of malondialdehyde as a generally accepted lipid oxidation indicator should be done with caution (Vandemoortele & De Meulenaer, 2015). Within proteins it is expected that especially the sulfhydryl, amino, guanidyl and imidazole functional groups are involved in the interaction with these reactive lipid oxidation products. Considering the secondary oxidation products, it is especially accepted that reactive carbonyl species are involved. However, Schaich suggested earlier the involvement of EFAs by binding to proteins to form adducts as well (Schaich, 2005, 2008). According to the reactivity of the epoxy ring, EFAs reactions may involve either electrophilic attack on the oxygen atom or a nucleophilic attack on one of the ring carbon atoms (Heath et al., 2005). Depending on the degree of unsaturation of the carbon chain (Frankel, 2005), reactivity of EFAs is expected to be similar to that of fatty acids whereby the polyunsaturates > monounsaturates > saturated EFAs. In presence of proteins, nucleophilic amines ($–NH_2$), sulfhydryl (–SH) or hydroxyl (–OH) groups could open the epoxy ring leading to formation of lipid-protein adducts (Cucu et al., 2011; Mestdagh et al., 2011) as illustrated in **Scheme 6-1**. If such a reaction occurs, a decrease in the EFAs content is expected to be observed.



Scheme 6-1: Potential adduct formation route during EFAs protein interactions

The objective of this study was to have an insight into the interaction between proteins and EFAs in protein stabilised and Tween 20 stabilised emulsions containing proteins as well. In Tween 20 stabilized emulsions it is expected that proteins are nearly absent in the water-oil interface at the surface of the oil droplets which could affect the potential interaction between EFAs and proteins. It was hypothesized that there is a significant decrease in the amount of EFAs due to their reaction with proteins present in the emulsions. Emulsions were made using three oxidation levels of oils, originating from the same type of oil but oxidized to a different extent before making the emulsion. Because the epoxy ring is known to be very reactive and since EFAs were already present in the emulsions, in this study the incubation time was limited to 24 hrs.

6.2 MATERIALS AND METHODS

6.2.1 Supplies and reagents

Potassium phosphate buffer (0.1 M, pH 7.4) consisted of K_2HPO_4 and KH_2PO_4 (Chem-Lab, Zedelgem, Belgium). Phosphate-buffered saline (PBS; pH 6.8) consisted of 0.135 M NaCl, 1.5 mM KH_2PO_4, 8 mM $NaH_2PO_4 \cdot 12H_2O$ and 2.7 mM KCl. Tween 20® was supplied by Sigma-Aldrich (Bornem, Belgium).

Whey protein isolate (Lacprodan DI-9224) and sodium caseinate (Microdan 30) were provided by Acatris Food Belgium (Londerzeel, Belgium) and Arla Foods (Wageningen, The

Netherlands). Soybean oil was purchased from a local store. The rest of the chemicals used are described in **chapter 2 section 2.2.1** and **chapter 3 section 3.2.1.**

6.2.2 Oil stripping

Triacylglycerols (TAGs) were isolated from soybean oil using a modified method as already described in **chapter 5 section 5.2.2.** To retard lipid oxidation during stripping, the collected triacylglycerols were held in an ice bath, which was covered with aluminum foil. The amount of C18:1, C18:2 and C18:3 in the soybean oils was as follows: 25.0, 51.6 and 6.4 g/100 g total fatty acids.

6.2.3 Thermoxidation of oil

For the oxidation of the soybean oil, samples (70 g) of stripped oil were weighed into a Pyrex heavy duty crystallizing dish (100 mm diameter x 50 mm height), placed in an oven and then heated at 60°C for maximum 14 h. The oils were analysed for the peroxide value (PV) and p-anisidine value (p-AV) as described below.

6.2.4 Preparation of casein and whey protein-stabilized emulsion

The emulsions were prepared using 6 mg mL^{-1} of protein in 0.1 M potassium phosphate buffer (K_2HPO_4, KH_2PO_4; pH = 7.4) (final concentration 3% oil), as described by (Waraho et al., 2009). A total of 200 mL of coarse emulsion (premix) was prepared by mixing with an Ultra-Turrax (Janke & Kunkel, IKA-Werk, Staufeb, Germany) for 2 min at 9200 rpm. The coarse emulsion was further homogenised in an APV-1000 homogeniser (SPX Flow Technology, Germany), at 250 bars in the first stage and 50 bars in the second stage to produce an emulsion of a more stable and uniform particle size. A total of 100 mL of emulsion was placed in a 250 mL transparent Schott glass bottle closed with a PTFE septum cap and stored in a cold room in the dark at 5°C for 24 h. Each emulsion preparation was prepared in triplicate. As oils, in a first set of experiments, soybean oils at three different levels of oxidation were used. In a second set of experiments, triheptanoin was used. The C7 triglyceride was spiked with a known amount of methyl *cis*-9,10-epoxystearate before making the emulsions.

6.2.5 Preparation of Tween 20-stabilised emulsion containing proteins

The preparation of these protein containing emulsions (respectively caseins and whey proteins at 6 mg mL^{-1}) followed the same protocol as above using triheptanoin as oil. The C7 triglyceride was spiked with 281.1 µg g^{-1} of oil of methyl *cis*-9,10-epoxystearate before making the emulsions. Tween 20 (0.3%) was added to prepare the pre-emulsion. A total of 10 mL of emulsion was placed in transparent SPME glass vials of 20 mL volume, closed with a PTFE septum cap and incubated at 5°C for 24 h. Each emulsion preparation was done in triplicate.

6.2.6 Peroxide and p-anisidine determination

The PV and p-AV of the oils prior to emulsification were determined. The PV was determined using an iron based spectrophotometric method as described previously in chapter 5 section 5.2.4.1. The p-AV measurement was according to the AOCS official method Cd 18-90 (AOCS, 1990).

6.2.7 EFA determination

6.2.7.1 EFA determination in oils

For determination of EFAs, the method followed is described in **chapter 2 section 2.2.5.**

6.2.7.2 EFA determination in emulsions

A sample of 8 mL of emulsion was accurately pipetted into the glass centrifuge tubes containing the previously dried internal standard (20 µg). 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 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 ≤2) was added and homogenized for 30 s. The tubes were centrifuged at 3500 rpm 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.

The C18 EFAs determination was based on a base-catalyzed transmethylation with sodium methoxide in methanol at room temperature. Finally, EFAs were analyzed using a gas chromatograph with flame ionization detector (GC-FID) after SPE pre-separation.

6.2.8 Statistical analysis

Results correspond to the mean of three replicates calculated from the collection of individual data obtained in three emulsions prepared independently. To test if the means of the different emulsions are statistically different, one-way ANOVA and post-hoc Turkey test were performed using SPSS 22 statistical package (IBM, SPSS, Inc). The significance level used was $p < 0.05$.

6.3 RESULTS AND DISCUSSION

6.3.1 Experiments with soybean oil based emulsions

In this study, EFAs were determined in the oils before making the emulsions and after incubating the emulsions for 24 h at 5°C. Stripped soybean was oxidized in an oven at 60°C and thus oils with different amounts of primary and secondary oxidation products, as assessed using the PV and p-AV were obtained (**Table 6.1**). In general, each oil was oxidized to have distinct PV and especially p-anisidine values. Although the p-anisidine value is typically related to the concentration of reactive carbonyl species, it was also expected that the more oxidized oils were characterized with a higher EFA content. This was confirmed (**Table 6.2**) and similar as in **Chapter 5**, it was obvious that because of the thermal oxidation a more equilibrated mix between the *cis* and *trans* isomer of each EFA was formed in the oils, while due to photooxidation a preference for the formation of *cis* EFAs was noticed.

Remarkably, no specific trend in the EFA content of the emulsions could be observed upon comparing with the initial EFA of the original oils. It was observed however that in case the low oxidation oil was used also the lowest levels of EFA was obtained in the emulsions. Similarly, the high oxidation degree oil resulted in the highest EFA amount.

Table 6.1: Initial peroxide (meq oxygen kg^{-1} oil) and p-anisidine values of soybean oils prior to emulsification

Oxidative status of oil	PV	p-AV
Fresh	0.50	0.17
Medium	21	50
High	26	97

Abbreviations: PV, peroxide value; p-AV, p-anisidine value

For instance, for the low oxidized soybean oil-based emulsion, *cis*-9,10-epoxystearate (44.1 µg g^{-1} of oil) dropped upon emulsification with casein and whey proteins to 20.5 and 21.9 µg g^{-1} of oil respectively, while the corresponding trans isomer increased. To the contrary, the same cis isomer increased to different levels in case of the medium and highly oxidized soybean oil. *Trans*-12,13-epoxylinoleic acid levels however in soybean oil-based emulsions dropped upon emulsification with casein or whey proteins irrespective of the initial oxidation status of the oils. No consistent trends and especially not a generally decreasing trend in the EFA content of the emulsified oils could be observed.

These observations can be explained by two potentially parallel occurring phenomena. The first is the ongoing oxidation process, which of course may have been influenced by the addition of proteins and the emulsification process. Recently in our research group it was also observed that because of emulsification of oil in the presence of dairy proteins and a short (24 h) storage period at 4°C, the concentration of specific secondary oxidation products such as malondialdehyde and hexanal tended to further increase as well (Obando, Soto, & De Meulenaer, 2018). A second phenomenon which could have occurred is that because of the competition between the other reactive secondary oxidation products present in the oils, the expected interaction between EFAs and proteins did not occur or only occurred to a limited extent, because of a too high competition for the reactive groups. It should be realized in this respect that the low molecular weight reactive carbonyls are expected indeed to be much more mobile compared to an EFA still attached to the triacylglycerol backbone. This phenomenon could be relevant however in the case of the medium and high oxidized oil but seems less likely in the case of the oil with the lowest oxidation level. Despite the fact that for this oil, a decrease for some EFAs was observed, others seemed to be formed.

Table 6.2: EFA content (µg g^{-1} of oil) in the original oils and the emulsion (after 24 h incubation in the dark)

		Epoxy fatty acids										
Oxidation level	**Model**	***trans*-9,10-ES**	***cis*-9,10-ES**	***trans*-12,13-EO**	***cis*-12,13-EO**	***trans*-9,10-EO**	***cis*-9,10-EO**	***trans*-12,13-EOL**	***cis*-12,13 &*trans*-9,10-EOL**	***cis*-9,10-EOL**	***trans*-15,16-EOL**	***cis*-15,16-EOL**
Low	Oil	3.3	44.1	44.6	11.5	8.0	9.0	5.6	0.0	0.0	8.2	0.0
	Casein-Emulsion	14.9	20.5	15.4	25.4	16.5	22.7	2.7	4.1	5.3	5.1	3.5
	Whey-Emulsion	16.8	21.9	27.7	28.3	26.4	34.3	2.3	7.4	9.9	2.2	11.6
	p value[a]	0.346	0.926	0.783	0.509	0.871	0.426	0.938	0.686	0.677	0.989	0.353
Medium	Oil	192.7	141.1	238.5	371.3	320.5	144.3	85.9	42.6	21.4	33.1	29.3
	Casein-Emulsion	314.5	215.2	546.8	244.1	627.8	196.2	60.2	61.3	41.8	53.5	25.3
	Whey-Emulsion	345.6	182.4	606.9	231.2	607.3	231.0	65.1	103.6	45.2	61.7	25.5
	p value[a]	0.127	0.269	0.125	0.77	0.043	0.149	0.883	0.148	0.338	0.072	0.954
High	Oil	285.8	365.1	401.2	463.9	471.3	376.4	258.2	61.2	46.6	44.3	68.0
	Casein-Emulsion	519.9	606.2	892.2	634.3	928.8	572.1	90.1	147.2	91.7	84.6	68.7
	Whey-Emulsion	486.5	551.3	865.1	560.0	923.1	535.3	83.8	145.8	74.8	74.4	47.4
	p value[a]	0.106	0.125	0.008	0.55	0.025	0.158	0.888	0.009	0.178	0.133	0.874

All the data are expressed as the mean of two independent replicates

[a]*p* value <0.05 is significant according Tukey test in one-way ANOVA in every oxidation level for all the models.

Abbreviations: ES, epoxystearate; EO, epoxyoleate; EOL, epoxylinoleate.

Consequently, it was concluded the experimental setup proved to be unsuitable to evaluate potential interactions between epoxy fatty and proteins and that a different approach was necessary.

6.3.2 Experiments with methyl *cis*-9,10-epoxystearate in C7-triglycerol oil

Because there was no definite trend in the behaviour of the EFAs that were being detected, a further experiment was performed which involved spiking a known amount of a single saturated EFA (*cis*-9,10-epoxystearate) isomer in a C7 triglyceride (triheptanoin). The systems investigated involved oil-in-water emulsions (3 vol% C7 triglyceride) stabilised by a mixture of either commercial sodium caseinate or whey protein isolate. A second set of emulsions was prepared as well containing the same proteins and the non-ionic emulsifier polyoxyethylene sorbitan monolaurate (Tween 20). The initial EFA content of the triheptanoin was analysed and it was found not to have any C18-EFAs. The results obtained in this experiment are shown in **Table 6.3**.

Table 6.3: Methyl *cis*-9,10-epoxystearate (µg g^{-1} of oil) content in 3% C7-triglyceride emulsions incubated in the dark at 5°C for 24 h

Sample	EFAs
Original EFA spiked	281.1 ± 2.77^{ab}
Casein emulsion	254.4 ± 18.60^{b}
Casein-Tween 20 emulsion	271.2 ± 16.59^{ab}
Whey emulsion	300.1 ± 18.94^{a}
Whey-Tween 20 emulsion	280.9 ± 18.10ab

Values with different superscripts in a same column are significantly different ($p < 0.05$)

As can be observed for none of the emulsions a significant decrease in the *cis*-9,10-epoxystearate content could be observed. It should be noted that also no other EFAs were observed in the chromatograms. In case of the Tween 20 stabilized emulsions less interaction between the EFAs and proteins was expected, as competitive displacement of for instance caseins by Tween 20 from interfaces in emulsions has been demonstrated (Courthaudon, Dickinson, & Dalgleish, 1991; Courthaudon, Dickinson, Matsumura, & Clark, 1991; Dickinson & Gelin, 1992; Mackie, Gunning, Wilde, & Morris, 2000; Wilde & Clark, 1993).

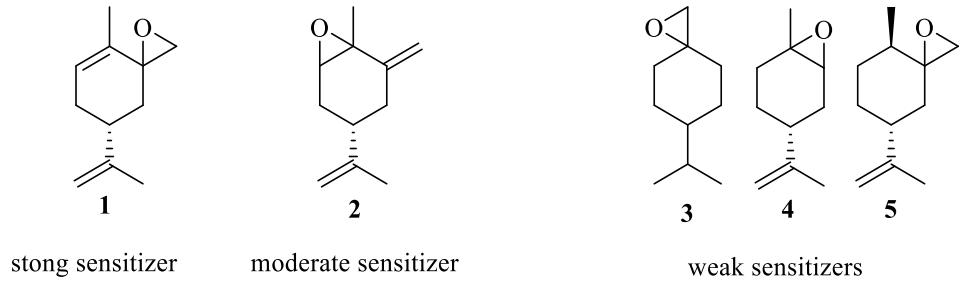
In absence of Tween 20 however, proteins are present in the interphase and as such should indeed be able to interact with the epoxystearate. No significant reduction however was observed, either using caseins or whey proteins. This observation was not expected based on the generally reported reactivity of epoxides as discussed in Chapter 1 (**section 1.6.2, Scheme 1-10**).

However, by a more detailed literature search with respect to the reactivity of epoxides, the observations could be explained. The reactivity of epoxides and other potentially electrophilic oxidized species originating from organic molecules has been carefully studied in the framework of allergic contact dermatitis towards organic components present in fragrances. Contact dermatitis develops after a primary sensitization of the skin to the compounds of interest. As the molecules involved are not immunogenic as such, because of their low molecular weight, they should enter the epidermis and react with the proteins present, due to which a hapten is produced. These immunogenic haptens trigger then the sensitization reaction, after which the contact allergy develops. A key element determining the allergic potential of the low molecular weight organics is consequently their reactivity to proteins (Karlberg, Bergström, Börje, Luthman, & Nilsson, 2008; Nilsson, Bergström, Luthman, Nilsson, & Karlberg, 2005; Roberts et al., 2017).

Proteins can be considered as nucleophiles, because of the presence of a variety of nucleophilic side chains in the amino acids: SH group (in cysteine), NH_2 group (in lysine and arginine), OH group (in tyrosine, threonine and serine) and the ε-NH group (in histidine). Therefore, it is obvious that the stronger the electrophilic character of the organic molecule present in for instance a fragrance, the more potent it can be as a contact allergen (Karlberg et al., 2008). Consequently, an LC-MS based assay was developed to evaluate the reactivity of a model peptide to chemicals in order to have an *in vitro* prediction of their skin sensitization potential (Natsch & Gfeller, 2008). Recently a quantitative mechanistic model has been developed as well specifically for epoxides to evaluate their structure-potency relationship in allergic contact dermatitis (Roberts et al., 2017). This model was based on plenty of experimental data from literature. These data allowed to derive the following conclusion with respect to the reactivity of epoxides towards proteins.

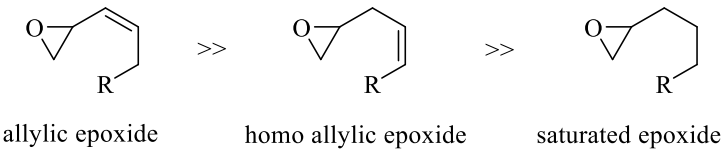
As already explained in **Scheme 1-10** (**chapter 1, section 1.6.2**), epoxides are typically S_N2 electrophiles and although the ether oxygen is a poor leaving group, its reactivity is enhanced

by the tension in the ring. Electronegative substituents however will also enhance the reactivity, because the developing negative charge on the oxygen atom during the nucleophilic substitution reaction is stabilized. Therefore, and also because of steric reasons, the reactivity is higher at primary carbons, compared to secondary and tertiary carbon atoms. In EFAs, the reactive carbon center is secondary. More important even is the observation that especially in open chain compounds, allylic (and benzylic) carbon centers are more reactive than saturated ones (Roberts et al., 2017). This can be illustrated with one particular example taken from the study of (Bergström, Luthman, Nilsson, & Karlberg, 2006), who evaluated the sensitizing capacity of a number of monoterpenes or similar compounds and their oxidation products (**Scheme 6-2**).



Scheme 6-2: Classification of various epoxides according to their skin sensitizing capacity. (7*R*)-7-isopropenyl-4-methyl-1-oxaspiro[2.5]oct-4-ene (1), (4*S*)-1,2-epoxy-4-isopropenyl-1-methyl-6-methylene-cyclohexane (2), 6-Isopropyl-1-oxa-spiro[2.5]octane (3), (+)-limonene oxide (4) and (4*R*,7*R*)-7-Isopropenyl-4-methyl-1-oxa-spiro[2.5]octane (5) (based on Bergström et al., 2006).

These observations most likely imply as well that the allylic epoxide in **Scheme 6-3** is much more reactive than the homo allylic epoxide. Saturated epoxides are probably even less reactive.



Scheme 6-3: Supposed reactivity of allylic, homo allylic and saturated epoxides towards nucleophiles

Given that the epoxy fatty acid evaluated in this study was a fully saturated one, it is probably not surprising that its reactivity to proteins in the model system used, was not significant. Similarly, in view of the EFAs formed and detected in all the samples investigated in this

study, it could be suggested that the EFAs formed in foods from most of the unsaturated fatty acids are expected to be rather stable towards proteins. The only exception could be the EFA which can be formed from conjugated linoleic acid, which would give rise indeed to a more reactive allylic epoxide.

6.4 CONCLUSIONS

Unexpectedly, methyl *cis*-9,10-epoxystearate present in triheptanoin proved to be stable when emulsified in the presence of proteins. If an oxidized oil was emulsified in the presence of proteins, an overall increase in the EFA content of the lipid phase was observed, although it should be noted that for some particular EFAs a decrease was observed. However, as observed also in other studies in our research group, the oxidation process is proceeding upon emulsification of an (oxidized) oil, so this experimental approach was considered unsuitable for its purpose.

In view of the studied reactivity of other epoxides present in biological matrices, i.e. produced during the oxidation of terpenes present in fragrances, the observed lack of reactivity of a fully saturated EFA towards proteins is however not surprising. In fact, allylic epoxides are only expected to show a high reactivity, which suggests that also unsaturated EFA, which are mostly homo allylic epoxides, would be rather stable in the presence of proteins. This seems to be in line with the relatively high levels of EFA observed in composite food matrices as reported in Chapters 3 and 4. Additional experiments using a similar experimental approach as the one used for the saturated EFA, but using instead an unsaturated, homo allylic EFA should corroborate this hypothesis.

CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

In the first part of this research the available literature on EFAs was evaluated and after a critical review of the available data and information it was found that there are many missing links especially in the area of analysis and mechanistic studies of EFAs in foods. These fatty acids occur naturally and some are produced during lipid oxidation as secondary products. In **chapter 1**, it was noted that EFAs had been identified in 1954 by Gunstone. It was observed that the available research about EFAs was old and yet reports indicate that these compounds form in high amounts during lipid oxidation especially at frying temperatures. It was also observed that the epoxidation reaction which leads to the synthesis of these fatty acids was a well-established and a simple reaction to perform at laboratory level. However, analytical methods available in literature indicated the necessity for improving the available methods. Although toxicity of these compounds had been elucidated by some researchers, no risk assessment study had been done. There was no mechanistic study on the formation and the reactivity or interaction of the EFAs identified. Based on these identified gaps**,** it was decided that the study should concentrate on development of better analytical methods and perform some mechanistic studies to investigate the formation and interaction of EFAs with other food components. Sensitive and reliable analytical methods would be a pillar in this study area which is still open to further research and this would facilitate investigations into the behavior of these compounds.

As described in **Chapter 2**, the developed improved analytical method was based on room temperature transmethylation using sodium methoxide of the fatty acids with a FAME as an IS, because the epoxy ring is known to be sensitive to acid and heat which lead to its opening. This was followed by a three step SPE separation of all the methyl esters present in order to remove the big part of the non-oxidized FAMEs which could co-elute with the EFAs and thus lead to over estimation of the analytes. Instrumentation for the analysis of EFAs was based on GC-FID with a polar capillary column equipped with a precolumn which reliably separated EFAs in fresh oils and led to accurate quantification. Qualitative analysis was based on a GC-MS full scan in electron ionization mode operating in nearly the same conditions as those of the GC-FID. The outcome was an accurate and reproducible analytical method that could provide reliable quantification data of analytes at low concentrations. Twelve C18 mono EFA isomers were well separated and could be identified on GC-MS. Thus, the separation proved to be isomer (place and stereochemistry) specific. Results obtained for the total EFAs content

in fresh oils were in the range 0.03-2 mg g^{-1} of oil with repeatability coefficient of variation (CV) ranging from 2.8 to 9.9% for duplicate analysis. Such a reliable method would be important in further studies focusing on the formation of specific isomers which would allow getting information on how EFAs form. Although this study was mainly aimed at quantifying EFAs, it laid a good foundation for the analysis of other oxygenated fatty acid especially hydroxyl and oxo fatty acids. Whereas EFAs could be analysed on a polar capillary column without further derivatisation, hydroxyl fatty acids required silylation to be analysed. Due to the presence of polar polymerized fatty acid methyl esters, it was always necessary to use a pre-column to protect the capillary column.

The presented method is expected to be easily extended to the analysis of other mono-EFAs. Especially the analysis of EFAs originating from long chain polyunsaturates present in fish and algae oil could be very interesting. Extension of the method to EFAs with multiple epoxy groups in their chain would require a more substantial elaboration. It is expected that the column used, would be too polar and thus multi-epoxy fatty acids would require very long elution times. Thus, the use of a less polar column would probably be necessary. In addition, it is expected that a further elaboration of the SPE cleanup procedure is required.

In **Chapter 3**, a universal analytical method that could analyse EFAs in different food matrices was developed. This was based on the Bligh and Dyer analytical method for the extraction of lipids from different matrices. The initial Bligh and Dyer method was meant to use chloroform, but due to the limitations of this compound which includes being toxic and carcinogenic, the method was optimised for use of dichloromethane which is a less toxic chemical. As an improvement from the first method which used a FAME as an IS, this method used a synthetic EFA methyl ester (C17:0), which was in-house synthetized using an epoxidation reaction. The advantage for using this IS was that more reliable analytical results could be got because the IS could be added at the beginning of the extraction process. This step would further correct all the analytical uncertainties. This method was in-house validated in several food matrices and it was judged as reliable. It is considered evident the approach presented would also fit for the purpose of analysing other oxygenated lipid species in foods, such as hydroxy or oxo-fatty acids, if appropriate internal standards are used.

An exposure assessment was one of the main parts of this research as presented in **Chapter 4**. To evaluate the dietary exposure of the population to the EFAs, consumption data of

specific food groups as well as their contamination levels in a representative and substantial number of samples were computed. Concentration data were obtained from analyzing 17 food groups which were categorized as either of animal or plant origin, resulting in almost 400 samples. Once the analytical methods were developed and validated, screening of specific food groups available on the Belgian market was performed and the amount of EFAs was computed. The analysed foods were purchased from the Belgian market as fresh when required. Consumption data were obtained from the Belgian National Food Consumption Survey (BNFCS) conducted in 2004. Based on the consumption data obtained from the BNFCS and the concentration data for each food, an exposure assessment was performed. At this point it should be noted that twelve isomers of mono EFAs can be detected in foods which originate from oleic, linoleic and linolenic fatty acids. This fact coupled with the natural occurrence of EFA already reported, may explain the high amounts of EFAs that can be quantified especially in oils that have more C18:1 and C18:2 fatty acids. The EFAs consisted especially of *cis* stereoisomers (these detailed results were not shown in the particular chapter).

Food categories with the highest contribution to the intake of EFA were mayonnaise, butter-margarine and ready to eat meals. On the other hand, consumption of meat products especially cooked meat, smoked salmon and raw ham had the lowest contribution to the intake. The intake from milk was high mainly because of the average consumption of milk which is high. Due to lack of specific toxicological data, the Threshold of Toxicological Concern value was applied to characterize the risk. EFAs are classified as group III compounds based on Cramers method and have a TTC value of 1.5 μg kg^{-1} bw day^{-1} which was used to infer about the risk posed. Based on the levels of exposure obtained from this approach, it was concluded that the consumers of the studied food categories might be at risk, because exposure was far above the defined TTC value for fourteen out of seventeen analysed foods. This study did not consider the presence of EFAs originating from very long chain PUFAs, which are expected to be present in fish and processed fish and similar products. It is obvious that this study revealed a potential safety concern with respect to the ubiquitous presence of EFAs in our diet, but it is obvious that a better insight in the toxicological properties of these compounds is necessary in order to come to more firm conclusions. Combined with the exposure of other lipid oxidation products however, it is obvious that lipid oxidation is a process of potential health concern.

To obtain an insight into the formation of EFAs in food systems, the study presented in **Chapter 5** was carried out. In this study, formation of epoxy fatty acids (EFAs) from oleic, linoleic and linolenic acids was investigated in different oil blends during photosensitized oxidation (at 6°C in the presence of chlorophyll and its degradation products), photoxidation (at 6°C) and autoxidation (at 70°C). Results indicated that initially hydroperoxides were produced, followed by the production of the EFAs. Formation of EFA proved to occur in a stereospecific manner and in addition only specific place isomers of EFA were formed. On basis of these observations, it was suggested that EFA are most likely produced as a result of a 1,3 cyclisation of the hydroperoxide radical or alkoxide radical to the double bond of the oxidizing fatty acid, resulting initially in the formation of *cis* stereoisomers. Upon progressive oxidation a radical supported *cis-trans* isomerization is suggested resulting into an increasing *trans/cis* ratio. The generally accepted route of formation of EFAs could not be supported on basis of our experimental data.

Many other lipid oxidation products especially aldehydes such as malonaldehyde are known to interact with biological materials to cause cellular damage by cross linking and reacting with amino groups of enzymes, proteins and DNA resulting in conjugated Schiff bases which lead to loss of functionality. Epoxy compounds are known to be highly reactive compounds due to the presence of the epoxy ring, so EFAs should further be investigated in some models especially where proteins are involved given their nucleophilic character. This will give insights into the reactivity of EFAs. In **chapter 6** preliminary experiments into this area revealed that when proteins were added to oxidizing lipids containing EFAs, that the final EFA content could either increase or decrease. This indicates that proteins have an impact on the formation of EFAs from hydroperoxides, which implies that the lipid oxidation pathway in pure oils differs from the pathway occurring in composite foods. If a pure EFA was added to a saturated oil in which no EFA could be generated, no interaction between the EFA and proteins upon emulsification and storage could be observed. This could imply that EFAs are unexpectedly and potentially quite stable indicators for the oxidation process.

As a future perspective, since the study focused on C18 mono EFAs, the optimisation of the analytical methods for determining C20 and longer chain EFAs is timely. Also, there is a need to develop a method that can analyse the free EFAs so as to be able to get the total EFAs that can be available in a food. Methods to analyse free fatty acids are available and these can be extended to free EFAs analysis. The challenge of separating these closely related

compounds can still be over come by optimizing the currently used solvent systems for the analysis of free fatty acids. The final quatification can be by GC analysis on a Nukol column without methylation or other columns after methylation.

EFAs are potentially stable final products resulting from the decomposition of primary oxidation compounds. However, because of the presence of double bonds, they can undergo further oxidation. The method could be extended to modified EFAs, but also to hydroxy or oxo fatty acid analysis although this will involve optimisation of nearly all the analytical conditions.

Furthermore, a great contribution for a more accurate risk assessment could be the availability of specific toxicological data, preferably involving in vivo and in vitro toxicity studies such that the final assessment is based on a more specific value than a TTC value. Since hydroxy fatty acids occur in high amounts according to literature, it would be interesting to do a risk assessment of these compounds and this can be extended to the oxo fatty acids as well.

Another further perspective on formation of EFAs, it is recommended that more detailed studies in the area of EFA formation be done. Also, such studies should involve kinetic studies on pure fatty acids to try and validate the integrated lipid oxidation pathways already referred to in literature. The current approach in this study was to begin with simple models, i.e. oils. In **Chapter 6** the first step towards studies in more complex matrices was made via the introduction of proteins in an emulsions system. It is clear that the formation of EFAs in such more complex systems via the various oxidation modes applicable is worthwhile to be investigated.

It is still not clear why fresh oils contain generally a relative high EFA content. The oil refining process is one of the possible routes of formation which should be studied. A detailed investigation into the oil processing chain to be able to give a material balance along the chain is recommended. This may reveal interesting formation routes, whether the EFAs are endogenous or whether they form down the chain during processing. Steps like bleaching which involve use of activated clays are known to remove hydroperoxides from the crude feedstock. However, it is obvious that disappearing does not exist. It implies that hydroperoxides are adsorbed or converted to other compounds. Given however the acidic

character of these bleaching earths and the known sensitivity of EFA hydrolysis in acidic conditions, it remains unclear if the bleaching step is part of the origin or potentially part of the solution of the presence of EFAs in oils and fats. If toxicological research indicates that EFAs are indeed a relevant food safety issue, it is clear that refining technology is a potential key element in the solution of it.

On interaction of EFAs with proteins, it is recommended that better experimental designs which should include factors like temperature, time, pH and antioxidants need to be investigated. The use of different pure EFAs isomers should be investigated separately since reactivity seem not only to depend on the position of the epoxy ring but also on the degree of unsaturation, position and type of other groups attached to the backbone fatty acid chain.

Finally, a totally different outreach can be suggested given the straightforward way in which epoxy fatty acid standards were made in the laboratory throughout this study. Hydroperoxides (i.e. hydrogen peroxide and peracetic acid) are frequently used disinfectant reagents in various biocides. Residues of these highly reactive substances may come in contact with foodstuffs, or foods may be even treated directly with these agents for microbiological reasons. It is basically impossible to trace back the use these peroxides as in view of their high reactive character no residues of the substances as such are remaining in the food. It would be interesting to explore if EFAs are eventually produced as a result of such a treatment or the presence of a residual amount, and thus could be used as a relevant indicator.

CHAPTER

8 APPENDIX

Table 8.1: Composition of the oil blends studied in Chapter 5

Sample type	Sample code	Composition
Stripped blends	A	Stripped refined olive oil + virgin olive oil
	B	Stripped sunflower oil + virgin olive oil
	C	Stripped linseed oil + virgin olive oil
	D	Stripped refined olive oil
	E	Stripped sunflower oil + stripped refined olive oil
	F	Stripped linseed oil + stripped refined olive oil
Non-stripped blends	A'	Refined olive oil + virgin olive oil
	B'	Sunflower oil + virgin olive oil
	C'	Linseed oil + virgin olive oil
	D'	Refined olive oil
	E'	Sunflower oil + refined olive oil
	F'	Linseed oil + refined olive oil

Table 8.2: EFAs content (µmol kg^{-1}) of stripped oils blends during oxidation in the dark at 6°C

		Epoxy fatty acids										
Blend[1]	**Storage time (days)**	***trans*-9,10-ES**	***cis*-9,10-ES**	***trans*-12,13-EO**	***cis*-12,13-EO**	***trans*-9,10-EO**	***cis*-9,10-EO**	***trans*-12,13-EOL**	***cis* -12,13 & trans- 9,10-EOL**	***cis*-9,10-EOL**	***trans*-15,16-EOL**	***cis*-15,16 -EOL**
A	0	47.4	542.6	<LOD	48.4	<LOD	77.1	<LOD	47.1	6.5	<LOD	7.8
	12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	19	23.1	453.8	<LOD	36.8	5.2	60.6	<LOD	30.2	7.8	<LOD	7.1
	27	19.6	335.9	2.6	24.2	1.9	41.3	7.8	27.6	5.2	<LOD	4.2
	43	28.5	478.8	3.9	43.9	4.8	68.4	<LOD	33.8	9.4	<LOD	5.2
	54	26.9	455.4	6.5	40.6	4.5	79.4	<LOD	31.8	10.1	<LOD	2.9
B	0	23.4	411.5	<LOD	159.7	<LOD	207.4	<LOD	32.5	0.0	<LOD	0.0
	12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	19	21.2	346.8	4.5	102.3	16.5	159.7	<LOD	33.4	0.0	<LOD	44.2
	27	34.0	270.2	<LOD	61.0	<LOD	111.6	25.0	39.3	<LOD	<LOD	<LOD
	43	26.0	394.2	<LOD	131.0	<LOD	185.8	<LOD	31.5	<LOD	<LOD	<LOD
	54	42.6	398.1	<LOD	127.4	<LOD	224.5	<LOD	41.6	<LOD	<LOD	<LOD
C	0	25.3	282.7	<LOD	36.8	<LOD	51.0	<LOD	115.9	118.5	<LOD	117.9
	12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	19	31.1	301.0	1.9	33.9	2.6	56.5	5.8	121.8	134.1	6.5	125.3
	27	26.3	213.8	2.6	19.0	2.3	37.1	1.3	66.9	72.7	1.6	67.2
	43	35.3	343.6	3.1	40.9	2.5	66.5	4.9	139.8	152.4	8.4	145.3
	54	33.0	308.3	2.9	33.2	5.5	69.0	3.2	119.2	143.2	7.1	118.5
D	0	7	67.6	<LOD	11.0	<LOD	19.4	<LOD	<LOD	<LOD	<LOD	<LOD
	12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	19	4	50.6	<LOD	6.8	<LOD	11.0	<LOD	<LOD	<LOD	<LOD	35.1
	27	4	44.2	3.2	5.2	<LOD	9.7	11.0	3.6	<LOD	0.0	<LOD
	43	5	55.1	1.6	6.5	<LOD	11.9	<LOD	<LOD	<LOD	<LOD	<LOD
	54	6	51.6	<LOD	11.0	<LOD	15.5	<LOD	<LOD	<LOD	<LOD	<LOD
E	0	18	62.8	<LOD	41.3	<LOD	65.8	<LOD	<LOD	<LOD	<LOD	<LOD
	12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	19	6	43.9	<LOD	24.8	<LOD	49.7	<LOD	<LOD	<LOD	<LOD	16.9
	27	14	40.1	<LOD	23.2	<LOD	39.7	<LOD	<LOD	<LOD	<LOD	<LOD
	43	8	41.3	<LOD	28.1	<LOD	38.1	<LOD	<LOD	<LOD	<LOD	<LOD
	54	<LOD	59.6	<LOD	33.9	<LOD	70.3	<LOD	<LOD	<LOD	<LOD	<LOD
F	0	14	64.7	<LOD	22.3	<LOD	35.5	9.1	43.8	52.3	6.2	48.4
	12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	19	30	86.5	10.3	21.6	12.3	41.6	16.6	86.0	92.9	22.1	87.7
	27	46	101.3	17.1	25.8	12.6	45.5	31.5	110.4	114.9	27.9	111.4
	43	84	133.0	32.6	36.5	31.9	51.9	63.3	162.3	151.0	74.0	129.5
	54	111	222.4	46.8	62.9	42.9	110.3	100.3	261.4	246.4	114.9	198.4

LOD, level of detection; ES, epoxystearate; EO, epoxyoleate; EOL, epoxylinoleate.

[1]Blend: A = stripped refined olive + virgin olive oil; B = stripped sunflower + virgin olive oil; C = stripped linseed + virgin olive oil; D = stripped refined olive oil; E = stripped sunflower + stripped refined olive oil; F = stripped linseed + stripped refined olive oil.

Table 8.3: EFAs content (µmol kg^{-1}) of stripped oils blends during oxidation under illumination at 6°C

Epoxy fatty acids												
Blend[1]	**Time (days)**	***trans*-9,10-ES**	***cis*- 9,10-ES**	***trans*-12,13-EO**	***cis*-12,13-EO**	***trans*-9,10-EO**	***cis*-9,10-EO**	***trans*-12,13-EOL**	***cis* -12,13 & *trans*-9,10-EOL**	***cis*-9,10-EOL**	***trans*-15,16-EOL**	***cis*-15,16 -EOL**
A	0	47.4	542.6	<LOD	48.4	<LOD	77.1	<LOD	47.1	6.5	<LOD	7.8
	12	37.8	583.3	<LOD	114.2	41.9	161.0	4.5	60.1	48.7	<LOD	22.4
	19	30.1	785.3	<LOD	83.5	36.8	147.7	15.9	47.1	115.9	<LOD	15.9
	27	66.3	921.5	7.1	145.2	117.4	191.0	14.3	76.0	87.0	89.6	42.9
	43	94.6	844.2	<LOD	144.2	80.3	175.2	<LOD	72.7	152.9	87.3	<LOD
	54	100.3	784.6	<LOD	122.3	107.7	158.7	<LOD	81.5	131.2	62.7	<LOD
B	0	23.4	411.5	<LOD	159.7	<LOD	207.4	<LOD	32.5	<LOD	<LOD	<LOD
	12	35.9	543.9	9.0	218.1	25.8	358.4	13.0	32.1	28.6	<LOD	16.6
	19	46.5	565.1	4.8	216.1	63.2	404.5	24.0	50.3	29.5	<LOD	48.4
	27	69.6	500.3	21.3	209.0	106.5	335.5	44.5	56.2	53.2	65.3	<LOD
	43	37.2	532.7	27.1	260.6	65.8	415.5	<LOD	69.2	173.1	96.1	<LOD
	54	51.0	605.8	30.0	337.4	103.9	538.4	<LOD	58.8	48.1	40.3	<LOD
C	0	25.3	282.7	<LOD	36.8	<LOD	51.0	<LOD	115.9	118.5	<LOD	117.9
	12	39.1	477.2	5.2	57.4	8.1	114.8	30.8	221.4	352.3	13.0	244.5
	19	42.6	454.2	3.9	65.8	29.4	114.2	24.4	223.1	346.1	17.2	237.7
	27	53.2	407.4	4.2	62.3	22.6	98.7	18.5	203.9	282.1	26.9	195.5
	43	62.5	485.3	20.3	59.0	29.4	118.7	44.8	239.0	300.0	32.1	230.8
	54	64.7	569.2	7.1	76.5	23.5	148.7	39.6	316.9	393.5	42.9	284.4
D	0	7.1	67.6	<LOD	11.0	<LOD	19.4	<LOD	<LOD	<LOD	<LOD	<LOD
	12	46.8	228.5	9.4	37.7	10.6	58.1	<LOD	6.2	7.5	<LOD	7.5
	19	77.2	192.0	18.7	29.4	23.2	49.7	<LOD	3.9	60.7	<LOD	22.1
	27	145.5	260.3	32.3	44.8	32.9	63.2	2.6	6.5	51.9	3.2	9.7
	43	325.0	397.4	67.1	71.6	66.8	98.4	<LOD	10.4	112.7	<LOD	18.2
	54	444.6	461.5	84.2	51.3	82.3	85.5	<LOD	<LOD	129.9	<LOD	8.4
E	0	17.6	62.8	<LOD	41.3	<LOD	65.8	<LOD	<LOD	<LOD	<LOD	<LOD
	12	9.0	131.4	8.1	72.6	15.5	134.5	<LOD	5.8	3.9	<LOD	19.5
	19	41.7	131.7	30.6	80.6	32.3	142.6	<LOD	<LOD	<LOD	<LOD	<LOD
	27	69.9	175.0	68.4	117.4	78.4	187.1	<LOD	<LOD	111.4	<LOD	<LOD
	43	168.9	301.3	156.8	212.3	175.2	311.0	<LOD	<LOD	<LOD	<LOD	<LOD
	54	222.4	321.8	215.2	223.9	225.2	351.6	<LOD	<LOD	<LOD	<LOD	<LOD
F	0	14.1	64.7	<LOD	22.3	<LOD	35.5	9.1	43.8	52.3	6.2	48.4
	12	66.0	163.5	21.9	36.1	16.5	60.3	44.8	166.9	180.8	51.3	163.0
	19	78.5	149.4	29.4	30.6	22.6	58.1	57.1	178.9	139.9	75.6	127.6
	27	133.3	199.7	48.4	57.1	52.3	80.0	120.1	253.2	205.5	127.6	171.8
	43	267.3	271.8	107.7	94.2	101.6	108.4	248.1	424.0	225.0	270.5	207.1
	54	320.5	344.9	122.3	100.6	124.2	143.9	307.5	510.4	339.6	332.8	280.2

LOD, level of detection; ES, epoxystearate; EO, epoxyoleate; EOL, epoxylinoleate.

[1]Blend: A = stripped refined olive + virgin olive oil; B = stripped sunflower + virgin olive oil; C = stripped linseed + virgin olive oil; D = stripped refined olive oil; E = stripped sunflower + stripped refined olive oil; F = stripped linseed + stripped refined olive oil.

Table 8.4: Epoxy fatty acids (µmol kg^{-1}), of stripped oils blends during autoxidation at 70°C

		Epoxy fatty acids										
Blend[1]	**Storage time (days)**	**tr*ans*-9,10-ES**	***cis*-9,10-ES**	**tr*ans*-12,13-EO**	***cis*-12,13-EO**	***trans*-9,10-EO**	***cis*-9,10-EO**	***trans*-12,13-EOL**	***cis* -12,13 & *trans*-9,10-EOL**	***cis*-9,10-EOL**	***trans*-15,16-EOL**	***cis*-15,16 -EOL**
A	0	11.9	262.2	<LOD	17.4	<LOD	27.1	<LOD	<LOD	<LOD	<LOD	<LOD
	1	15.7	242.9	<LOD	18.1	<LOD	27.4	<LOD	<LOD	<LOD	<LOD	<LOD
	3	37.2	333.3	5.8	27.4	13.2	43.5	<LOD	24.4	0.0	0.0	<LOD
	5	59.3	334.3	6.5	26.1	<LOD	36.8	<LOD	31.2	<LOD	<LOD	<LOD
B	0	14.1	206.4	<LOD	66.5	<LOD	92.3	<LOD	<LOD	<LOD	<LOD	<LOD
	1	34.0	219.2	<LOD	69.0	<LOD	90.6	<LOD	<LOD	<LOD	<LOD	<LOD
	3	151.6	370.2	46.1	126.8	50.6	175.2	<LOD	26.6	<LOD	<LOD	<LOD
	5	136.2	342.3	71.6	130.3	74.8	164.8	<LOD	112.3	<LOD	<LOD	<LOD
C	0	11.2	184.6	<LOD	21.9	<LOD	33.2	<LOD	63.0	65.9	53.9	<LOD
	1	67.0	184.6	11.0	18.1	11.3	27.1	26.0	89.0	50.3	44.2	27.9
	3	244.2	387.5	51.9	62.6	60.6	81.3	140.3	314.3	193.8	159.4	148.7
	5	391.0	534.3	132.6	124.5	139.7	124.8	329.5	590.6	284.7	269.8	343.5
D	0	<LOD	26.6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	1	330.1	162.5	54.2	21.0	54.2	27.1	<LOD	<LOD	<LOD	<LOD	<LOD
	3	3599.0	1415.1	542.3	202.9	611.6	196.5	64.0	159.1	121.4	36.7	25.3
	5	6184.3	2664.7	911.9	403.5	936.1	319.0	100.0	311.0	205.5	60.1	38.6
E	0	<LOD	30.1	<LOD	18.7	<LOD	30.3	<LOD	<LOD	<LOD	<LOD	<LOD
	1	268.6	173.4	193.9	90.0	195.5	122.9	<LOD	<LOD	<LOD	<LOD	<LOD
	3	1552.9	1050.3	1291.9	646.1	1332.9	666.1	26.0	97.4	79.5	14.0	12.0
	5	1928.2	1587.5	1676.8	1032.6	1713.2	952.6	<LOD	159.4	<LOD	<LOD	<LOD
F	0	<LOD	21.8	<LOD	<LOD	<LOD	9.7	<LOD	14.6	20.1	13.6	<LOD
	1	246.8	144.9	84.2	35.2	85.5	42.6	211.4	299.0	99.0	83.4	217.5
	3	1151.9	1226.3	442.6	369.7	437.7	342.9	984.1	1605.2	693.5	672.1	978.6
	5	1836.9	2754.2	679.0	781.3	677.4	703.5	1533.1	2752.3	1556.2	1471.8	1527.9

LOD, level of detection; ES, epoxystearate; EO, epoxyoleate; EOL, epoxylinoleate.

[1]Blend: A = stripped refined olive + virgin olive oil; B = stripped sunflower + virgin olive oil; C = stripped linseed + virgin olive oil; D = stripped refined olive oil; E = stripped sunflower + stripped refined olive oil; F = stripped linseed + stripped refined olive oil

Table 8.5: Epoxy fatty acids (µmol kg^{-1}) of non stripped oil blends during autoxidation at 70°C

		Epoxy fatty acids										
Blend	Time (days)	trans-9,10-ES	cis-9,10-ES	trans-12,13-EO	cis-12,13-EO	trans-9,10-EO	cis-9,10-EO	trans-12,13-EOL	cis -12,13 & trans-9,10-EOL	cis-9,10-EOL	trans-15,16-EOL	cis-15,16-EOL
	0	63.1	437.5	7.4	36.5	6.8	41.3	0.0	294.5	12.0	0.0	7.1
	1	68.9	505.8	8.1	50.0	3.9	51.9	0.0	314.9	13.3	0.0	33.4
	3	68.3	431.1	7.7	32.3	3.9	36.8	0.0	300.0	13.6	0.0	6.2
A'	5	75.3	549.4	7.4	43.2	4.2	48.1	0.0	308.4	17.2	0.0	7.5
	0	40.4	1559.0	15.8	389.7	16.8	3542.6	0.0	41.9	17.5	0.0	13.6
	1	53.2	1635.9	18.1	389.0	16.8	3615.5	0.0	71.1	16.6	0.0	10.7
	3	45.2	1576.3	20.6	371.9	22.6	3573.2	0.0	48.1	20.8	0.0	10.1
B'	5	66.3	1673.4	30.6	401.9	33.5	3636.8	0.0	50.3	22.1	0.0	14.0
	0	34.6	114.7	0.0	18.4	0.0	23.2	0.0	72.4	23.7	0.0	1709.4
	1	33.7	149.0	0.0	20.3	0.0	33.9	0.0	76.3	68.2	3.2	1763.0
	3	59.6	160.3	11.0	25.2	7.4	34.2	29.9	116.2	63.0	29.5	1721.4
C'	5	135.9	302.6	31.0	57.7	28.4	61.3	92.2	263.0	149.4	99.0	1817.5
	0	79.2	366.7	9.7	39.7	9.0	32.9	0.0	411.4	14.9	0.0	8.4
	1	84.6	367.0	8.7	38.1	4.8	38.1	0.0	422.4	17.9	0.0	70.5
	3	88.8	334.3	8.4	33.9	5.8	30.0	0.0	402.3	14.6	0.0	5.5
D'	5	102.2	351.3	11.9	36.8	7.4	35.2	0.0	444.5	15.3	0.0	5.2
	0	61.9	1589.4	16.1	351.9	19.4	3632.6	0.0	167.5	20.1	0.0	10.7
	1	65.1	1539.4	17.1	354.8	19.0	3580.3	0.0	159.4	18.5	0.0	10.7
	3	67.6	1638.1	22.3	359.0	24.5	3787.4	0.0	149.7	21.8	0.0	11.7
E'	5	92.3	1702.6	36.1	381.3	34.8	3814.5	0.0	174.0	22.4	0.0	11.7
	0	49.4	119.2	6.8	30.6	4.5	13.9	0.0	165.6	33.4	0.0	1733.1
	1	46.2	163.3	4.3	28.3	3.1	121.2	0.00	154.71	45.26	3.17	1631.3
	3	96.8	170.5	15.8	27.7	14.2	25.8	34.4	231.2	48.4	34.4	1783.4
F'	5	180.1	308.7	38.7	62.6	36.1	56.8	106.5	374.7	137.0	112.0	1809.7

LOD, level of detection; ES, epoxystearate; EO, epoxyoleate; EOL, epoxylinoleate.

[1]Blend: A' = refined olive oil + virgin olive oil; B' = sunflower oil + virgin olive oil; C' = linseed oil + virgin olive oil; D' = refined olive oil; E' = sunflower oil + refined olive oil; F' = Linseed oil + refined olive oil.

Table 8.6: Percentage isomeric distribution (ID) of ES, EO and EOL on mole basis of the different non stripped oil blends during autoxidation at 70°C

Isomeric Distribution												
		Oleic acid[a]		Linoleic acid[a]				Linolenic acid[a]				
Blend[1]	Storage time (days)	*trans*-9,10-ES	*cis*-9,10-ES	*trans*-12,13-EO	*cis*-12,13-EO	*trans*-9,10-EO	*cis*-9,10-EO	*trans*-12,13-EOL	*cis*-12,13 & *trans*-9,10-EOL	*cis*-9,10-EOL	*trans*-15,16-EOL	*cis*-15,16 -EOL
A'	0	13	87	8	40	7	45	0	94	4	0	2
	1	12	88	7	44	3	46	0	87	4	0	9
	3	14	86	10	40	5	46	0	94	4	0	2
	5	12	88	7	42	4	47	0	93	5	0	2
B'	0	3	97	0	10	0	89	0	57	24	0	19
	1	3	97	0	10	0	90	0	72	17	0	11
	3	3	97	1	9	1	90	0	61	26	0	13
	5	4	96	1	10	1	89	0	58	26	0	16
C'	0	23	77	0	44	0	56	0	4	1	0	95
	1	18	82	0	38	0	63	0	4	4	0	92
	3	27	73	14	32	10	44	2	6	3	2	88
	5	31	69	17	32	16	34	4	11	6	4	75
D'	0	18	82	11	43	10	36	0	95	3	0	2
	1	19	81	10	42	5	42	0	83	3	0	14
	3	21	79	11	43	7	38	0	95	3	0	1
	5	23	77	13	40	8	39	0	96	3	0	1
E'	0	4	96	0	9	0	90	0	84	10	0	5
	1	4	96	0	9	0	90	0	85	10	0	6
	3	4	96	1	9	1	90	0	82	12	0	6
	5	5	95	1	9	1	89	0	84	11	0	6
F'	0	29	71	12	55	8	25	0	9	2	0	90
	1	22	78	21	31	18	30	0	9	2	0	89
	3	36	64	19	33	17	31	2	11	2	2	84
	5	37	63	20	32	19	29	4	15	5	4	71

ES, epoxystearate; EO, epoxyoleate; EOL, epoxylinoleate.

[1]Blend: A' = Refined olive oil + virgin olive oil; B' = Sunflower oil + virgin olive oil; C' = Linseed oil + virgin olive oil; D' = Refined olive oil; E' = Sunflower oil + refined olive oil; F' = Linseed oil + refined olive oil.

[a] As% total of the originating fatty acid for the EFA

REFERENCES

Aerts, H.A., & Jacobs, P.A. (2004). Epoxide yield determination of oils and fatty acid methyl esters using ^{1}H NMR. *Journal of the American Oil Chemists' Society, 81*(9), 841-846.

Aguirre, M., Dobarganes, M.C., Marmesat, S., & Ruiz Méndez, M.V. (2010). Application of high-temperature gas chromatography to the analysis of used frying fats. *Grasas y Aceites, 61*, 197-202.

Aidos, I., Jacobsen, C., Jensen, B., Luten, J.B., van der Padt, A., & Boom, R.M. (2002). Volatile oxidation products formed in crude herring oil under accelerated oxidative conditions. *European Journal of Lipid Science and Technology, 104*(12), 808-818.

Akoh, C.C., & Min, D.B. (2008). *Food lipids: Chemistry, nutrition, and biotechnology* (Third ed.): Taylor & Francis.

Andersen, L.T., Lund, M.N., Glyager, R., Jensen, S.B., Mortensen, G., & Skibsted, L.H. (2006). Light-induced deterioration of reduced-fat cream cheese. Relative importance of protein and lipid oxidation. *Milchwissenschaft, 61*(2), 161-165.

AOCS. (1990). Official method Ce 1b-89 for marine oils: The American Oil Chemists' Society.

AOCS. (1998). *Official methods and recommended practices of the American Oil Chemists' Society* (Fifth ed.): Champaign, Illinois: The American Oil Chemists' Society.

AOCS. (2011). Official method Cd 8b-90. Peroxide value acetic acid-isooctane method: The American Oil Chemists' Society.

Badami, R.C., & Patil, K.B. (1980). Structure and occurrence of unusual fatty acids in minor seed oils. *Progress in Lipid Research, 19*(3), 119-153.

Barlow, S., Renwick, A., Kleiner, J., Bridges, J., Busk, L., Dybing, E., Edler, L., Eisenbrand, G., Fink-Gremmels, J., & Knaap, A. (2006). Risk assessment of substances that are both genotoxic and carcinogenic: Report of an international conference organized by EFSA and WHO with support of ILSI Europe. *Food and chemical toxicology, 44*(10), 1636-1650.

Barnes, D.G., Dourson, M., Preuss, P., Bellin, J., Derosa, C., Engler, R., Erdreich, L., & Farber, T. (1988). Reference dose (RfD): Description and use in health risk assessments. *Regulatory Toxicology and Pharmacology, 8*(4), 471-486.

Belitz, H.-D., Grosch, W., & Schieberle, P. (2009). *Food chemistry* (Fourth ed.): Springer: Berlin.

Benford, D., Bolger, P.M., Carthew, P., Coulet, M., DiNovi, M., Leblanc, J.-C., Renwick, A.G., Setzer, W., Schlatter, J., & Smith, B. (2010). Application of the margin of exposure (MOE) approach to substances in food that are genotoxic and carcinogenic. *Food and Chemical Toxicology, 48*, S2-S24.

Berdeaux, O., Dutta, P., Dobarganes, M., & Sebeio, J.L. (2009). Analytical methods for quantification of modified fatty acids and sterols formed as a result of processing. *Food Analytical Methods, 2*(1), 30-40.

Berdeaux, O., Fontagné, S., Sémon, E., Velasco, J., Sébédio, J.L., & Dobarganes, C.M. (2012). A detailed identification study on high-temperature degradation products of oleic and linoleic acid methyl esters by GC–MS and GC–FTIR. *Chemistry and Physics of Lipids, 165*(3), 338-347.

Berdeaux, O., Marquez-Ruiz, G., & Dobarganes, C.M. (1999a). Selection of methylation procedures for quantitation of short-chain glycerol-bound compounds formed during thermoxidation. *Journal of Chromatography A, 863*(2), 171-181.

Berdeaux, O., Marquez-Ruiz, G., & Dobarganes, M.C. (1999b). Characterization, quantitation and evolution of monoepoxy compounds formed in model systems of fatty acid methyl esters and monoacid triglycerides heated at high temperature. *Grasas y Aceites, 50*(1), 53-59.

Berdeaux, O., Velasco, J., Márquez-Ruiz, G., & Dobarganes, M.C. (2002). Evolution of short-chain glycerol-bound compounds during thermoxidation of fame and monoacid tag. *Journal of the American Oil Chemists' Society, 79*(3), 279-285.

Bergström, M.A., Luthman, K., Nilsson, J.L.G., & Karlberg, A.-T. (2006). Conjugated dienes as prohaptens in contact allergy: In vivo and in vitro studies of structure− activity relationships, sensitizing capacity, and metabolic activation. *Chemical Research in Toxicology, 19*(6), 760-769.

Bhardwaj, H.L., Hamama, A.A., & Dierig, D.A. (2007). Fatty acids in Vernonia produced in the Mid-Atlantic region of the United States. *Journal of the American Oil Chemists' Society, 84*(4), 393-397.

Bligh, E.G., & Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*(8), 911-917.

Buettner, G.R. (1993). The pecking order of free radicals and antioxidants: Lipid peroxidation, alpha-tocopherol, and ascorbate. *Archives of Biochemistry and Biophysics, 300*(2), 535-543.

Burns, D.T., Danzer, K., & Townshend, A. (2002). Use of the terms "recovery" and "apparent recovery" in analytical procedures - (IUPAC recommendations 2002). *Pure and Applied Chemistry, 74*(11), 2201-2205.

Cahoon, E.B., & Kinney, A.J. (2005). The production of vegetable oils with novel properties: Using genomic tools to probe and manipulate plant fatty acid metabolism. *European Journal of Lipid Science and Technology, 107*(4), 239-243.

Cahoon, E.B., Ripp, K.G., Hall, S.E., & McGonigle, B. (2002). Transgenic production of epoxy fatty acids by expression of a cytochrome P450 enzyme from *Euphorbia lagascae* seed. *Plant Physiology, 128*(2), 615-624.

Cardenia, V., Waraho, T., Rodriguez-Estrada, M.T., McClements, D.J., & Decker, E.A. (2011). Antioxidant and prooxidant activity behavior of phospholipids in stripped soybean oil-in-water emulsions. *Journal of the American Oil Chemists' Society, 88*(9), 1409-1416.

Carlson, K.D., & Chang, S.P. (1985). Chemical epoxidation of a natural unsaturated epoxy seed oil from *Vernonia galamensis* and a look at epoxy oil markets. *Journal of the American Oil Chemists' Society, 62*(5), 934-939.

Castle, L., Mayo, A., & Gilbert, J. (1990). Migration of epoxidised soya bean oil into foods from retail packaging materials and from plasticised pvc film used in the home. *Food Additives and Contaminants, 7*(1), 29-36.

Cequier-Sánchez, E., Rodríguez, C., Ravelo, A.G., & Zárate, R. (2008). Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. *Journal of Agricultural and Food Chemistry, 56*(12), 4297-4303.

Chacon, J.N., McLearie, J., & Sinclair, R.S. (1988). Singlet oxygen yields and radical contributions in the dye-sensitised photo-oxidation in methanol of esters of polyunsaturated fatty acids (oleic, linoleic, linolenic and arachidonic). *Photochemistry and Photobiology, 47*(5), 647-656.

Chaiyasit, W., Elias, R.J., McClements, D.J., & Decker, E.A. (2007). Role of physical structures in bulk oils on lipid oxidation. *Critical Reviews in Food Science and Nutrition, 47*(3), 299-317.

Chen, B., McClements, D.J., & Decker, E.A. (2011). Minor components in food oils: A critical review of their roles on lipid oxidation chemistry in bulk oils and emulsions. *Critical Reviews in Food Science and Nutrition, 51*(10), 901-916.

Chen, B., McClements, D.J., & Decker, E.A. (2013). Oxidation in different food matrices: How physical structure impacts lipid oxidation in oil-in-water emulsions and bulk oils. In Amy Logan (Ed.), *Lipid oxidation: Challenges in food systems* pp. 129-154. Urbana, Illinois, USA: AOCS Press.

Choe, E. (2008). Effects and mechanisms of minor compounds in oil on lipid oxidation. *Food Lipids: Chemistry, Nutrition, and Biotechnology*, 449-474.

Choe, E., & Min, D.B. (2006). Mechanisms and factors for edible oil oxidation. *Comprehensive Reviews in Food Science and Food Safety, 5*(4), 169-186.

Chow, C.K. (2007). *Fatty acids in foods and their health implications* (Third ed.): Taylor & Francis.

Christie, W.W. (2003). *Lipid analysis: Isolation, separation, identification and strucutral analysis of lipids* (Third ed.). England, UK: The Oily Press.

Christie, W.W. (2011a). *The lipid library* CHRISTIE (Ed.) *Gas Chromatography and Lipids: The Analysis of fatty acids:The Preparation of derivatives of fatty acids* Retrieved from http://lipidlibrary.aocs.org/GC_lipid/04_deriv/index.htm

Christie, W.W. (2011b). Preparation of ester derivatives of fatty acids for chromatographic analysis. Retrieved from http://lipidlibrary.aocs.org/topics/ester_93/index.htm.

Christie, W.W. (2012). *Mass spectrometry of fatty acid derivatives*. Retrieved from http://lipidlibrary.aocs.org/ms/masspec.html

Chu, I., Villeneuve, D.C., Nestmann, E.R., Douglas, G., Becking, G.C., Lough, R., & Matula, T.I. (1980). Subacute toxicity and mutagenicity of *cis*-9,10-epoxystearic acid. *Bulletin of Environmental Contamination and Toxicology, 25*(1), 400-403.

Codex Alimentarius, C. (1999). Principles and guidelines for the conduct of microbiological risk assessment. *CAC/GL, 30*, 1-6.

Costa, R., & Kristbergsson, K. (2008). *Predictive modeling and risk assessment* (Vol. 4): Springer Science & Business Media.

Courthaudon, J.L., Dickinson, E., & Dalgleish, D.G. (1991). Competitive adsorption of β-casein and nonionic surfactants in oil-in-water emulsions. *Journal of Colloid and Interface Science, 145*(2), 390-395.

Courthaudon, J.L., Dickinson, E., Matsumura, Y., & Clark, D.C. (1991). Competitive adsorption of β-lactoglobulin + Tween 20 at the oil-water interface. *Colloids and Surfaces, 56*, 293-300.

Cramer, G.M., Ford, R.A., & Hall, R.L. (1978). Estimation of toxic hazard—a decision tree approach. *Food and Cosmetics Toxicology, 16*(3), 255-276.

Cucu, T., Devreese, B., Mestdagh, F., Kerkaert, B., & De Meulenaer, B. (2011). Protein–lipid interactions during the incubation of whey proteins with autoxidizing lipids. *International Dairy Journal, 21*(6), 427-433.

Cui, L., & Decker, E.A. (2016). Phospholipids in foods: Prooxidants or antioxidants? *Journal of the Science of Food and Agriculture, 96*(1), 18-31.

Cui, P.H., Duke, R.K., & Duke, C.C. (2008a). Monoepoxy octadecadienoates and monoepoxy octadecatrienoates 1: NMR spectral characterization. *Chemistry and Physics of Lipids, 152*(2), 122-130.

Cui, P.H., Duke, R.K., Tattam, B.N., & Duke, C.C. (2008b). Monoepoxy octadecadienoates and monoepoxy octadecatrienoates 2: Mass spectral characterization. *Chemistry and Physics of Lipids, 152*(2), 65-70.

Cui, P.H., Zhang, W.V., Hook, J., Tattam, B.N., Duke, C.C., & Murray, M. (2009). Synthesis and NMR characterization of the methyl esters of eicosapentaenoic acid monoepoxides. *Chemistry and Physics of Lipids, 159*(1), 30-37.

Daelman, J., Jacxsens, L., Membré, J.-M., Sas, B., Devlieghere, F., & Uyttendaele, M. (2013). Behaviour of Belgian consumers, related to the consumption, storage and preparation of cooked chilled foods. *Food Control, 34*(2), 681-690.

Damodaran, S., Parkin, K.L., & Fennema, O.R. (2007). *Fennema's food chemistry* (Fourth ed.). Boca Raton: CRC press.

Daun, J.K. (2012). Spectrophotometric analysis of chlorophyll pigments in canola and rapeseed oils. *Lipid Technology, 24*(6), 134-136.

de Boer, E.J., Slimani, N., van't Veer, P., Boeing, H., Feinberg, M., Leclercq, C., Trolle, E., Amiano, P., Andersen, L.F., & Freisling, H. (2011). The European food consumption validation project: Conclusions and recommendations. *European Journal of Clinical Nutrition, 65*, S102-S107.

De Vries, J. (1996). *Food safety and toxicity*: Boca Raton. CRC Press.

Delmonte, P., Kia, A.-R.F., Hu, Q., & Rader, J.I. (2009). Review of methods for preparation and gas chromatographic separation of trans and cis reference fatty acids. *Journal of AOAC International, 92*(5), 1310-1326.

deMan, J.M. (1999). *Principles of food chemistry* (Third ed.): Aspen Publishers.

Derelanko, M.J., & Hollinger, M.A. (2001). *Handbook of toxicology* (Second ed.): CRC Press.

Dickinson, E., & Gelin, J.L. (1992). Influence of emulsifier on competitive adsorption of α_s-casein + β-lactoglobulin in oil-in-water emulsions. *Colloids and Surfaces, 63*(3), 329-335.

Dobarganes, M.C. (2009). *Frying oils-chemistry formation of epoxy-, keto- and hydroxy-fatty acids* Christie (Ed.) *Frying Oils- Chemistry Formation of Epoxy-, Keto- and Hydroxy-Fatty Acids* Retrieved from http://lipidlibrary.aocs.org/frying/c-epoxy/index.htm

Dodds, E.D., McCoy, M.R., Rea, L.D., & Kennish, J.M. (2005). Gas chromatographic quantification of fatty acid methyl esters: Flame ionization detection vs. Electron impact mass spectrometry. *Lipids, 40*(4), 419-428.

Earle, F.R. (1970). Epoxy oils from plant seeds. *Journal of the American Oil Chemists Society, 47*(12), 510-513.

EFSA. (2004). Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the commission related to the use of epoxidised soybean oil in food contact materials (Vol. 64, pp. 1-17).

EFSA. (2005). Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. *EFSA Journal, 3*(10), 282.

Esselman, W.J., & Clagett, C.O. (1969). Gas-liquid chromatography-mass spectrometry of hydroxylated octadecanols derived from stearic acids. *Journal of Lipid Research, 10*(2), 234-239.

European Commission. (2002). Commission decision 2002/657/EC of 12 August 2002 implementing council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Communities, 221*, 8-36.

Fankhauser-Noti, A., Fiselier, K., Biedermann-Brem, S., & Grob, K. (2006). Assessment of epoxidized soy bean oil (ESBO) migrating into foods: Comparison with ESBO-like epoxy fatty acids in our normal diet. *Food and Chemical Toxicology, 44*(8), 1279-1286.

FDA. (1995). Food additives: Threshold of regulation for substances used in food-contact articles *Federal Register, 60*(136), 36582-36596.

Fennema, O.R. (1996). *Food chemistry* (3 ed.): Taylor & Francis.

Fer, M., Goulitquer, S., Dréano, Y., Berthou, F., Corcos, L., & Amet, Y. (2006). Determination of polyunsatured fatty acid monoepoxides by high performance liquid chromatography–mass spectrometry. *Journal of Chromatography A, 1115*(1–2), 1-7.

Frankel, E.N. (2005). *Lipid oxidation* (Second ed. Vol. 18): Cambridge, UK: The Oily Press.

Gardner, H.W. (1979). Lipid hydroperoxide reactivity with proteins and amino acids: A review. *Journal of Agricultural and Food Chemistry, 27*(2), 220-229.

Gardner, H.W. (1989). Oxygen radical chemistry of polyunsaturated fatty acids. *Free Radical Biology and Medicine, 7*(1), 65-86.

Gardner, H.W., Weisleder, D., & Nelson, E.C. (1984). Acid catalysis of a linoleic acid hydroperoxide: Formation of epoxides by an intramolecular cyclization of the hydroperoxide group. *The Journal of Organic Chemistry, 49*(3), 508-515.

Gérard, H.C., Moreau, R.A., Fett, W.F., & Osman, S.F. (1992). Separation and quantitation of hydroxy and epoxy fatty acid by high-performance liquid chromatography with an evaporative light-scattering detector. *Journal of the American Oil Chemists' Society, 69*(4), 301-304.

German Institute of Human Nutrition Potsdam-Rehbrücke (DIFE). (2013). The multiple source method (MSM).

Gilbert, J., Shepherd, M.J., Startin, J.R., & Eagles, J. (1981). Dimeric epoxy fatty acid methyl esters: Formation, chromatography and mass spectrometry. *Chemistry and Physics of Lipids, 28*(1), 61-68.

Giuffrida, F., Destaillats, F., Robert, F., Skibsted, L.H., & Dionisi, F. (2004). Formation and hydrolysis of triacylglycerol and sterols epoxides: Role of unsaturated triacylglycerol peroxyl radicals. *Free Radical Biology and Medicine, 37*(1), 104-114.

Goicoechea, E., & Guillen, M.D. (2010). Analysis of hydroperoxides, aldehydes and epoxides by ^{1}H Nuclear Magnetic Resonance in sunflower oil oxidized at 70 &100 °c. *Journal of Agricultural and Food Chemistry, 58*(10), 6234-6245.

Gray, J.I. (1978). Measurement of lipid oxidation: A review. *Journal of the American Oil Chemists' Society, 55*(6), 539-546.

Greene, J.F., Newman, J.W., Williamson, K.C., & Hammock, B.D. (2000a). Toxicity of epoxy fatty acids and related compounds to cells expressing human soluble epoxide hydrolase. *Chemical Research in Toxicology, 13*(4), 217-226.

Greene, J.F., Williamson, K.C., Newman, J.W., Morisseau, C., & Hammock, B.D. (2000b). Metabolism of monoepoxides of methyl linoleate: Bioactivation and detoxification. *Archives of Biochemistry and Biophysics, 376*(2), 420-432.

Gunstone, F.D. (1954). Fatty acids. Part ii. The nature of the oxygenated acid present in *Vernonia anthelmintica* (willd.) seed oil. *Journal of the Chemical Society* 1611-1616.

Gunstone, F.D. (2004). *The chemistry of oils and fats : Sources, composition, properties, and uses*. Oxford, UK : Boca Raton, FL: Blackwell Pub. ; CRC Press.

Gunstone, F.D., Harwood, J.L., & Dijkstra, A.J. (2007). *The lipid handbook with cd-rom* (Third ed.). Boca Raton, Florida, USA: CRC Press, Taylor & Francis.

Gunstone, F.D., & Jacobsberg, F.R. (1972). Fatty acids, part 35 the preparation and properties of the complete series of methyl epoxyoctadecanoates. *Chem. Phys. Lipids, 9*(1), 26-34.

Gunstone, F.D., & Schuler, H.R. (1975). Fatty acids, part 45 epoxyoctadecenoates, dihydroxyoctadecenoates, and diepoxyoctadecanoates: Preparation, chromatographic properties, and reaction with boron trifluoride etherate. *Chemistry and Physics of Lipids, 15*(2), 174-188.

Gutiérrez-Rosales, F., Garrido-Fernández, J., Gallardo-Guerrero, L., Gandul-Rojas, B., & Minguez-Mosquera, M.I. (1992). Action of chlorophylls on the stability of virgin olive oil. *Journal of the American Oil Chemists Society, 69*(9), 866-871.

Halarnkar, P.P., Wixtrom, R.N., Silva, M.H., & Hammock, B.D. (1989). Catabolism of epoxy fatty esters by the purified epoxide hydrolase from mouse and human liver. *Archives of Biochemistry and Biophysics, 272*(1), 226-236.

Hammarling, L., Gustavsson, H., Svensson, K., Karlsson, S., & Oskarsson, A. (1998). Migration of epoxidized soya bean oil from plasticized PVC gaskets into baby food. *Food Additives & Contaminants, 15*(2), 203-208.

Hammond, E.W. (2002). Lipid analysis -a 20th century success? *Journal of the Science of Food and Agriculture, 82*(1), 5-11.

Harttig, U., Haubrock, J., Knueppel, S., Boeing, H., & Consortium, EFCOVAL. (2011). The MSM program: Web-based statistics package for estimating usual dietary intake using the multiple source method. *European Journal of Clinical Nutrition, 65*, S87-S91.

Harvey, D. (2000). *Modern analytical chemistry*. Boston, USA: The McGraw-Hill Companies, Inc.

Haubrock, J., Noethlings, U., Volatier, J.-L., Dekkers, A., Ocke, M., Harttig, U., Illner, A.-K., Knueppel, S., Andersen, L.F., & Boeing, H. (2011). Estimating usual food intake distributions by using the multiple source method in the EPIC-Potsdam calibration study. *Journal of Nutrition, 141*(5), 914-920.

Hayakawa, M., Kosaka, K., Sugiyama, S., Yokoo, K., Aoyama, H., Izawa, Y., & Ozawa, T. (1990). Proposal of leukotoxin, 9,10-epoxy-12-octadecenoate, as a burn toxin. *Biochemistry International, 21*(3), 573-579.

Hayakawa, M., Sugiyama, S., Takamura, T., Yokoo, K., Iwata, M., Suzuki, K., Taki, F., Takahashi, S., & Ozawa, T. (1986). Neutrophils biosynthesize leukotoxin, 9, 10-epoxy-12-octadecenoate. *Biochemical and Biophysical Research Communications, 137*(1), 424-430.

Heath, R.J., Di, Y., Clara, S., Hudson, A., & Manock, H. (2005). Epoxide tannage: A way forward. *Journal of the Society of Leather Technologists and Chemists, 89*(5), 186-193.

Hennes, E.C. (2012). An overview of values for the threshold of toxicological concern. *Toxicology Letters, 211*(3), 296-303.

Holser, R.A. (2008). Transesterification of epoxidized soybean oil to prepare epoxy methyl esters. *Industrial Crops and Products, 27*(1), 130-132.

Huybrechts, I., Matthys, C., Vereecken, C., Maes, L., Temme, E.H.M., Van Oyen, H., De Backer, G., & De Henauw, S. (2008). Food intakes by preschool children in Flanders compared with dietary guidelines. *International Journal of Environmental Research and Public Health, 5*(4), 243-257.

IARC. (1999). Monographs on the evaluation of carcinogenic risks to humans: Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide. *Vol. 71. Lyon, France*.

IARC/WHO. (1987). *Overall evaluations of carcinogenicity: An updating of IARC monographs* (Vol. Vol. 7.): International Agency for Research on Cancer. Lyon, France.

Iverson, S.J., Lang, S.L.C., & Cooper, M.H. (2001). Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. *Lipids, 36*(11), 1283-1287.

Iwahama, T., Hatta, G., Sakaguchi, S., & Ishii, Y. (2000). Epoxidation of alkenes using alkyl hydroperoxides generated in situ by catalytic autoxidation of hydrocarbons with dioxygen. *Chemical Communications*(2), 163-164.

Jung, M., Yoon, S., & Min, D. (1989). Effects of processing steps on the contents of minor compounds and oxidation of soybean oil. *Journal of the American Oil Chemists' Society, 66*(1), 118-120.

Kalogeropoulos, N., Salta, F.N., Chiou, A., & Andrikopoulos, N.K. (2007). Formation and distribution of oxidized fatty acids during deep- and pan-frying of potatoes. *European Journal of Lipid Science and Technology, 109*(11), 1111-1123.

Karel, M. (1973). Protein-lipid interactions. *Journal of Food Science, 38*(5), 756-763.

Karel, M. (1980). Lipid oxidation, secondary reactions, and water activity of foods. In Simic & Karel (Eds.), *Autoxidation in food and biological systems* (pp. 191-206): Springer US.

Karlberg, A.-T., Bergström, M.A., Börje, A., Luthman, K., & Nilsson, J.L.G. (2008). Allergic contact dermatitis-formation, structural requirements, and reactivity of skin sensitizers. *Chemical Research in Toxicology, 21*(1), 53-69.

Kent, J.A. (2013). *Handbook of industrial chemistry and biotechnology* (Vol. 1 and 2): Springer Science & Business Media.

Khan, M.A., & Shahidi, F. (2002). Photooxidative stability of stripped and non-stripped borage and evening primrose oils and their emulsions in water. *Food Chemistry, 79*(1), 47-53.

Kittipongpittaya, K., Panya, A., McClements, D.J., & Decker, E.A. (2014). Impact of free fatty acids and phospholipids on reverse micelles formation and lipid oxidation in bulk oil. *Journal of the American Oil Chemists' Society, 91*(3), 453-462.

Kleiman, R., & Spencer, G. (1973). Gas chromatography-mass spectrometry of methyl esters of unsaturated oxygenated fatty acids. *Journal of the American Oil Chemists' Society, 50*(2), 31-38.

Knothe, G. (2006). NMR spectroscopy of fatty acids and their derivatives. The AOCS Lipid Library, Cardiff, Wales, UK.

Koga, T., & Terao, J. (1994). Antioxidant activity of a novel phosphatidyl derivative of vitamin E in lard and its model system. *Journal of Agricultural and Food Chemistry, 42*(6), 1291-1294.

Kroes, R., Kleiner, J., & Renwick, A. (2005). The threshold of toxicological concern concept in risk assessment. *Toxicological Sciences, 86*(2), 226-230.

Kroes, R., Müller, D., Lambe, J., Löwik, M.R.H., van Klaveren, J., Kleiner, J., Massey, R., Mayer, S., Urieta, I., Verger, P., & Visconti, A. (2002). Assessment of intake from the diet. *Food and Chemical Toxicology, 40*(2–3), 327-385.

Kroes, R., Renwick, A.G., Cheeseman, M., Kleiner, J., Mangelsdorf, I., Piersma, A., Schilter, B., Schlatter, J., van Schothorst, F., Vos, J.G., & Wurtzen, G. (2004). Structure-based thresholds of toxicological concern (TTC): Guidance for application to substances present at low levels in the diet. *Food and Chemical Toxicology, 42*(1), 65-83.

Kubow, S. (1992). Routes of formation and toxic consequences of lipid oxidation-products in foods. *Free Radical Biology and Medicine, 12*(1), 63-81.

Kumarathasan, R., Rajkumar, A.B., Hunter, N.R., & Gesser, H.D. (1992). Autoxidation and yellowing of methyl linolenate. *Progress in Lipid Research, 31*(2), 109-126.

Labuza, T.P., & Dugan Jr, L.R. (1971). Kinetics of lipid oxidation in foods. *Critical Reviews in Food Science & Nutrition, 2*(3), 355-405.

Laguerre, M., Lecomte, J., & Villeneuve, P. (2007). Evaluation of the ability of antioxidants to counteract lipid oxidation: Existing methods, new trends and challenges. *Progress in Lipid Research, 46*(5), 244-282.

Le Quéré, V., Plée-Gautier, E., Potin, P., Madec, S., & Salaün, J.-P. (2004). Human CYP4F3s are the main catalysts in the oxidation of fatty acid epoxides. *Journal of Lipid Research, 45*(8), 1446-1458.

Lethuaut, L., Métro, F., & Genot, C. (2002). Effect of droplet size on lipid oxidation rates of oil-in-water emulsions stabilized by protein. *Journal of the American Oil Chemists' Society, 79*(5), 425-430.

Liu, Y., Cheng, Y., Li, J., Wang, Y., & Liu, Y. (2018). Epoxy stearic acid, an oxidative product derived from oleic acid, induces cytotoxicity, oxidative stress and apoptosis in HepG2 cells. *Journal of agricultural and food chemistry*.

Mackie, A.R., Gunning, A.P., Wilde, P.J., & Morris, V.J. (2000). Orogenic displacement of protein from the oil/water interface. *Langmuir, 16*(5), 2242-2247.

Mariod, A., Matthäus, B., & Hussein, I.H. (2011). Effect of stripping methods on the oxidative stability of three unconventional oils. *Journal of the American Oil Chemists' Society, 88*(5), 603-609.

Marmesat, S., Velasco, J., & Dobarganes, M.C. (2008). Quantitative determination of epoxy acids, keto acids and hydroxy acids formed in fats and oils at frying temperatures. *Journal of Chromatography A, 1211*(1–2), 129-134.

Martínez-Yusta, A., Goicoechea, E., & Guillén, M.D. (2014). A review of thermo-oxidative degradation of food lipids studied by ^{1}H NMR spectroscopy: Influence of degradative conditions and food lipid nature. *Comprehensive Reviews in Food Science and Food Safety, 13*(5), 838-859.

McClements, D.J., & Decker, E.A. (2000). Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science, 65*(8), 1270-1282.

Medeiros Vinci, R., Jacxsens, L., Van Loco, J., Matsiko, E., Lachat, C., de Schaetzen, T., Canfyn, M., Van Overmeire, I., Kolsteren, P., & De Meulenaer, B. (2012). Assessment of human exposure to benzene through foods from the Belgian market. *Chemosphere, 88*(8), 1001-1007.

Merzougui, A., Hasseine, A., Kabouche, A., & Korichi, M. (2011). LLE for the extraction of alcohol from aqueous solutions with diethyl ether and dichloromethane at 293.15 k, parameter estimation using a hybrid genetic based approach. *Fluid Phase Equilibria, 309*(2), 161-167.

Mestdagh, F., Kerkaert, B., Cucu, T., & De Meulenaer, B. (2011). Interaction between whey proteins and lipids during light-induced oxidation. *Food Chemistry, 126*(3), 1190-1197.

Min, D.B., Smouse, T.H., & Society, A.O.C. (1985). *Flavor chemistry of fats and oils*: American Oil Chemists' Society.

Mitchell, L.A., Moran, J.H., & Grant, D.F. (2002). Linoleic acid, *cis*-epoxyoctadecenoic acids, and dihydroxyoctadecadienoic acids are toxic to Sf-21 cells in the absence of albumin. *Toxicology Letters, 126*(3), 187-196.

Moghaddam, M.F., Grant, D.F., Cheek, J.M., Greene, J.F., Williamson, K.C., & Hammock, B.D. (1997). Bioactivation of leukotoxins to their toxic diols by epoxide hydrolase. *Nature Medicine, 3*(5), 562-566.

Morales, A., Dobarganes, C.M., Márquez-Ruiz, G., & Velasco, J. (2010). Quantitation of hydroperoxy-, keto- and hydroxy-dienes during oxidation of fames from high-linoleic and high-oleic sunflower oils. *Journal of the American Oil Chemists' Society, 87*(11), 1271-1279.

Morales, A., Marmesat, S., Dobarganes, M.C., Márquez-Ruiz, G., & Velasco, J. (2012). Quantitative analysis of hydroperoxy-, keto- and hydroxy-dienes in refined vegetable oils. *Journal of Chromatography A, 1229*(0), 190-197.

Moran, J.H., Mitchell, L.A., Bradbury, J.A., Qu, W., Zeldin, D.C., Schnellmann, R.G., & Grant, D.F. (2000). Analysis of the cytotoxic properties of linoleic acid metabolites produced by renal and hepatic P450s. *Toxicology and Applied Pharmacology, 168*(3), 268-279.

Moran, J.H., Mon, T., Hendrickson, T.L., Mitchell, L.A., & Grant, D.F. (2001). Defining mechanisms of toxicity for linoleic acid monoepoxides and diols in Sf-21 cells. *Chemical Research in Toxicology, 14*(4), 431-437.

Morris, L.J., Holman, R.T., & Fontell, K. (1961). Naturally occurring epoxy acids: I. Detection and evaluation of epoxy fatty acids by paper, thin-layer, and gas-liquid chromatography. *Journal of Lipid Research, 2*(1), 68-76.

Morris, L.J., & Wharry, D.M. (1965). Chromatographic behaviour of isomeric long-chain aliphatic compounds : I. Thin-layer chromatography of some oxygenated fatty acid derivatives. *Journal of Chromatography A, 20*, 27-37.

Mubiru, E., Shrestha, K., Papastergiadis, A., & De Meulenaer, B. (2013). Improved gas chromatography-flame ionization detector analytical method for the analysis of epoxy fatty acids. *Journal of Chromatography A, 1318*, 217-225.

Mubiru, E., Shrestha, K., Papastergiadis, A., & De Meulenaer, B. (2014). Development and validation of a gas chromatography–flame ionization detection method for the determination of epoxy fatty acids in food matrices. *Journal of Agricultural and Food Chemistry, 62*(13), 2982-2988.

Mungroo, R., Pradhan, N.C., Goud, V.V., & Dalai, A.K. (2008). Epoxidation of canola oil with hydrogen peroxide catalyzed by acidic ion exchange resin. *Journal of the American Oil Chemists' Society, 85*(9), 887-896.

Munro, I.C., Renwick, A.G., & Danielewska-Nikiel, B. (2008). The threshold of toxicological concern (TTC) in risk assessment. *Toxicology Letters, 180*(2), 151-156.

Natsch, A., & Gfeller, H. (2008). LC-MS–based characterization of the peptide reactivity of chemicals to improve the in vitro prediction of the skin sensitization potential. *Toxicological Sciences, 106*(2), 464-478.

Neff, W.E., & Byrdwell, W.C. (1998). Characterization of model triacylglycerol (triolein, trilinolein and trilinolenin) autoxidation products via high-performance liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry. *Journal of Chromatography A, 818*(2), 169-186.

Neff, W.E., Frankel, E.N., Scholfield, C.R., & Weisleder, D. (1978). High press liquid chromatography of autoxidized lipids.1. Methyl oleate and linoleate. *Lipids, 13*(6), 415-421.

Neff, W.E., Frankel, E.N., & Weisleder, D. (1981). High pressure liquid chromatography of autoxidized lipids: II. Hydroperoxy-cyclic peroxides and other secondary products from methyl linolenate. *Lipids, 16*(6), 439-448.

Nilsson, A.-M., Bergström, M.A., Luthman, K., Nilsson, J.L.G., & Karlberg, A.-T. (2005). A conjugated diene identified as a prohapten: Contact allergenic activity and chemical reactivity of proposed epoxide metabolites. *Chemical Research in Toxicology, 18*(2), 308-316.

Nuchi, C.D., Hernandez, P., McClements, D.J., & Decker, E.A. (2002). Ability of lipid hydroperoxides to partition into surfactant micelles and alter lipid oxidation rates in emulsions. *Journal of Agricultural and Food Chemistry, 50*(19), 5445-5449.

O'Brien, J., Renwick, A.G., Constable, A., Dybing, E., Müller, D.J.G., Schlatter, J., Slob, W., Tueting, W., Van Benthem, J., & Williams, G.M. (2006). Approaches to the risk assessment of genotoxic carcinogens in food: A critical appraisal. *Food and Chemical Toxicology, 44*(10), 1613-1635.

Obando, M., Papastergiadis, A., Li, S., & De Meulenaer, B. (2015). Impact of lipid and protein co-oxidation on digestibility of dairy proteins in oil-in-water (o/w) emulsions. *Journal of Agricultural and Food Chemistry, 63*(44), 9820-9830.

Obando, M., Soto, E., & De Meulenaer, B. (2018). Influence of oxidized oils on digestibility of caseins in o/w emulsions. *European Journal of Lipid Science and Technology*, 1700331.

Orellana-Coca, C., Adlercreutz, D., Andersson, M.M., Mattiasson, B., & Hatti-Kaul, R. (2005). Analysis of fatty acid epoxidation by high performance liquid chromatography coupled with evaporative light scattering detection and mass spectrometry. *Chemistry and Physics of Lipids, 135*(2), 189-199.

Osborn, H.T., & Akoh, C.C. (2004). Effect of emulsifier type, droplet size, and oil concentration on lipid oxidation in structured lipid-based oil-in-water emulsions. *Food Chemistry, 84*(3), 451-456.

Papastergiadis, A., Fatouh, A., Jacxsens, L., Lachat, C., Shrestha, K., Daelman, J., Kolsteren, P., Langenhove, H., & De Meulenaer, B. (2014). Exposure assessment of malondialdehyde, 4-hydroxy-2-(E)-nonenal and 4-hydroxy-2-(E)-hexenal through specific foods available in belgium. *Food and Chemical Toxicology, 73*(2014), 51-58.

Papastergiadis, A., Mubiru, E., Van Langenhove, H., & Meulenaer, B. (2013). Development of a sensitive and accurate stable isotope dilution assay for the simultaneous determination of free 4-hydroxy-2-(E)-nonenal and 4-hydroxy-2-(E)-hexenal in various food matrices by gas chromatography–mass spectrometry. *Food Analytical Methods, 7*(4), 836-843.

Pauli, G.F., Godecke, T., Jaki, B.U., & Lankin, D.C. (2012). Quantitative ^{1}H NMR. Development and potential of an analytical method: An update. *Journal of natural products, 75*(4), 834-851.

Pedersen, G.A., Jensen, L.K., Fankhauser, A., Biedermann, S., Petersen, J.H., & Fabech, B. (2008). Migration of epoxidized soybean oil (ESBO) and phthalates from twist closures into food and enforcement of the overall migration limit. *Food Additives & Contaminants: Part A, 25*(4), 503-510.

Perdue, R.E., Carlson, K.D., & Gilbert, M.G. (1986). *Vernonia-galamensis*, potential new crop source of epoxy acid. *Economic Botany, 40*(1), 54-68.

Piazza, G.J., Nunez, A., & Foglia, T.A. (2003a). Epoxidation of fatty acids, fatty methyl esters, and alkenes by immobilized oat seed peroxygenase. *Journal of Molecular Catalysis B: Enzymatic, 21*(3), 143-151.

Piazza, G.J., Nunez, A., & Foglia, T.A. (2003b). Isolation of unsaturated diols after oxidation of conjugated linoleic acid with peroxygenase. *Lipids, 38*(3), 255-261.

Picot, C., & Roudot, A.C. (2012). A practical example of risk assessment: Risk assessment to phycotoxins in a recreational shellfish harvester's subpopulation. In Novel approaches and their applications in risk assessment. InTech.

Pokorny, J., Kalinova, L., & Dysseler, P. (1995). Determination of chlorophyll pigments in crude vegetable oils: Results of a collaborative study and the standardized method (Technical report). *Pure and Applied Chemistry, 67*(10), 1781-1787.

Poli, E., Clacens, J.M., Barrault, J., & Pouilloux, Y. (2009). Solvent-free selective epoxidation of fatty esters over a tungsten-based catalyst. *Catalysis Today, 140*(1–2), 19-22.

Psomiadou, E., & Tsimidou, M. (2002). Stability of virgin olive oil. 2. Photooxidation studies. *Journal of Agricultural and Food Chemistry, 50*(4), 722-727.

Ricard, B., Alicia, M., Jordi, R., & Rius, F.X. (2002). Validation of analytical methods. *Grasas y Aceites, 53*(1), 128-143.

Roberts, D.W., Aptula, A., & Api, A.M. (2017). Structure–potency relationships for epoxides in allergic contact dermatitis. *Chemical Research in Toxicology, 30*(2), 524-531.

Ruxton, C.H.S., Calder, P.C., Reed, S.C., & Simpson, M.J.A. (2005). The impact of long-chain n-3 polyunsaturated fatty acids on human health. *Nutrition Research Reviews, 18*(01), 113-129.

Saurabh, T., Patnaik, M., Bhagt, S.L., & Renge, V.C. (2011). Epoxidation of vegetable oils: A review. *International Journal of Advanced Engineering Technology, 2*(4), 491-501.

Schaich, K.M. (2005). Lipid oxidation: Theoretical aspects. In F.Shahidi (Ed.), *Bailey's industrial oil and fat products* (Sixth ed., pp. 269-355). New Jersey, USA: John Wiley & Sons.

Schaich, K.M. (2008). Co-oxidations of oxidizing lipids: Reactions with proteins. *Lipid Oxidation Pathways, 2*, 183-274.

Schaich, K.M. (2012). Thinking outside the classical chain reaction box of lipid oxidation. *Lipid Technology, 24*(3), 55-58.

Schaich, K.M. (2013). Challenges in elucidating lipid oxidation mechanisms: When, where, and how do products arise? In Amy L., Uwe N. & Xiangqing (Shawn) (Eds.), *Lipid oxidation: Challenges in food systems* (pp. 1-52). Urbana, Illinois, USA: AOCS, Press.

Schelbert, S., Aubry, S., Burla, B., Agne, B., Kessler, F., Krupinska, K., & Hörtensteiner, S. (2009). Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in arabidopsis. *The Plant Cell, 21*(3), 767-785.

Severiano, A., Hagström, A., Hatti-Kaul, R., & Da Fonseca, M.M.R. (2008). Chemoenzymatic epoxidation of rapeseed methyl esters: Parameters influencing the reaction and enzyme stability.

Shahidi, F. (2005). *Bailey's industrial oil and fat products, 6 volume set*: Wiley.

Shahidi, F., & Spurvey, S.A. (1996). Oxidative stability of fresh and heat-processed dark and light muscles of mackerel (Scomber scombrus). *Journal of Food Lipids, 3*(1), 13-25.

Shahidi, F., & Zhong, Y. (2010). Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews, 39*(11), 4067-4079.

Shrestha, K., & De Meulenaer, B. (2014). Antioxidant activity of Maillard type reaction products between phosphatidylethanolamine and glucose. *Food Chemistry, 161*(0), 8-15.

Shrestha, K., Gemechu, F.G., & De Meulenaer, B. (2013). A novel insight on the high oxidative stability of roasted mustard seed oil in relation to phospholipid, Maillard type reaction products, tocopherol and canolol contents. *Food Research International, 54*(1), 587-594.

Smith, C.R. (1980). Unusual seed oils and their fatty acids. *Journal of the American Oil Chemists Society, 57*(2), A175-A175.

Smith Jr, C.R., Bagby, M.O., Lohmar, R.L., Glass, C.A., & Wolff, I.A. (1960). The epoxy acids of *Chrysanthemum coronarium* and *Clarkia elegans* seed oils. *The Journal of Organic Chemistry, 25*(2), 218-222.

Spitzer, V. (1999). Screening analysis of unknown seed oils. *Lipid / Fett, 101*(1), 2-19.

Steele, R. (2004). *Understanding and measuring the shelf-life of food*: Cambridge, UK: Woodhead Publishing.

Suter, B., Grob, K., & Pacciarelli, B. (1997). Determination of fat content and fatty acid composition through 1-min transesterification in the food sample; principles. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A, 204*(4), 252-258.

Swern, D. (1955). Oxygenated fatty acids. *Progress in the Chemistry of Fats and other Lipids, 3*, 213-239.

Synek, V. (2008). Evaluation of the standard deviation from duplicate results. *Accreditation and Quality Assurance, 13*(6), 335-337.

Tallent, W.H., Cope, D.G., Hagemann, J.W., Earle, F.R., & Wolff, I.A. (1966). Identification and distribution of epoxyacyl groups in new, natural epoxy oils. *Lipids, 1*(5), 335-340.

Tian, F., Decker, E.A., & Goddard, J.M. (2013). Controlling lipid oxidation of food by active packaging technologies. *Food & Function, 4*(5), 669-680.

Usuki, R., Endo, Y., & Kaneda, T. (1984). Prooxidant activities of chlorophylls and pheophytins on the photooxidation of edible oils. *Agricultural and Biological Chemistry, 48*(4), 991-994.

Van Loco, J., Elskens, M., Croux, C., & Beernaert, H. (2002). Linearity of calibration curves: Use and misuse of the correlation coefficient. *Accreditation and Quality Assurance, 7*(7), 281-285.

Vandemoortele, A., Babat, P., Yakubu, M., & De Meulenaer, B. (2017). Reactivity of free malondialdehyde during in vitro simulated gastrointestinal digestion. *Journal of Agricultural and Food Chemistry, 65*(10), 2198-2204.

Vandemoortele, A., & De Meulenaer, B. (2015). Behavior of malondialdehyde in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 63*(23), 5694-5701.

Vandevijvere, S., De Vriese, S., Huybrechts, I., Moreau, M., Temme, E., De Henauw, S., De Backer, G., Kornitzer, M., Leveque, A., & Van Oyen, H. (2009). The gap between food-based dietary guidelines and usual food consumption in Belgium, 2004. *Public Health Nutrition, 12*(03), 423-431.

Velasco, J., Berdeaux, O., Márquez-Ruiz, G., & Dobarganes, M.C. (2002). Sensitive and accurate quantitation of monoepoxy fatty acids in thermoxidized oils by gas–liquid chromatography. *Journal of Chromatography A, 982*(1), 145-152.

Velasco, J., & Dobarganes, M.C. (2002). Oxidative stability of virgin olive oil. *European Journal of Lipid Science and Technology, 104*(9-10), 661-676.

Velasco, J., Marmesat, S., Bordeaux, O., Márquez-Ruiz, G., & Dobarganes, C.M. (2004). Formation and evolution of monoepoxy fatty acids in thermoxidized olive and sunflower oils and quantitation in used frying oils from restaurants and fried-food outlets. *Journal of Agricultural and Food Chemistry, 52*(14), 4438-4443.

Velíšek, J., & Cejpek, K. (2006). Biosynthesis of food constituents: Lipids. 1. Fatty acids and derived compounds–a review. *Czech Journal of Food Sciences, 24*, 193-216.

Viljanen, K., Kivikari, R., & Heinonen, M. (2004). Protein-lipid interactions during liposome oxidation with added anthocyanin and other phenolic compounds. *Journal of Agricultural and Food Chemistry, 52*(5), 1104-1111.

Vose, D. (2008). *Risk analysis: A quantitative guide*. West Sussex, England: John Wiley & Sons.

Vromman, V., Waegeneers, N., Cornelis, C., De Boosere, I., Van Holderbeke, M., Vinkx, C., Smolders, E., Huyghebaert, A., & Pussemier, L. (2010). Dietary cadmium intake by the Belgian adult population. *Food additives & Contaminants: Part A, 27*(12), 1665-1673.

Waraho, T. (2011). *Effects of free fatty acids, mono and diacylglycerols on oxidative stability of soybean oil-in-water emulsions.* (Doctoral dissertation), University of Massachusetts Amherst.

Waraho, T., Cardenia, V., Rodriguez-Estrada, M.T., McClements, D.J., & Decker, E.A. (2009). Prooxidant mechanisms of free fatty acids in stripped soybean oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 57*(15), 7112-7117.

Waraho, T., McClements, D.J., & Decker, E.A. (2011). Mechanisms of lipid oxidation in food dispersions. *Trends in Food Science & Technology, 22*(1), 3-13.

WHO/FAO. (1985). Guidelines for the study of dietary intakes of chemical contaminants. *World Health Organization. Offset publication No. 87. Geneva*.

WHO/FAO. (2009). Principles and methods for the risk assessment of chemicals in food: Dietary exposure assessment of chemicals in food. *World Health Organization. Geneva*.

WHO/IPCS. (2004). *Risk assessment terminology: Harmonization of approaches to the assessment of risk from exposure to chemicals*: International Programme on Chemical Safety. Geneva.

Wilde, P.J., & Clark, D.C. (1993). The competitive displacement of β-lactoglobulin by Tween 20 from oil-water and air-water interfaces. *Journal of Colloid and Interface Science, 155*(1), 48-54.

Williams, P.L., James, R.C., & Roberts, S.M. (2000). *Principles of toxicology: Environmental and industrial applications* (Second ed.). New York: John Wiley & Sons.

Wilson, I.D., & Brinkman, U.A.T. (2003). Hyphenation and hypernation: The practice and prospects of multiple hyphenation. *Journal of Chromatography A, 1000*(1–2), 325-356.

Wilson, R., Fernie, C.E., Scrimgeour, C.M., Lyall, K., Smyth, L., & Riemersma, R.A. (2002). Dietary epoxy fatty acids are absorbed in healthy women. *European Journal of Clinical Investigation, 32*(2), 79-83.

Xia, W. (2017). *Determination of epoxides and alcohols in edible oils* (Doctoral dissertation), Dalhousie University.

Xia, W., Budge, S.M., & Lumsden, M.D. (2015). New ^{1}H NMR-based technique to determine epoxide concentrations in oxidized oil. *Journal of agricultural and food chemistry, 63*(24), 5780-5786.

Xia, W., Budge, S.M., & Lumsden, M.D. (2016). ^{1}H-NMR characterization of epoxides derived from polyunsaturated fatty acids. *Journal of the American Oil Chemists' Society, 93*(4), 467-478.

Yam, K.L. (2009). *The wiley encyclopedia of packing technology*. New Brunswick, New Jersey, USA: John Wiley & Sons.

Zamora, R., & Hidalgo, F.J. (2005). Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Critical Reviews in Food Science and Nutrition, 45*(1), 49-59.

Zheng, J., Plopper, C.G., Lakritz, J., Storms, D.H., & Hammock, B.D. (2001). Leukotoxin-diol - a putative toxic mediator involved in acute respiratory distress syndrome. *American Journal of Respiratory Cell and Molecular Biology, 25*(4), 434-438.

CURRICULUM VITAE
Edward Mubiru

edmubiru@cns.mak.ac.ug; Mubirued@gmail.com

Edward Mubiru was born on 7th February, 1969 in Nkozi, Uganda. In 1993, he graduated with a Diploma in Chemistry at Kyambogo University, Uganda. In November, 2000 he graduated with a Bachelor's degree in Food Science and Technology at Makerere University, Uganda. His thesis entitled "Effect of processing, storage methods and time on the development of Aflatoxins in groundnuts was a result of original research, and the results were defended at the University. After working with the Makerere University at the department of chemistry, he joined "Department of Food Science and Technology Makerere University for a Masters of Food Science and Technology and graduated in 2008. His master thesis entitled "Pesticide use and residues in cabbages and tomatoes: A case of Kiboga, Mbarara and Mukono districts" was carried out in Uganda was successfully defended.

Since October 2010, he has been working on his PhD project entitled "Production of lipid oxidation products in foods: Formation and analysis of oxygenated polar fatty acids with emphasis on epoxy fatty acids" with support from Geconcerteerde Onderzoeksacties / Concerted research actions (GOA) of Ghent University. This research has been carried out in the NutriFOODchem laboratory, Department of Food technology, Safety and Health (Faculty of Bioscience Engineering, Ghent University) under the guidance of Prof. dr. ir. Bruno De Meulenaer. The findings of this research project were published in peer-reviewed scientific journals and presented during national and international meetings. During this period, he also supervised three students for the fulfillment of their Master thesis**.**

Publications of MSc Edward Mubiru

Published in A1 Journals:

Papastergiadis, A., Mubiru, E., Van Langenhove, H., & De Meulenaer, B. (2012). Malondialdehyde measurement in oxidized foods: Evaluation of the spectrophotometric thiobarbituric acid reactive substances (TBARS) test in various foods. *Journal of Agricultural and Food Chemistry, 60*(38), 9589-9594.

Papastergiadis, Antonios, Mubiru, E., Van Langenhove, H., & Meulenaer, B. (2013). Development of a sensitive and accurate stable isotope dilution assay for the simultaneous determination of free 4-hydroxy-2-(E)-nonenal and 4-hydroxy-2-(E)-

hexenal in various food matrices by gas chromatography–mass spectrometry. *Food Analytical Methods, 7*(4), 836-843.

Mubiru, E., Shrestha, K., Papastergiadis, A., & De Meulenaer, B. (2013). Improved gas chromatography-flame ionization detector analytical method for the analysis of epoxy fatty acids. *Journal of Chromatography A, 1318,* 217-225.

Mubiru, E., Shrestha, K., Papastergiadis, A., & De Meulenaer, B. (2014). Development and validation of a gas chromatography–flame ionization detection method for the determination of epoxy fatty acids in food matrices. *Journal of Agricultural and Food Chemistry, 62*(13), 2982-2988.

Mubiru, E., Liesbeth Jacxsens, Antonios Papastergiadis, Carl Lachat, Kshitij Shrestha, N. H. M. Rubel Mozumder & Bruno De Meulenaer (2017): Exposure assessment of epoxy fatty acids through consumption of specific foods available in Belgium, Food Additives & Contaminants: Part A, 34:6, 1000-1011, DOI: 10.1080/19440049.2017.1310399

Oral Presentations:

Mubiru, E., Shrestha, K., Papastergiadis, A., & De Meulenaer, B. (2013). "Improved gas chromatography-flame ionization detector analytical method for the analysis of epoxy fatty acids. Abstract of Scientific Papers of the 105th AOCS Annual Meeting & Expo, San Antonio, Texas, USA.

Mubiru, E., Shrestha, K., Papastergiadis, A., & De Meulenaer, B. (2014). "Development and Validation of a Gas Chromatography–Flame Ionization Detection Method for the Determination of epoxy fatty acids in Food Matrices. Abstract of Scientific Papers of the 105th AOCS Annual Meeting, San Antonio, Texas, USA.

Flash Poster Presentation:

Gas chromatographic analytical method for the analysis of oxygenated polar fatty acids. Mubiru, E., Shrestha, K., Papastergiadis, A., & De Meulenaer, B. (2014). 13th International symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-13) and 3rd International symposium on Hyphenated Techniques for Sample Preparation (HTSP-3), Book of abstracts, Brugges, Belgium.

Participation at International Meetings:

January 29th-31st, 2014: International Symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-13) and 3rdInternational Symposium on Hyphenated Techniques for Sample Preparation (HTSP-3, Book of abstracts, Brugges, Belgium. May 4 - 7th, 2014: 105th AOCS Annual Meeting & Expo, San Antonio, Texas, USA.

Supervised dissertations as a tutor

1. Buyle, Z (2014). Formation of epoxy fatty acids during autoxidation of oils at elevated temperature. Master dissertation, Ghent University
2. Ciko, T (2014). Evolution of epoxy fatty acids during photo and autoxidation of vegetable oils. Master dissertation, Ghent University.
3. Rubel Mozumder, N.H.M. (2014). Quantitative determination of epoxy fatty acid s in oils and foods by gas-liquid chromatography. Master dissertation, Ghent University.

Professional Memberships

American Oil Chemists Society