Analysis of lipid oxidation during digestion by liquid chromatography–mass spectrometric and nuclear magnetic resonance spectroscopic techniques

MARKO TARVAINEN

Department of Biochemistry and Food Chemistry
University of Turku
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From the Department of Biochemistry and Food Chemistry
University of Turku, Turku Finland

Supervised by

Professor Heikki Kallio, Ph.D.
Department of Biochemistry and Food Chemistry
University of Turku
Turku Finland

Professor Arnis Kuksis, Ph.D.
Banting and Best Department of Medical Research
University of Toronto
Toronto Canada

Jukka-Pekka Suomela, Ph.D.
Department of Biochemistry and Food Chemistry
University of Turku
Turku Finland

Reviewed by

Professor Carmen M. Dobarganes, Ph.D.
Consejo Superior de Investigaciones Científicas
Instituto de la Grasa
Sevilla Spain

Professor Paavo Kalo, Ph.D.
Department of Food and Environmental Sciences
University of Helsinki
Helsinki Finland

Opponent

Professor Joseph J. Kanner, Ph.D.
Department of Food Science
Israel Agricultural Research Organization (ARO), Volcani Center
Beit Dagan, Israel

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In memory of my father

CONTENTS

ABSTRACT

Lipid autoxidation is an unwanted process that affects the quality of food and has impact on human health. Lipid oxidation has been studied extensively, but oxidation during digestion has largely been ignored. Formation of oxidized lipids increases rapidly when protective antioxidants are exhausted. On the other hand, the nature of antioxidants can lead to problems when fortifying foods with too much antioxidants. Pro-oxidative effects of several antioxidants have been observed when used in excessive amounts.

Methods for studying lipid oxidation are numerous. Among them are unspecific titrimetric methods and highly specialized chromatographic and mass spectrometric methods. Nuclear magnetic resonance (NMR) spectroscopy, especially the proton (^{1}H) NMR, is a promising technique for fast screening of lipid samples as it is non-destructive and because of the large dynamic scale of the technique. Drawbacks of NMR are that relatively large amount of sample is required for the analysis and that specific molecular structures may be difficult to identify from complex spectrum. This thesis focuses on the study of in vitro lipid oxidation by different chromatographic, mass spectrometric and nuclear magnetic resonance spectroscopic methods.

The most significant findings of the studies in this thesis centre around oxidation, hydrolysis, and behaviour of lipids in an artificial digestion model used in the studies. The model simulates the digestion processes of human and can be used to study lipid oxidation in vitro. Also of importance, are the lipid analysis techniques developed for the experiments, as the techniques can be adopted to other fields of scientific studies as well for industrial uses.

Four major studies were conducted in this thesis: first an in vitro digestion model was adopted to study the behaviour of differently oxidized rapeseed oils. Simultaneously, a novel HPLC–evaporative light scattering detector–MS analysis technique was developed, which enabled the analysis of native and oxidized free fatty acids, monoacylglycerols, diacylglycerols, and triacylglycerols in the chyme produced by the digestion model. The main findings of the study were that thermally oxidized rapeseed oil, chemically oxidized rapeseed oil and unoxidized rapeseed oil were hydrolyzed in a similar manner. No hydroperoxides were detected in the digested samples, even though they were present in the undigested oils. Also, the finding of

large amounts of *sn*-1(3) monoacylglycerols was surprising, questioning the long believed mechanism of triacylglycerol digestion and absorption.

In the second study, an ultra-high performance liquid chromatography (UHPLC) analysis technique was developed to replace the previous HPLC method. Analysis time was reduced by a factor of 5.5 without the loss of chromatographic resolution or detection sensitivity. Over 150 compounds were detected from digested and undigested oxidized rapeseed oils with the method. Most significant finding was that toxic core aldehydes present in the undigested oxidized oils were not detected in the extracted chyme. This implies that the aldehydic functions were either lost during the hydrolysis of lipids or that the compounds formed various complexes with other components of the chyme and were not detectable by the analysis technique used.

In the third study, a series of antioxidants were assessed for the effects in the artificial digestion model. An improved UHPLC–ESI–MS analysis method was developed, which used lithium salt to greatly enhance the ionization and therefore the detection limits of the low level analytes in electrospray ionization–mass spectrometry. The main findings were that native (unoxidized) rapeseed oil can be oxidized during the digestion processes and that none of the used antioxidants could completely prevent this oxidation. L-ascorbic acid, 6-palmitoyl-*O*-L-ascorbic acid, 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), DL-α-tocopherol, and DL-α-tocopheryl acetate had different kinds of effects against this oxidation, as measured by the concentration of oxidized lipids in the samples.

The findings of our second study were supported by the fourth study in where ^{1}H NMR spectroscopy was used along UHPLC–ESI–MS analyses to study the behaviour of core aldehyde-rich oils in the artificial digestion model. Again, no compounds with aldehydic functions were detected by UHPLC–ESI–MS analyses of the digested oils even when high amounts of core aldehydes were present in the original oil. However, ^{1}H NMR analyses of several samples revealed that there were some remaining carbonyl functions in the digested samples. The combined results of these analyses techniques strongly hinted that Schiff bases and Michael addition products were formed in the digestion mixture. Overall, the scientific studies conducted in this thesis have increased the knowledge of lipid oxidation and especially provided more detailed information on possible oxidation during lipid digestion. The findings merit for more research in the field.

LIST OF ABBREVIATIONS

AA, arachidonic acid
ABTS, 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid
ACN, acyl carbon number
ALDH, aldehyde dehydrogenase
AnV, p-anisidine value
APCI, atmospheric pressure chemical ionization
BBI, inverse broadband
BHA, butylated hydroxyaniline
BHT, butylated hydroxytoluene
CAD, collision assisted dissosiation
CD, conjugated diene
CE, cholesterol ester
CLA, conjugated linoleic acid
COSY, correlation spectroscopy
COX, cyclooxygenase
CT, conjugated triene
DAD, diode array detector
DBN, double bond number
DGLA, dihomogammalinoleic acid
DHA, docosahexaenoic acid
DNA, deoxyribonucleic acid
DNPH, 2,4-dinitrophenylhydrazine
DPPH, 2,2-diphenyl-1-picrylhydrazyl
EDE, 4,5-epoxy-2-decenal
EET, epoxy-eicosatetraenoic acid
ELSD, evaporative light scattering detector
EPA, eicosapentaenoic acid
EpHODE, epoxyoctadecenoate
ESI, electrospray ionization
ESR, electron spin resonance
ET, electron transfer
FA, fatty acid
FAB, fast atom bombardment
FAME, fatty acid methyl ester
FFA, free fatty acid
FID, flame ionization detector
FOX, ferrous-oxide xylenol orange
FRAP, ferric reducing antioxidant power
FTIR, Fourier transform infrared
GC, gas chromatography
gHSQC, gradient heteronuclear single quantum coherence
gHMBC, gradient heteronuclear multiple bond correlation
GPC, glycerophosphocholine
GPx, glutathione peroxidase
GSH, glutathione
GSSG, glutathione disulfide
HAT, hydrogen atom transfer
H_2O_2, hydrogen peroxide
HETE, hydroxy-eicosatetraenoic acid
HILIC, hydrophilic interaction chromatography
HMP, 2-hydrazino-1-methylpyridine
HMQC, heteronuclear multiple quantum coherence
HNE 4-hydroxy-*trans*-2-nonenal
HpHODE, hydroperoxyoctadecenoate
HO_2·, hydroperoxyl radical
HODE, hydroxy-octadecadienoic acid
HOOA-PC , hydroxy-oxo-octenoyl-phosphocholine
HPLC, high performance liquid chromatography
IL-6, interleucine-6
IU, international unit
KODE, keto-octadecadienoic acid
L, lipid
·L, lipid radical
LDL, low-density lipoprotein
LOO·, lipid hydroperoxy radical
LOOH, hydroperoxidized lipid
LOX, lipoxygenase
MAG, monoacylglycerol
MALDI, matrix assisted laser desorption ionization
MDA malondialdehyde
MRM, multiple reaction monitoring
MS, mass spectrometry
MS/MS, tandem mass spectrometry (quadrupole equipment)
MS^2, tandem mass spectrometry (ion trap or similar equipment)
Me, methyl
NADPH, nicotinamide adenine dinucleotide phosphate
NMR, proton nuclear magnetic resonance
·NO, nitric oxide
NP, normal phase
O_2, molecular oxygen
O_3, ozone
·O_2-, superoxide radical
·OH, hyroxyl radical
$^1O_2$, singlet oxygen
$^3O_2$, triplet oxygen
ODS, octadecylsilyl
ONE, 4-oxo-2-nonenal
9-ONC, 9-oxononanoyl cholesteryl ester core aldehyde
ORAC, oxygen radical absorbance capacity
oxoHODE, oxo-octadecenoate
oxoODE, oxo-octadecadienoic acid
5-OVC, 5-oxovaleroyl cholesteryl ester core aldehyde
PC, phosphatidyl choline
PDA, photodiode array
PE, phosphoethanolamine
PID, photo ionization detector
PIOX, pathogen-induced oxygenase
PV, peroxide value
RCT, relayed coherence transfer
RI, refractive index
ROS, reactive oxygen species
RP, reversed phase
sn, stereospecific numbering
SOD, superoxide dismutase
SPE, solid phase extraction
SPME, solid phase microextraction
SRM, selected reaction monitoring
TAG, triaclyglycerol
TBARS, thiobarbituric acid reactive substances
TEAC, trolox equivalent antioxidant capacity
THF, tetrahydrofurane
TLC, thin layer chromatography
TMS, trimethylsilyl (or tetramethysilyl)
TOCSY, total correlation spectroscopy
TOTOX, total oxidation
TPP, triphenylphosphine
TRAP, total peroxyl radical trapping potential
UHPLC, ultra-high performance liquid chromatography
UFA, unsaturated fatty acid
UV, ultra violet
VLDL, very-low-density lipoprotein

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following four original publications referred in the text by roman numerals (**I**–**IV**).

(**I**) Tarvainen M., Suomela J.-P., Kuksis A., and Kallio H. (2010) Liquid chromatography–light scattering detector–mass spectrometric analysis of digested oxidized rapeseed oil. *Lipids*. **45**: 1061–1079.

(**II**) Tarvainen M., Suomela J.-P., and Kallio H. (2011) Ultra high performance liquid chromatography–mass spectrometric analysis of oxidized free fatty acids and acylglycerols. *European Journal of Lipid Science and Technology*. **113**: 409–422.

(**III**) Tarvainen M., Phuphusit A., Suomela J.-P., Kuksis A., and Kallio H. (2012) The effects of antioxidants on rapeseed oil oxidation in an artificial digestion model analyzed by UHPLC–ESI–MS. *Journal of Agricultural and Food Chemistry*. **60**: 3564–3579.

(**IV**) Tarvainen M., Jokioinen H., Suomela J.-P., Sinkkonen J., Kuksis A., and Kallio H. Core aldehydes in an artificial digestion model analyzed by UHPLC–ESI–MS and ^{1}H NMR. *Manuscript.*

1 INTRODUCTION

Unwanted lipid oxidation is a major concern for human health. Lipid oxidation is an almost inevitable process taking place during food manufacturing, storage and even consumption. The food industry has many ways to lower the oxidation of lipids e.g. by the use of antioxidants, modified atmosphere, low temperature storage by freezing etc. Consumers, however, have little ability to affect the oxidation of lipids other than using proper food preparation techniques and by using only fresh ingredients. It is particularly important to avoid unnecessarily high temperatures while cooking and to minimise the time that high fat ingredients are held in elevated temperatures.

The consumption of unsaturated fats and oils is increasing as the current healthy eating guidelines emphasise the avoidance of saturated fats for the prevention of coronary and heart diseases. The Finnish nutritional guideline published in 2005 states that fats and oils should constitute approximately 25−35% of the daily energy intake (E%). More specifically, saturated fats should provide 10 E%, monounsaturated fats 10−15 E% and polyunsaturated fats 5−10 E%. While it is certainly true that unsaturated fatty acids have important and essential functions in human metabolism, it is also true that saturated fatty acids also do. Palmitic acid is an essential component of lung epithelial phosphatidylcholines (Balint et al. 1980, Burnell et al. 1978), and heart muscle cells preferentially utilise stearic acid for β-oxidation (Lawson et al. 1979). The one good attribute of saturated fats, which is largely overlooked, is that they are much less likely to oxidise than unsaturated fats and oils and thus are more suited to high temperature food preparation.

All food must be eaten if its constituents are to be used in metabolism of the human body. The digestion processes of major macronutrients involve a series of hydrolysis reactions taking place in the mouth, stomach and intestines. Parietal cells in the stomach wall lining secrete hydrochloric acid to begin the hydrolysis of proteins, and abundant amount of oxygen is present in the stomach as saliva is ingested constantly (Soll et al. 1979, Dunn et al. 1923, Catalán et al. 2009). This makes the stomach a very active site of oxidative reactions, and, indeed, it has been shown that the stomach acts as a bioreactor (Kanner et al. 2001). The efficacy of several antioxidants against this oxidation has been studied before, but the methods used have been general in nature (Kanner et al. 2001, Lapidot et al. 2005a, Lapidot et al. 2005b). The behaviour of oxidised lipids in the stomach and intestines is still largely unknown, although it has been shown that the primary oxidation products, hydroperoxides, are decomposed to aldehydes and alcohols in the stomach before transfer to the small intestine (Kanazawa et al. 2008a, Kanazawa et al. 2008b).

This thesis focuses on lipid oxidation, lipid digestion and analysis of lipid oxidation. A brief review of the literature on lipid oxidation and digestion, the mammalian antioxidative defence system and methods of analysis precedes a summary of the four major studies that were conducted on the topic.

2 REVIEW OF THE LITERATURE

2.1 Fatty acids and triacylglycerols

The human diet is composed of three major macronutrients: carbohydrates, fats, and proteins. Fats contain the highest amount of energy per weight and are mainly composed of triacylglycerols. There are three fatty acids (FAs) esterified to a glycerol backbone in a triacylglycerol (TAG) molecule (see **Figure 1**). TAGs are part of larger group of compounds called lipids. Major lipids in animal adipose tissues are triacylglycerols (90−95%), different phospholipids, free and esterified cholesterol, and, as minor components, lipophilic antioxidants. These compounds serve many functions in the body; for example, fatty acids can be used as fuel in energy metabolism (Large et al. 2004) or as local hormones after transformation to eicosanoids, which can even modulate adipose tissue inflammation and oxidative stress (Fan et al. 2012). Cholesterol and phospholipid species are essential components of the cell wall lipid bilayer structures (Nagle et al. 2000) and different antioxidants protect the unsaturated fatty acids from oxidation (Gutteridge 1995). In addition, it has recently been discovered that eicosanoids can be esterified into membrane phospholipids providing short-term storage (Hammond et al. 2012).

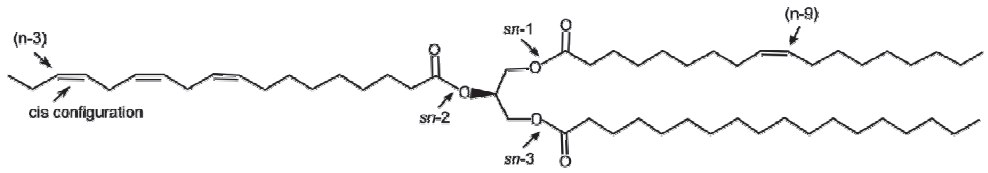


Figure 1. Example of a triacylglycerol molecule (1-oleoyl-2-α-linolenoyl-3-stearoyl-*sn*-glycerol). The triacylglycerol contains oleic acid (18:1 n−9) in the *sn*-1 position, α-linolenic acid (18:3 n−3) in the *sn*-2 position and stearic acid (18:0) in the *sn*-3 position.

Fats can be divided into several groups according to the physical behaviour of the fat. Fat is called oil if it is liquid at room temperature. Natural fats and oils are very rarely, if ever, pure compounds; instead, they are mixtures of different length fatty acids with different amounts of double bonds, and bound in different positions in the glycerol backbone (see **Figure 1**). This gives numerous possible structures for TAGs composed of some twenty common fatty acids (see **Table 1**). Sixteen and eighteen carbon fatty acids are the most abundant in nature, but many other chain lengths (even

and odd numbered) exist as branched and cyclical fatty acids (Shorland et al. 1955, Kaneda 1977). Commonly, FAs exist without functional groups other than the carboxyl group; however, some FAs do contain, for example, hydroxyl, ketone, and epoxide groups (Prostenik et al. 1978, O'Leary 1962, Stahl et al. 1996, Brechany et al. 1994). Unsaturation is common in all animal, vegetable and algae fatty acids, although vegetable and algae FAs tend to be more highly unsaturated. Monounsaturated fatty acids are a major component of most mammalian milk and fatty tissue triacylglycerols (Månsson 2008, Yahas et al. 2008, Rohman et al. 2012, Codex Standard for named vegetable oils 2011).

Table 1. Common fatty acids

ACN:DBN[1]	**Trivial name (abbreviation)**	**IUPAC name**
6:0	caproic acid	hexanoic acid
8:0	caprylic acid	octanoic acid
10:0	capric acid	decanoic acid
12:0	lauric acid	dodecanoic acid
14:0	myristic acid	tetradecanoic acid
16:0	palmitic acid (P)	hexadecanoic acid
16:1 (n−7)	palmitoleic acid (Po)	(Z)-hexadec-7-enoic acid
18:0	stearic acid (S)	octadecanoic acid
18:1 (n−9)	oleic acid (O)	(Z)-octadec-9-enoic acid
18:2 (n−6)	linoleic acid (L)	(Z,Z)-octadeca-9,12-dienoic acid
18:3 (n−3)	alpha-linolenic acid (Ala)	(Z,Z,Z)-octadeca-9,12,15-trienoic acid
18:3 (n−6)	gamma-linolenic acid (Gla)	(Z,Z,Z)-octadeca-6,9,12-trienoic acid
20:0	arachidic acid (A)	eicosanoic acid
20:1 (n−9)	gondoic acid	(Z)-eicosa-12-enoic acid
20:3 (n-6)	dihomogammalinoleic acid, DGLA	(Z,Z,Z)-eicosa-8,11,14-trienoic acid
20:4 (n−6)	arachidonic acid (Aa)	(Z,Z,Z,Z)-eicosa-5,8,11,14-tetraenoic acid
20:5 (n−3)	timnodonic acid (EPA)	(Z,Z,Z,Z,Z)-eicosa-5,8,11,14,17-pentaenoic acid
22:0	behenic acid	docosanoic acid
22:1 (n−9)	erucic acid	(Z)-docosa-9-enoic acid
22:6 (n−3)	cervonic acid (DHA)	(Z,Z,Z,Z,Z,Z)-docosa-4,7,10,13,15,18-hexaenoic acid
24:1 (n−9)	nervonic acid	(Z)-tetracosa-15-enoic acid

[1] Acyl carbon number: double bond number (position of the first double bond calculated from the last carbon)

2.2 Rapeseed oil

Vegetable oils are produced in large amounts around the world (see **Figure 2**). Rapeseed (spring rape/*Brassica napus* L. v. oleifera subv. annua in Finland) and turnip rapeseed (spring turnip rape/*Brassica rapa* L. v. oleifera subv. annua in Finland) are the second most commonly cultivated oilseeds in the world and the most cultivated in Finland (Foreign Agricultural Service/USDA 2010, www.agronet.fi 2012). Consequently, low erucic acid rapeseed oils, including both rapeseed and turnip rapeseed oils, are commonly consumed oils in the Finnish diet, which contributes to the high intake of n-3 polyunsaturated fatty acids in Finns (The national FINDIET 2007 survey). Natural rapeseed oil has up to 50% erucic acid in it, but commonly cultivated, low erucic acid rapeseed varieties have very low amounts of erucic acid (<1%), and high contents of α-linolenic acid (5−15%), linoleic acid (15−30%), and oleic acid (51−70%) (Kallio et al. 1993, Codex standard for named vegetable oils 2011). Compared to olive oil, which usually has less than 1% of α-linolenic acid, rapeseed oil is much more prone to oxidation. However, natural tocopherols in rapeseed oil protect it from oxidation in storage (Isnardy et al. 2003). There is approximately 44−119 mg of γ-tocopherol, 19−69 mg α-tocopherol, and 0−3 mg of δ-tocopherol in 1 kg of rapeseeds depending on the variety and growth conditions (Seker et al. 2008). Commercial oil contains usually between 19 and 30 mg of combined tocopherols (calculated as vitamin E equivalents) per 100 g of oil, but cold pressed oil can contain up to 51 mg α-tocopherol per 100 g oil (Schwartz et al. 2008, Codex standard for named vegetable oils 2011).

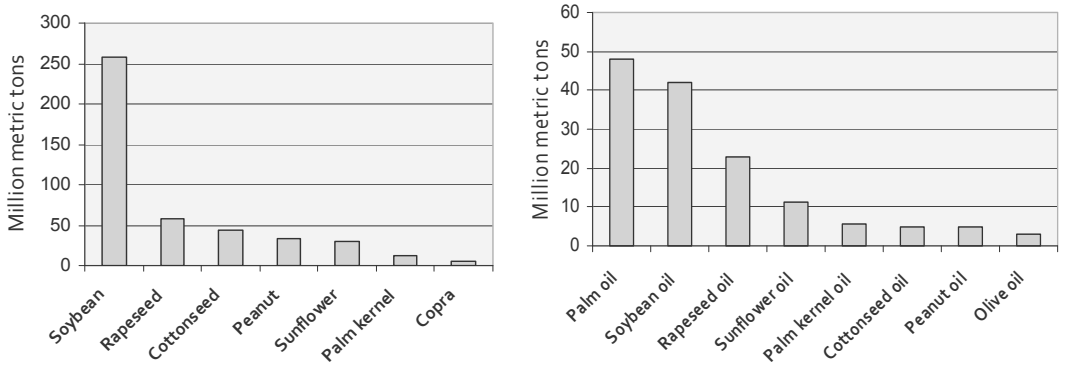


Figure 2. Yearly oilseed and vegetable oil production in the world in 2011 (rapeseed production contains all of the Brassica species based oilseeds including turnip rapeseed) (Foreign Agricultural Service/USDA 2010).

2.3 Lipid oxidation and reactive oxygen species

Lipid oxidation is an unwanted process in which double bonds of unsaturated fatty acids are attacked by reactive oxygen species (ROS), and oxygenated lipids are formed. This alters the sensory and physiological properties of the fat or oil, and causes health concerns for individuals consuming oxidised fats (Kanner 2007). Usually, lipophilic antioxidants protect the triacylglycerols and other lipids from oxidation, but the antioxidants are consumed by ROS after a while and primary oxidation products start to form (Kamal-Eldin 2003). This lag period is not present if there are no antioxidants in the oil (Isnardy et al. 2003). Oxidation processes can be explained in a more precise manner by spin systems in molecular orbitals. Normal atmospheric oxygen, O_2, is in the triplet state ($^3O_2$) that is relatively stable and unreactive. When the dioxygen molecule absorbs energy, it can be excited to the so called singlet state ($^1O_2$), which is the principal ROS in photo-oxidation (DeRosa et al. 2002). In the singlet state, the highest occupied molecular orbitals of $^1O_2$ contain the same amount of electrons (two) as in the triplet state, but they are now both located in the same anti-bonding π_{2px^*} orbital with opposing spins (see **Figure 3**).

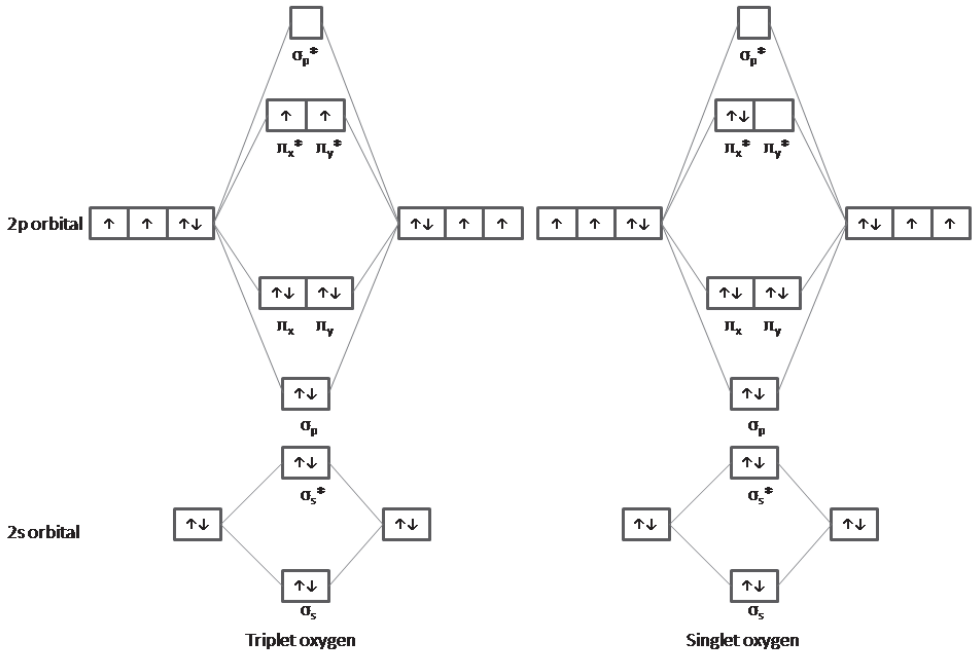


Figure 3. The atomic and molecular orbitals of triplet and singlet oxygen.

Oxygen in the singlet state is very reactive and has a lifetime of up to 100 µs in solvents. However, in a gas atmosphere, singlet oxygen can have a lifetime of up to 70 minutes (Wilkinson et al. 1995). The required energy is brought to the system usually either as heat or UV light. Certain photosensitisers and other compounds such as metal ions increase the formation of singlet oxygen by lowering the energy required for electron excitation (DeRosa et al. 2002). Oxygen may also be split into atomic oxygen, which occurs commonly in the atmosphere. Atomic oxygen is radical and radicals have unpaired and extremely reactive outer orbital electrons. In a single bond between carbon atoms, σ bonds are formed from sp2 orbitals. Double bonds have, in addition to σ bonds, delocalised electrons from p orbitals that form a π orbital. The energy required to interact with a double bond is much lower than with a single bond. Furthermore, if the molecule has more than one double bond in close proximity with each other, the energy required is even smaller (Flemming 2010).

Triacylglycerols in saturated fats such as bovine milk fat (butter), pig lard or coconut oil contain more fatty acids with no double bonds or only a single double bond than common vegetable oils or fish oils. This makes saturated fats more resistant to autoxidation by heat and UV light exposure. Docosahexaenoic acid, which is found abundantly in fatty fish species such as salmon and herring, oxidises much more easily than α-linolenic acid, and linoleic acid oxidises more easily than oleic acid (Cosgrove et al. 1987). Stearic acid is essentially resistant to autoxidation.

The natural methyl interrupted double bond system is affected by oxidation in such a way that conjugated dienes and trienes are also formed, as double bonds are able to migrate during the formation of intermediate structures. These intermediate structures in free radical oxidation include delocalised carbon-centred radicals (Pratt et al. 2011). In triacylglycerols, the position of fatty acids in the glyceryl backbone can have an effect on the susceptibility of an FA to be oxidised. The *sn*-2 position may be slightly more protected against oxidation than the *sn*-1 and *sn*-3 positions (Neff et al. 1996).

There are several ROS that can be formed. The most abundant are singlet oxygen ($^1O_2$), ozone (O_3), superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$). The pKa of superoxide radical is 4.88, so the more reactive hydroperoxyl ($HO_2\cdot$) form is dominant in acidic conditions such as in the stomach (Bielski et al. 1985). While the majority of ROS are hydrophilic, hydrogen peroxide is lipid soluble, enabling it to readily cross lipid membranes (Seaver et al. 2001). Hydroxyl radicals are very strong and short-lived (10^{-9} s) oxidising reagents and are formed *e.g.* by Fenton reaction (Lipinski 2011):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

Reactive oxygen species are formed constantly *e.g.* in the atmosphere by UV radiation, in mitochondria and endoplasmic reticulum during the normal metabolism of cells, during the action of specialised defence cells of the immune system such as macrophages and phagocytes, and during apoptosis (Alfadda et al. 2012, Circu et al. 2010). Certain tissues generate ROS to dispose of unwanted chemicals and toxins in cell organelles called peroxisomes and lysosomes (Schrader et al. 2008, Nohl et al. 2005).

2.3.1 Formation of lipid hydroperoxides

After most of the antioxidants in the system are consumed, the formation of hydroperoxides begins. The exact mechanism of the beginning of peroxidation is a much debated issue and several mechanisms have been proposed. The formation methods of hydroperoxides can be roughly separated into three groups: autoxidation by thermal energy, photo-oxidation, and enzymatic oxidation (Tejero et al. 2004). Both thermal oxidation and photo-oxidation require catalysts of some kind to initiate the peroxidation cascade (Privett et al. 1962). Unfortunately, small amounts of metal ions and other compounds are usually present in oil containers and in oils.

The first step in autoxidation is called *initiation*; there, a lipid molecule (L) is attacked by an ROS that subtracts a hydrogen atom from the lipid molecule, producing a lipid radical (·L). This radical then reacts with molecular oxygen in the *propagation* step, producing a lipid peroxyl radical (LOO·). LOO· reacts further with an unoxidised lipid molecule (L) and propagates the peroxidation reaction by producing L· and LOOH. This propagation is continued until the *termination* step, where radicals are quenched by other radicals as their concentration increases sufficiently, and stable end-products are formed (Porter et al. 1995, Kamal-Eldin 2003, Pratt 2011).

Autoxidation of oleic acid produces 8-, 9-, 10-, and 11-hydroperoxides in nearly equal amounts. The double bond in *cis* configuration commonly isomerises to *trans* configuration and the end-products include 8-hydroperoxy-*cis*-9-octadecenoic acid, 8-hydroperoxy-*trans*-9-octadecenoic acid, 9-hydroperoxy-*trans*-9-octadecenoic acid, 10-hydroperoxy-*trans*-9-octadecenoic acid, 11-hydroperoxy-*cis*-9-octadecenoic acid, and 11-hydroperoxy-*trans*-9-octadecenoic acid. Also, some rearrangements of the position of the hydroperoxyl group and the double bond are possible after the initial formation (Porter et al. 1995).

The recent discovery of *bis*-allylic 11-hydroperoxide along the main oxidation products, conjugated diene hydroperoxides, after the autoxidation of linoleic acid, has revealed that antioxidants have an active role in some oxidation pathways (Brash 2000). A strong antioxidant such as α-tocopherol is required as a hydrogen donor for the formation of *bis*-allylic 11-hydroperoxide as the intermediate bis-allylic peroxyl radical is very fleeting (Schneider 2009).

Photo-oxidation can happen via two different mechanisms. In type I photo-oxidation the photosensitiser gets excited by UV light and extracts an electron from the substrate molecule. A photosensitiser radical and substrate radical are formed, which react further with molecular oxygen to produce hydrogen peroxide and superoxide radical (Niki et al. 2005, Tejero 2004, Terao 1977). In type II photo-oxidation, the singlet oxygen formed by UV radiation directly attacks a double bond in a lipid molecule (Rontani 2012). Four hydroperoxide isomers are produced from linoleic acid by type II photo-oxidation: (**I**) 9-hydroperoxy-*trans*-10-*cis*-12-octadecadienoic acid, (**II**) 10-hydroperoxy-*trans*-8-*cis*-12-octadecadienoic acid, (**III**) 12-hydroperoxy-*cis*-9-*trans*-13-octadecadienoic acid, and (**IV**) 13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid (see **Figure 4**.) (Tejero 2004).

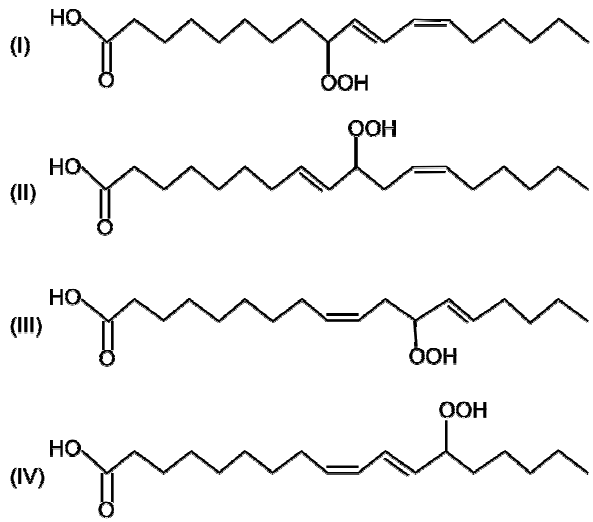


Figure 4. Primary photo-oxidation products of linoleic acid.

The enzymatic peroxidation of fatty acids is common in plant and animal tissues, e.g. for the production of eicosanoids in animal cells and the regulation of growth factors in plants (Harizi et al. 2008, Skórzyńska 2007). The production of eicosanoids from DGLA, AA, EPA and DHA by cyclo-

oxygenases (COX-1 and COX-2) is regulated and not an auto-oxidative process (Fitzpatrick et al. 2001). Plant-derived oxygenases (lipoxygenases, LOX) can sometimes be responsible for the unwanted peroxidation of vegetable oils, if the oil extraction procedure has not deactivated the enzymes (Dwiecki et al. 2012). This is the main problem for different cold pressed oils. Pathogen-induced oxygenase (PIOX) is a recent discovery among plant enzymes; it closely resembles animal cyclooxygenase and produces signalling molecules when plants experience oxidative burst during pathogen invasion and cell death (Sanz et al. 1998).

Also, different bacteria and moulds contain and produce peroxidising and other oxidising enzymes (e.g. cyclooxygenase-like enzymes and LOX), but this is not usually an issue for foods as spoiling is readily detected by off-flavours (Tsitsigiannis et al. 2005). Fermented cheese is known to contain both volatile and non-volatile lipid oxidation products (Arora et al. 1995, Brechany et al. 1992); however, these are usually considered the desired aroma compounds. It is, however, unknown how e.g. the intestinal microbes affect the small amount of lipids that escape the small intestine unabsorbed and enter the large intestine, and what the possible health implications of any bacterial lipid oxidation products are. Some common oxidised fatty acids and advanced lipid oxidation products are presented in **Table 2** and **Figure 5**.

Table 2. Common oxidised fatty acids and their degradation products[a].

ACN:DBN	Trivial name	IUPAC name	Source
3-OH-4:0	3-hydroxybutyric acid	3-hydroxybutanoic acid	bacterial fatty acid
-	2,4-hexadienal	(E,E)-hexa-2,4-dienal	heated oil
-	malondialdehyde	propanedienal	heated oil
2-diOH-18:0	β-dihydroxystearic acid	2-dihydroxyoctadecanoic acid	bacterial fatty acid
2-OH-18:0	2-hydroxystearic acid	2-hydroxyoctadecanoic acid	higher fungi
12-OH-18:1 (n-9)	ricinoleic acid	(Z)-12-hydroxyoctadec-9-enoic acid	castor oil
9-OOH-18:1 (n-9)	9-monohydroperoxy oleic acid	(E)-9-hydroperoxy-octadec-9-enoic acid	oxidised sunflower oil
5-CO-18:0	5-oxostearic acid	5-oxodecanoic acid	cheese lipids
12-O-18:1 (n-9)	vernolic acid	(Z)-12,13-epoxy-octadec-(Z)-9-enoic acid	*Vernonia anthelmintica*
-	prostaglandin E_2	(5*Z*,11α,13*E*,15*S*)-7-[3-hydroxy-2-(3-hydroxyoct-1-enyl)- 5-oxo-cyclopentyl] hept-5-enoic acid	arachidonic acid metabolite

[a] Collected from following sources: Prostenik et al. 1978, O'Leary 1962, Stahl et al. 1996, Brechany et al. 1994, Negre-Salvayre et al. 2008., Hsu et al. 2006).

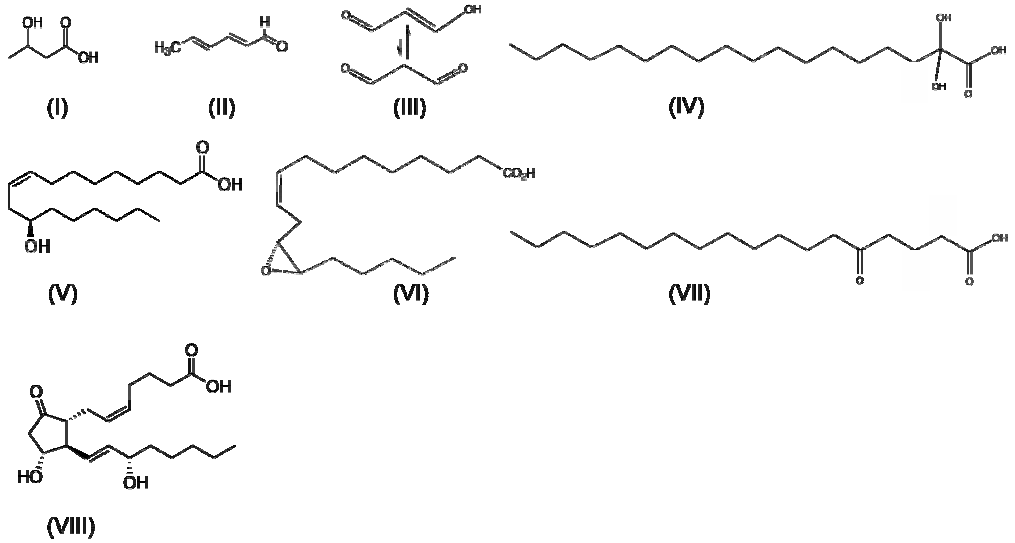


Figure 5. Selected oxidised fatty acids and advanced lipid oxidation products. (**I**) 3-hydroxybutyric acid, (**II**) 2,4-hexadienal, (**III**) resonance structure of malondialdehyde, (**IV**) β-dihydroxystearic acid, (**V**) ricinoleic acid, (**VI**) vernolic acid, (**VII**) 5-oxostearic acid, and (**VIII**) prostaglandin E_2.

2.3.2 Secondary lipid oxidation products

Secondary lipid oxidation products start to form after the hydroperoxides reach high enough concentrations. Formation pathways include the direct decomposition of hydroperoxides by β-scission (see **Figure 6**), Hock-cleavage and additional oxidation (Frankel 1983, Frankel 1984). The common end-products of peroxidised fatty acids include hydroxy fatty acids, oxo fatty acids, epoxy fatty acids, monocyclic peroxides, aldehydes and any combinations of these. Many of the oxidation products are volatile, some semi-volatile, and others non-volatile (Frankel 1984). Volatile lipid oxidation products give the rancid smell of oxidised fats and oils. Some of these compounds, such as malondialdehyde, 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal, and 4-oxo-2-hexenal, are found for example in the cooking vapours of heated oils (Kawai et al. 2006, Esterbauer 1991, Goicoechea et al. 2010, 2011).

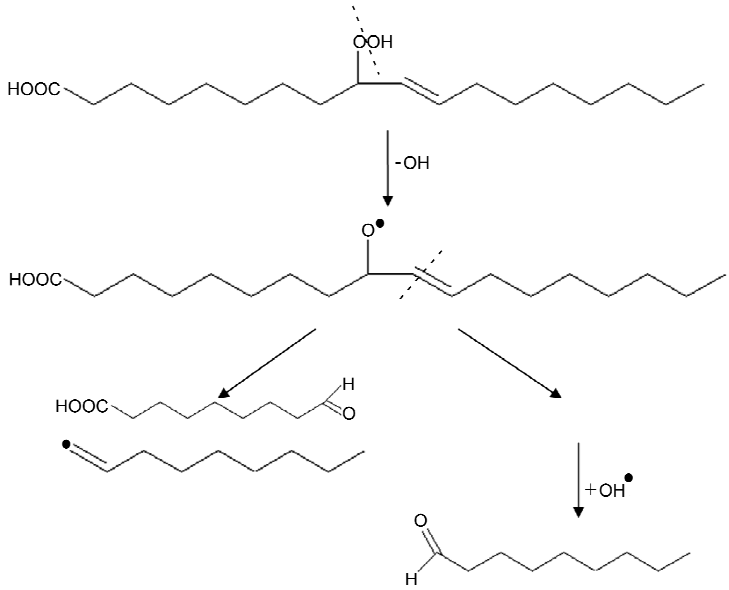
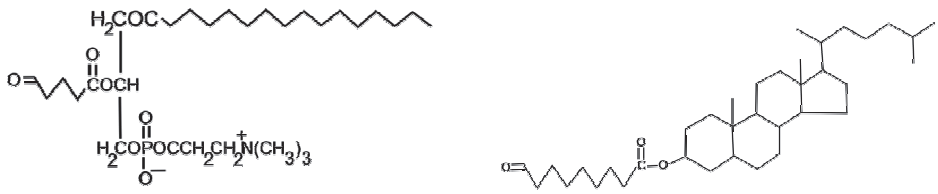


Figure 6. 1-Nonanal and 9-oxononanoic acid as end-products of the β-scission of 9-hydroperoxy-(*E*)-octadec-10-enoic acid.

The non-volatile secondary oxidation products of lipid autoxidation include oxidised triacylglycerols, diacylglycerols, monoacylglycerols and fatty acids, triacylglycerol polymers, and core aldehydes. Triacylglycerol polymers are a complex group of compounds formed under high heat (Byrdwell et al. 1999). The structures of polymerised TAGs may render them unhydrolysable by gastric and pancreatic lipases, enabling polymerised TAGs to enter the large intestine (Henderson 1993). The intestinal microbes may however possess enzymes that are capable of hydrolysing these polymer structures and possibly release smaller oxylipids that can then interact with intestinal wall cells.

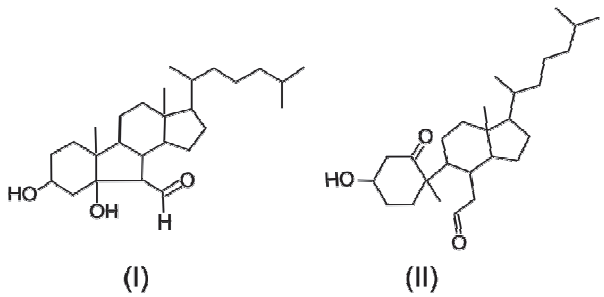
Core aldehydes represent an important group of non-volatile secondary oxidation products. Core aldehydes can be formed from several different lipid classes such as TAGs, phospholipids, and cholesteryl esters (Kuksis 1990). Typical core aldehyde structures formed from common lipids can be seen in **Figure 7**. (**I**) 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-glycerophosphocholine is found in human atheromas (Kamido et al. 2002) and (**II**) cholesterol 9-oxononanoate was detected from human lipoproteins after copper-induced peroxidation (Kamido et al. 1995).



(I) (II)

Figure 7. Typical core aldehydes formed from different common lipids.

Cholesterol itself can be oxidised by ozone in a manner that produces cholesteryl aldehydes such as (**I**) cholesterol carboxyaldehyde (3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxyaldehyde) and (**II**) cholesterol secocholestenal (3β-hydroxy-5-oxo-5,6-secocholestan-6-al) as seen in **Figure 8** (Mansano et al. 2010). Ozone is produced in atherosclerotic arteries, and a major cholesterol oxidation product and precursor for cholesterol carboxyaldehyde is cholesterol 5α-hydroperoxide (Uemi et al. 2009).



(I) (II)

Figure 8. Cholesterol carboxyaldehyde (**I**) and cholesterol secocholestenal (**II**).

Aldehyde groups are generally very reactive. Various carbonyl groups containing oxylipids have been observed to rapidly complex with other substances, such as amino acids, proteins, and nucleic acid bases in biological environments (Kamido et al. 1995, Kamido et al. 2002, Kikugawa 1987, Kotsovolou 2002, Kurvinen et al. 1999, Esterbauer 1999), while, in the chyme, aldehydic lipids have the possibility to be incorporated into many other compounds by forming adducts with amino acids, peptides, and proteins (see **Figure 9** for an example of the reduced Schiff base). The covalent bonds formed in the adduct molecules are sometimes reversible, depending on the pH and other factors (Kamido et al. 1995). The resulting Schiff bases and Michael addition products may be partially cleaved by digestive enzymes, and possibly absorbed in some form. If the aldehydic functions are retained after

absorption, there may be severe health affects as e.g. new covalent bonds between critical enzymes or DNA are formed.

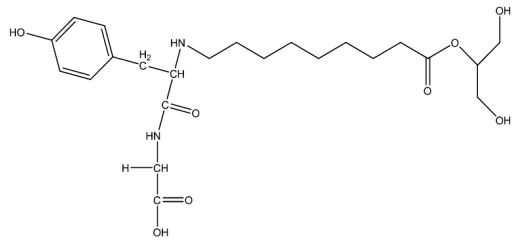


Figure 9. A reduced Schiff base formed from 2-(9-oxo)-nonanoyl-*sn*-glycerol and tyrosine−glycine dipeptide.

2.4 Antioxidants and their effects

Antioxidants protect lipids (and proteins) against oxidation by the quenching of radicals. They commonly act as electron donors, reducing the ROS that are present (Kamal-Eldin et al. 1996). They also form stable end-products with lipid radicals, preventing the propagation of any new radicals (Mäkinen et al. 2000, Niki 2005). Common natural antioxidants include carotenoids, tocopherols, ascorbic acid, and many phenolic compounds such as polyphenols (see examples in **Figure 10**).

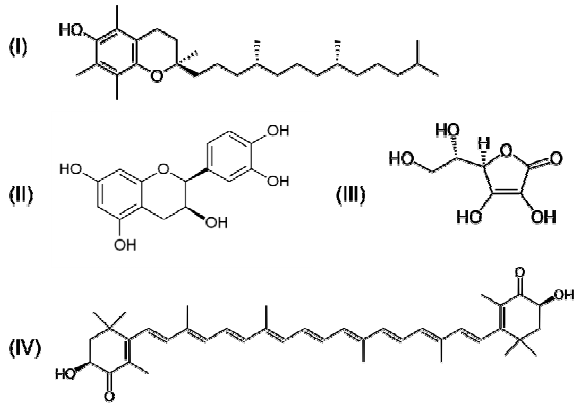


Figure 10. Common antioxidants. (**I**) α-Tocopherol, (**II**) epicatechin, (**III**) ascorbic acid, and (**IV**) astaxanthin.

The solubility of an antioxidant is an important property. Biological fluids are aqueous systems in which oxygen is readily soluble. Fats on the other hand are mainly hydrophobic, and in the case of TAGs they are highly hydrophobic. Oxidised fats commonly have highly polar functional groups in the molecular structure, making them amphiphilic. This usually results in a change in the three-

dimensional structure of the molecule in biological systems. For example, the hydroperoxyl group has high affinity to water and can force a normally lipophilic molecule to bend drastically if water–lipid surfaces are present (Sun et al. 2011).

2.4.1 Ascorbic acid

Ascorbic acid or vitamin C (see **Figure 10**) is probably the most recognised antioxidant. It is abundant in fruits and berries and other vegetables (Szeto et al. 2002). As a sole antioxidant, ascorbic acid may not be very effective against lipid peroxidation due to its hydrophilic nature, although it is an active scavenger of active and stable radicals (Niki 1991). If present with other lipid soluble antioxidants, ascorbic acid can regenerate other antioxidants such as tocopherols (Niki 1987). The sparing effect has also been observed *in vivo*, but the interactions are more complex, and move in both ways (Tanaka et al. 1997). Interestingly, ascorbic acid has been observed to be able to produce nitric oxide from nitrite in acidic environments such as in the stomach (Nagler 2002). This can reduce the probability of the formation of carcinogenic nitrosamines.

The efficacy of ascorbic acid against oxidative stress *in vivo* is a much debated issue. Liu et al. (2010) observed the protecting and restoring effect of ascorbic acid and α-lipoic acid when administered together, but not alone, against oxidative stress induced by arsenic exposure in rats. A protective effect against alcohol-induced liver and brain toxicity, as shown by decreased malondialdehyde levels, was observed when ascorbic acid, quercetin and thiamine were administered. Ascorbic acid was found to be the most effective of the tested antioxidants (Ambadath et al. 2010). However, Fumeron et al. (2005) did not find any protective effect of oral vitamin C supplementation (250 mg three times per week for 2 months) against oxidative stress as measured by plasma protein carbonyl compounds or inflammation markers in haemodialysis patients.

A known interaction between ascorbic acid and glutathione exists. They work as a redox pair in which oxidised ascorbic acid, dehydroascorbic acid, is regenerated in two-electron transfer by glutathione via the dehydroascorbate reductase enzyme (Wells et al. 1994). Glutathione is thought to maintain a reduced cellular environment and its metabolism and homeostasis is important in health and many diseases (Townsend et al. 2003).

2.4.2 Carotenoids

Carotenoids are a large group of lipid soluble, colourful antioxidants. They are found in chloroplasts and in the light harvesting centres of plant leaves and algae, in many fruits and berries, and in some fish and crustaceans (Scheer 2004, Edge et al. 1997). Probably the most abundant carotenoid, β-carotene, is a free radical scavenger and acts in a protective role in the plant light harvesting complexes as excess light energy is absorbed (Xiao et al. 2011). Carotenoids also work by quenching singlet oxygen. The number of conjugated double bonds increases the quenching ability of a carotenoid (Hirayama et al. 1994). Dietary β-carotene is considered safe, but as an excessively-consumed supplement may pose pro-oxidative effects in certain population groups such as smokers (Paiva et al. 1999, Goralzyk 2009).

Lycopene is red carotene found abundantly in tomato and tomato products. Native lycopene is usually in the all-*trans* configuration, but, as it is easily isomerised and oxidised, various *cis*-isomers and oxidation products are formed during food preparation and storage. Interestingly, the bioavailability of *cis*-isomerised lycopene is better than the all-*trans* form (Chi et al. 2000). High serum lycopene levels have been discovered to decrease the risk of stroke in men (Karppi et al. 2012). Lycopene (from tomatoes) has also been observed to have a positive effect on plasma antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, and glutathione reductase, and the lipid peroxidation rate measured as malondialdehyde in patients with grade-I hypertension and elevated oxidative stress status (Subhash et al. 2007). In some studies, high dietary lycopene intake is negatively correlated with certain cancers such as prostate cancer, but recent critical reviews of epidemiological studies have been critical to such claims (Wei et al. 2012).

Astaxanthin (see **Figure 10**) is a xanthophyl, oxygen containing carotenoid. It is found e.g. in a green algae *Haematococcus pluvialis* and in many fatty fish such as salmon, trout and char, thus protecting the highly unsaturated fats in the fish from oxidation (Boussiba et al. 1991, Lambertsen et al. 1971). Some evidence exists for the *in vivo* efficacy of astaxanthin supplementation. Plasma 12- and 15-hydroxy fatty acids were significantly reduced compared with placebo after daily 4 mg astaxanthin administration (Karppi et al. 2007).

Interactions of carotenoids, phenolic compounds, and tocopherols and tocotrienols have been studied and clear synergism was observed by Schroeder et al. (2006). Interestingly, tocotrienols had a stronger synergistic antioxidant effect with carotenoids against oxidation as measured by conjugated diene formation in phospholipid liposomes than in α-tocopherol.

2.4.3 Tocopherols and tocotrienols

Tocopherols and tocotrienols are a group of lipophilic antioxidants found in many vegetable oils and fish (Syväoja et al. 1985, Schwartz et al. 2008). Some tocopherols and tocotrienols also have vitamin E activity, while others only possess antioxidant properties. Natural α-tocopherol (RRR-α-tocopherol, see **Figure 10**) is the most active as vitamin E, while β-, γ-, δ-tocopherols have only part of the activity of the α-form. Tocotrienols differ from tocopherols by possessing an unsaturated farnesyl isoprenoid tail in place of saturated phytyl tail (Kamal-Eldin et al. 1996).

Tocopherols and tocotrienols (tocols) are added to refined oils to restore the lost oxidative stability of the oils. In this role, tocols work sufficiently well, as demonstrated by Rossi et al. (2007) by free radical scavenging efficacy measurements by 2,2-diphenyl-1-picrylhydrazyl (DPPH) of deep-fried vegetable oils. Total tocol concentration correlated strongly to radical scavenging activity. Lampi et al. (1999) found that γ-tocopherol is a more potent antioxidant that the α-form, and that they both help to protect rapeseed oil triacylglycerols from oxidation in the dark at 40°C.

α-Tocopherol is known to affect the decomposition pathways of lipid hydroperoxides. Mäkinen et al. (2000) discovered that at low levels α-tocopherol inhibited the breakdown of methyl linoleate hydroperoxides, and at higher levels inhibited the isomerisation of *cis*,*trans*-hydroperoxides to *trans*,*trans*-hydroperoxides and increased the production of hydroxy compounds.

Traditionally, tocols are thought to act as simple chain-terminating radical scavengers, but other functions also exist (Kamal-Eldin et al. 1996, Kamal-Eldin 2003). Tocols are very likely incorporated in biological membranes in non-random ways, and thus may have very different real-life efficacy as antioxidants *in vivo* when compared with *in vitro* models. It is already known that membranes of different tissues contain very different amounts of tocols and that different stereoisomers behave differently, especially when stereoselective proteins are present in the membranes (Atkinson et al. 2008).

The bioavailability of tocopherols has been observed to vary between individuals. In a study by Roxborough et al. (2000) delta-6-α-tocopherol concentrations of plasma were measured after vitamin E supplementation; the results showed that 40-fold differences existed between study subjects in tocopherol concentration in the blood, which indicates differences in absorption or metabolism.

Meta-analyses of previous clinical studies have raised concerns over high-dosage vitamin E supplementation. All-cause mortality was statistically significantly increased in populations consuming high amounts (≥400 IU/d) of vitamin E supplements. The study covered 19 randomised controlled trials with more than 135 000 participants. (Miller et al. 2005). In another study covering over 35 000 healthy men in North-America, a similar high dose of all-rac-α-tocopherol supplementation increased the risk of prostate cancer significantly (Klein et al. 2011). However, some researchers claim that diets rich in γ- and δ-tocopherols, and even supplementation with these isomers, may provide protection against several cancers, as demonstrated by studies with experimental animals (Yang et al. 2012).

2.4.4 Phenolic compounds

Phenolic compounds are a diverse and loose group of compounds found mainly in plants. Many phenolic compounds can be classified as antioxidants due to their structure and observed effects. Phenolic compounds work as antioxidants by scavenging radicals and by binding free metal ions, thus preventing the initiation of oxidation (Perron et al. 2009). Such antioxidants include e.g. simple phenolic compounds such as caffeic acid (3,4-dihydroxycinammic acid), other hydroxycinammic acids, gallic acid (3,4,5-trihydroxybenzoic acid), similar hydroxybenzoic acids, colourful anthocyanins (glycosides of anthocyanidins), polyphenols such as ellagitannins (hydrolysable tannin), gallotannins and flavonoids such as epicatechin (a flavan-3-ol, see **Figure 10**) (Dai et al. 2010).

Good dietary sources for various phenolic compounds are fruits, berries, vegetables, cereals, cocoa, tea, and red wine. The epidemiological studies on high consumption of good sources of phenolic compounds are unanimous on the positive effects of these foods in human health. The efficacies of several individual phenolic compounds, as also natural mixtures and extracts, have been investigated. In a study, polyphenols extracted from extra virgin olive oil were compared with artificial antioxidants for the inhibition of lipid oxidation of canned tuna. The peroxide value determination and headspace GC analysis of volatiles indicated that polyphenol extract is stronger antioxidant than butylated hydroxytoluene (BHT) or butylated hydroxyaniline (BHA) (Medina et al. 1999).

Red wine polyphenols include anthocyanins and caffeic acid-rich cathecins. They can increase the formation of nitric oxide (·NO) from nitrite in the acidic environment of the stomach (Gago et al. 2007). As ·NO is a radical molecule, it is regarded as a somewhat harmful compound and may be

involved in neurodegenerative diseases. In addition to negative health effects, certain positive health effects seem to exist on the human body such as vasodilation. ·NO remains a controversial compound (Knott et al. 2009). Red wine polyphenols have been observed to inhibit low-density lipoprotein oxidation, possibly explaining the French paradox of the low incidence of cardiovascular diseases in France in spite of the high consumption of saturated fats (Frankel et al. 1993). The antioxidant effect of red wine polyphenols is also observed in the gastric environment, as lipid peroxidation was reduced significantly when polyphenols were added to the model simulating food digestion (Kanner 2001). Kerem et al. (2006) investigated the effects of epichatechin, trans-rasveratrol, gallic acid and caffeic acid on lipid oxidation in the intestinal model system. Epicatechin was the only antioxidant that prevented the formation of primary oxidation products, hydroperoxides, but all of the tested phenolic compounds decreased the amount of hexenal.

2.4.5 Artificial antioxidants

Artificial antioxidants that are approved for food use include ascorbyl palmitate (E304) and butylated hydroxytoluene (BHT / E321) (Commission regulation (EU) No 1129/2011). Esterification of ascorbic acid with long chain fatty acids is performed to change the highly hydrophilic molecule to a more lipophilic form. Some evidence exists that ascorbyl palmitate can have advantages over regular ascorbic acid. The esterified form has been observed to penetrate the blood-brain barrier and access biomembranes that are not normally available to ascorbic acid (Pokorski et al. 2003). BHT is a controversial antioxidant, as it has been observed to increase the risk of some cancers based on *in vitro* and animal experiments (Kensler et al. 1985). The risk is, however, not regarded as significant by the WHO (IARC monographs 1986).

Tocopheryl acetate is not currently an approved antioxidant for human food use in the EU, but it is widely used in topical applications. The acetate group esterified on the phenolic hydroxyl of α-tocopherol inhibits any direct antioxidant activity as the hydrogen donor and resonance structure formation abilities are lost. The acetate group is however thought to be cleaved slowly, thus enabling antioxidant activity in a regulated manner. The acetate form is known to be absorbed by humans in a similar manner as free tocopherol but only after pancreatic lipases hydrolyse the acetate (Cheeseman et al. 1995).

2.4.6 Pro-oxidative effect of antioxidants

Most antioxidants have upper limits of concentration before negative effects start to take place. Tocopherols are known to work both as antioxidants and as pro-oxidants when too much is present. Tocopherols increased low-density lipoprotein (LDL) oxidation markedly when tocopherols were incubated with isolated LDL particles. The mechanism of pro-oxidative behaviour was speculated to arise from the extended presence of tocopheryl radicals inside the LDL particles, thus enabling the propagation of peroxides rather than termination reactions with other radicals (Upston et al. 1999).

Also, carotenoids have been shown to exhibit pro-oxidative effects in certain conditions. It is known that the very high intake of supplemental β-carotene can lead to pro-oxidative effects. Simultaneously present flavonoids may decrease the pro-oxidative effects markedly. Naringin, rutin and quercetin reduced DNA-strand breaks in mouse fibroblasts by UV light in the presence of β-carotene (Yeh et al. 2005). Also, lycopene has been proven to have pro-oxidative effects on *in vitro* models. In a study with human foreskin fibroblasts (Hs68 cells) that were incubated with lycopene and carotene in the presence of known oxidants, both antioxidative and pro-oxidative effects were observed after thiobarbituric acid reactive substance (TBARS) measurements (Yeh et al. 2000).

Ascorbic acid can interact with catalytically active metals such as iron and copper ions and thus can contribute to oxidative damage through the production of hydroxyl and alkoxyl radicals (Almaas et al. 1997, Jansson et al. 2003). In combination with carotene, ascorbic acid showed pro-oxidative effects up to concentrations of 10^{-3} M. Fe^{3+} and Co^{2+} ions behaved synergistically with ascorbic acids pro-oxidative effects (Kanner 2006). Also, in a study by Lapidot et al. (2005a), ascorbic acid demonstrated pro-oxidant effects at low levels and when "free" iron was present. This was, however, reversed in the presence of metmyoglobin; ascorbic acid then demonstrated antioxidative properties.

Kanner et al. (2001) studied the efficacy of several antioxidants, including ascorbic acid, in a gastric environment. The authors used the ferrous ion oxidation-xylenol orange (FOX2) method for the estimation of lipid peroxidation and high performance liquid chromatography (HPLC) for the determination of lipid hydroperoxides. Ascorbic acid as the sole antioxidant was found to be pro-oxidative in the presence of ferrous ions. However, red wine polyphenols were found to be effective antioxidants in gastric conditions.

Oliveira et al. (2012) studied the effects of low and high doses of β-carotene, α-tocopherol, and ascorbic acid on blood mononuclear cells from healthy donors. The results showed that pro-

oxidative effects were present in high dose samples, as demonstrated by ROS and interleucine-6 (IL-6) production. The authors concluded that vitamins can exert both antioxidant and pro-oxidant properties depending on the concentration.

2.4.7 Compartmentalisation of antioxidant systems

Systemic antioxidants that provide cells and tissues protection against ROS include uric acid, catalase, peroxiredoxins, glutathione, glutathione peroxidase, and family of superoxide dismutases (SODs). Three different SODs are found in humans: SOD1 is present in the cytoplasm and SOD2 in mitochondria; SOD3, however, is secreted to the extracellular matrix. They all perform similar functions, and neutralise superoxide radicals that are formed under oxidative stress (Johnson et al. 2005). SODs are essential for the protection of lungs against ROS, as demonstrated by Kinnula et al. (2003).

Glutathione (GSH) is a three amino acid peptide that effectively neutralises ROS in cytosol and cell organelles. It is found in large amounts in the gastric mucosa (Robert et al. 1984, Aw 1997). Glutathione is oxidised by ROS to glutathione disulphide (GSSG), which in turn is reverted back to active glutathione by glutathione reductase. The active part of glutathione is the sulphydryl residue of cysteine, which is able to act as the hydrogen donor, (Shigeoka et al. 1987, Aw 1997). There is evidence that the glutathione redox cycle in the intestines, when reducing GSSG back to GSH, receives reducing power from nicotinamide adenine dinucleotide phosphate (NADPH), and that it is fuelled mainly by exogenous glucose intake (Aw 2005). An important function of glutathione is found in the glutathione-ascorbate cycle, where hydrogen peroxide is neutralised to water by glutathione and ascorbic acid (Noctor et al. 1998). Glutathione peroxidase 4 is an enzyme that actively reduces hydroperoxidised lipids into lipid alcohols; it is mainly active in the phospholipid membranes (Seiler et al. 2008). Parenteral glutathione has been observed to significantly reduce ethanol-induced gastric mucosal damage in humans (Loguecio et al. 1993). The glutathione levels in the stomach have been noted to markedly affect the absorption of peroxidised lipids in a rat model (Aw et al. 1992). The glutathione reserves of different tissues can be increased via supplementation (Aw 1997).

Catalase breaks down hydrogen peroxide to water and oxygen, but is found mainly in peroxisomes (Reddan et al. 1996, Zhou et al. 2000). Peroxisomes are vital to all animal cells and any disruption in their assembly or faults in the enzymes acting inside the peroxisomes usually leads to severe problems (Sheikh et al. 1998).

Peroxiredoxins are a group of thiol-containing enzymes that work in a similar manner as glutathione and reduce peroxidised lipids by hydrogen donation (Yao et al. 2007, Boulos et al. 2007). Interestingly, peroxiredoxin II levels of nerve cells increase simultaneously with amyloid-β-protein and amyloid binding alcohol reductase as Alzheimer's disease progresses. This indicates that oxidative damage is a major contributor to the disease and that the body tries to combat the deleterious effects of ROS (Yao et al. 2007).

Uric acid is the most abundant antioxidant in the bloodstream and is estimated to possess 60% of the antioxidant capacity of plasma antioxidants (Benzie et al. 1996). It is also present in the saliva, gastric and intestinal fluids (Inoue et al. 2003, Powell et al. 1992). Uric acid has been found in high concentrations in tissues under oxidative stress such as the liver and lungs (Glantzounis et al. 2005, Moison et al. 1997). An important function of uric acid is that it can markedly inhibit the formation of nitrogen dioxide radical in the stomach (Pietraforte et al. 2006). Nitrogen dioxide radicals are highly toxic to cells (Kirsch et al. 2002). Nitric oxide ($NO^{\cdot}$) on the other hand has been shown to inhibit the pro-oxidative effects of uric acid during copper-mediated LDL oxidation in the presence of tocopherol (Sanguinetti et al. 2004). The authors proposed that $NO^{\cdot}$/uric acid could protect the arterial wall structures from oxidation. More detailed, it was proposed that uric acid reduces Cu(II) to Cu(I) and subsequently enables the Cu(I) to participate in the radical decomposition of lipid peroxides and propagation reactions. $NO^{\cdot}$ has also other effects on the human body, such as the modulation of host defence, blood flow, mucus formation, and motility (Rocha et al. 2011).

Aldehyde dehydrogenases (ALDHs) are a family of proteins that serve the function of oxidising a variety of aldehydes into corresponding acids. ALDH3A1, ALDH1A1 and ALDH2 are expressed in the stomach and cornea of several mammals. Their main function seems to be to neutralise any aldehydes that may be present in the stomach or formed e.g. by UV induced oxidation in the cornea (Pappa et al. 2002). Despite the presence and activation of aldehyde dehydrogenases in the stomach, carcinogenic effects are induced in experimental animals, if e.g. 2,4-hexadienal is consumed for a prolonged period of time (Lee et al. 2003, Nyska et al. 2001).

While not a direct antioxidant, albumin has been noted to clear carbonyl compounds from human plasma (Aldini et al. 2008). In the study, human plasma was spiked with 4-hydroxy-*trans*-2-nonenal (HNE) and the initial high concentration of HNE was rapidly decreased while no known metabolites were detected. The analysis of the plasma protein fraction revealed the formation of

Michael addition products of albumin and HNE. Cys34 and Lys199 were the active adduct-forming sites. Albumin is present in the circulating blood and in smaller amounts in the digestive juices.

Another intrinsic antioxidative mechanism (although not traditionally considered as such) that is distributed throughout the body, is based on a group of molecules called plasmalogens. Plasmalogens are glycerophospholipids constructed of choline or ethanolamine as the head group and ether bound fatty acid in the *sn*-1 position and esterified fatty acid in the *sn*-2 position (See **Figure 11**). Plasmalogens are found abundantly in the cardiovascular and nervous system (Maulik et al. 1993, Engelman 2004). Recent evidence suggests that plasmalogens may serve as lipophilic antioxidants as the vinyl ether bond makes them more susceptible to oxidation than the corresponding ester-bound glycerophospholipids (Braverman et al. 2012). Decreased circulating plasmalogen levels are strongly correlated to the functional decline of Alzheimer's disease patients (Wood et al. 2010).

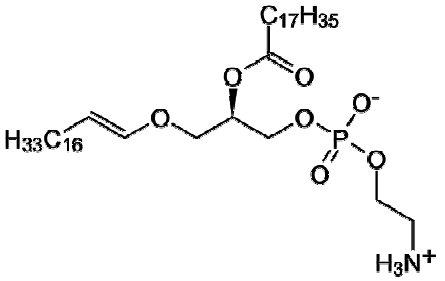


Figure 11. Plasmalogen phosphatidylethanolamine.

Epoxide hydrolases are a family of enzymes that are responsible for the oxidation of any epoxide-containing fatty acids in the cells that need to be discarded. Mammalian epoxide hydrolases are mainly found in the liver cells (Newman et al. 2005). Many local hormones, eicosanoids, contain epoxide structures; therefore, it is expected that epoxide hydrolases are also found elsewhere.

Many of these antioxidant systems work in the compartmentalised environments of cells and cannot effectively reduce or prevent oxidation in other parts of the body. Also, many functions of enzymes must be carefully regulated. Neurons are very sensitive to oxidative damage, for example, and mainly have two antioxidant defence mechanisms: the neuronal antioxidant GSH system and peroxiredoxins (Dringen et al. 1999). The latter acts as the main hydroperoxide-reducing enzyme in the brain. The release of peroxiredoxins in brain tissue after stroke may contribute to the delayed phase

of ischemic injury, which is a real life example of the importance of enzyme compartmentalisation (Garcia-Bonilla et al. 2012).

2.5 Lipid digestion and absorption

Lipid digestion involves a series of hydrolysis reactions catalysed by several specific enzymes. Ultimately, free fatty acids (FFAs) and monoacylglycerols (MAGs) are produced from TAG precursors (Larson et al. 1991, Lowe et al. 1994). Other lipids, such as cholesteryl esters and phospholipids, are also hydrolysed prior to absorption (Howles 2010, Cohn et al. 2010). Lipophilic antioxidants, carotenoids, tocopherols and tocotrienols are absorbed along FFAs and MAGs and their bioavailability is often influenced by the fat content of a meal and the properties of the food matrix (Parker 1996, Jeanes 2004).

2.5.1 Main stages of lipolysis

Food needs to be digested to release the principal components, sugars, fatty acids, and amino acids, from the more complex macronutrients carbohydrates, glycerolipids, and proteins. **Figure 12** shows the overview of the human digestive system. The first part of this digestion happens in the mouth. The taste and feeling of food affect the secretion of saliva and digestive enzymes from the parotid gland (Engelen et al. 2003). Interestingly, contradictory results have been obtained on the stimulating effects of odours (Lee et al. 1992, Engelen et al. 2003). There are several specialised receptors for different tastes in the mouth. Sweet, bitter, salty, acidic and umami are traditionally recognised basic tastes. In addition to these, the mouth also has sensors which register the temperature (and pungency) of food and the texture, as well as astringency. The newest receptors found in the human mouth are lipid-sensing receptors and certain alkaline earth metal sensitive receptors. The CD36 gene has been recognised to influence the fat tasting ability of human test subjects (Chevrot et al. 2012). As food is chewed and eaten, the smell and taste is registered as the combination of these, flavour. All of this stimulates the secretion of digestive juices from the specialised cells of the mouth, pharynx, stomach and intestines.

In humans, the digestion of fats begins in the stomach, when gastric and lingual lipase start to hydrolyse the fatty acids in triacylglycerols. Lingual lipase is secreted by von Ebner glands in the zymogen granules of the tongue. Activity of the lingual lipase is, however, reduced by the neutral pH of the saliva and true activity is reached only in the acidic stomach (Fink et al. 1984). Lingual and

gastric lipases prefer the *sn*-3 position over the *sn*-1 position of TAGs and usually cleave only one fatty acid of a TAG molecule, producing diacylglycerols and free fatty acids (Rogalska et al. 1990). Also, some fatty acids in the *sn*-2 position are hydrolysed, but to a much lesser extent (Jensen et al. 1982). Gastric and lingual lipases differ from other mammalian lipases in a way that they are able to penetrate lipid droplets (*e.g.* milk fat globules) for hydrolysis (Bernbäck et al. 1990). It is estimated that 10 to 30% of TAGs can be hydrolysed to diacylglycerols in the stomach because of the inhibition process induced by the cleaved long chain fatty acids (Pafumi et al. 2002). Lipid hydrolysis in the stomach is very important for infants, as pancreatic enzymes are not yet secreted properly (Bernbäck et al. 1990). Genetic variance in the expression of gastric lipase as also pancreatic lipases exists and there are diseases in which the function of lipases is reduced or completely inhibited (Fieker et al. 2011).

Smooth muscles in the stomach contract in a way that siphons the content of the stomach (Schulze-Delrieu et al. 1998). This starts the emulsification of lipids and water. Food is gradually transformed into chyme during this process. The stomach wall has specialised cells called parietal cells that secrete hydrochloric acid, and gastric chief cells that release enzymes such as pepsinogen and gastric lipase. Bicarbonate is also released into the stomach to regulate the pH and to protect the stomach lining (Kopic et al. 2009). The pH of stomach fluid can be as low as 0.8, but depends on the secretion of bicarbonate and the buffering capacity of the ingested food; this varies between 1.7−5.0 (Ovesen et al. 1086). The low pH enables a certain kind of oxidation to take place (Kanner et al. 2001). Oxygen is always present in the stomach (Dun et al. 1923). Small quantities of dissolved oxygen and gaseous oxygen are swallowed constantly as saliva secreted by the salivary glands in the mouth is ingested. Oxygen is also incorporated into chewed food and swallowed. After the formation of crude chyme, it is released in small quantities into the small intestine.

A round muscle named the Pyloric sphincter regulates the release of chyme from the stomach. Only small amounts of chyme and only small enough particles are released into the intestines at a time. The first part of the small intestine is called the duodenum. Bicarbonate is secreted into the duodenum to neutralise the highly acidic chyme. Typical pH of the chyme in duodenum is between 7.0−8.0 though slightly lower (5.0-6.0) in the duodeno-jejunal junction (Ovesen et al. 1986). To enhance the formation of lipid micelles, bile salts are introduced into chyme by the biliary gland via a common bile duct (Jones et al. 1979). The pancreas excretes other components, such as digestive enzymes. These enzymes include pancreatic triglyceride lipase, which is the main enzyme responsible for triacylglycerol digestion (Lowe 1994). Procolipase is also secreted and quickly

transformed to active colipase by the removal of the pentapeptide chain (Larsson et al. 1991). TAGs as well as diacylglycerols (DAGs) formed in the stomach are emulsified to smaller and smaller droplets by the bile salts. Pancreatic lipase cleaves fatty acids from the *sn*-1 or *sn*-3 position and leaves the *sn*-2 position intact. Thus, the resulting molecular species are free fatty acids and *sn*-2-monoacylglycerols. The most difficult step in lipid absorption is the crossing of hydrophobic compounds from the lipid phase of mixed micelles over the water-lipid interface on enterocytes (see **Figure 13**). Bile acids have an essential role in this crossing (Wilde et al. 2011). Enterocytes extend glycocalyx-covered microvilli structures into the small intestine, thus enlarging the surface area and improving the absorption of compounds (Maury et al. 1995).

Short- and medium-chain free fatty acids can be absorbed directly by the blood veins in the stomach and small intestine walls (Saunders 1991). All of the blood coming from the stomach and intestines flows through the liver via the hepatic portal vein; most of the fatty acids transported by albumin are transported into the liver and consumed in the beta-oxidation (Guillot et al. 1993). In the intestine, specialised lymphatic veins called lacteal absorb the longer-chain free fatty acids and monoacylglycerols from the chyme. Triacylglycerols, cholesteryl esters, and phospholipids are reassembled in the enterocytes in a non-randomised manner, as proven by studies by Yli-Jokipii et al. (2004, 2005). The reassembled triacylglycerols have a larger portion of oleic acid in the *sn*-2 position than in the ingested TAGs. The assembly of TAGs and chylomicrons takes place in the smooth endoplasmic reticulum (Cartwright et al. 2000).

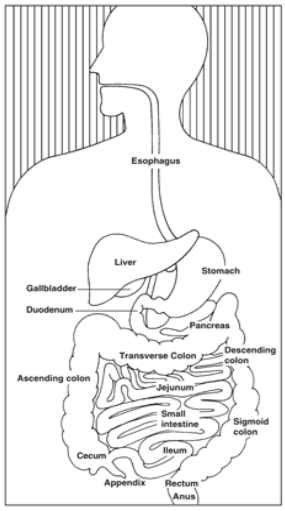


Figure 12. Overview of human digestive system.

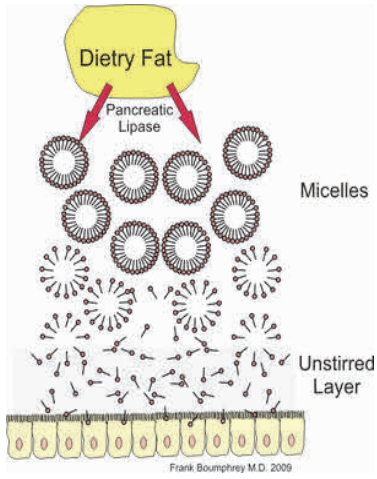


Figure 13. Fatty acid absorption starting from emulsification.

2.5.2 Simulating human digestion

Many methods exist for the simulation of human digestion. The more complicated methods include artificial stomachs and intestines equipped with expanding sacks and tubular structures, with dedicated lines for the addition of digestive juices and sample collection (Kong et al. 2010). More simple approaches utilise test tubes where pre-composed digestive juices are mixed with the study material at intervals. An essential part of the simulation is proper mixing and incubation at body temperature.

Much of the early work was done by Miller et al. (1981) for studying the bioavailability of iron from meals. More recently, extensive work on the simulation of human digestion has been done by Versantwoort et al. (2004, 2005). The *in vitro* model accounts most of the components found in digestive juices and takes into account the changing composition of the digestive juices between fasting and fed states. A recent review by McClements et al. (2010) covers many of the protocols that are used in the literature.

2.5.3 Absorption of oxidised lipids

Several experiments have been conducted to study the possible absorption of oxidised lipids. Burhke et al. (2012) found that furan fatty acids that are formed by the oxidation of conjugated linoleic acid (CLA), are readily absorbed into Caco-2 cells. There seemed to be no toxic effects against the intestinal cells up to a level of 100 μM. Interestingly, the 9,11-furan fatty acid was incorporated and stored as triglycerides in cellular lipid droplets. In a previous study, carbon-14-labelled 13-hydroperoxylinoleic acid and 13-hydroxylinoleic acid were incubated with Caco-2 cells (Penumetcha et al. 2000). Absorption was dependent on brush border structure, and the authors concluded that both hydroperoxy and hydroxy fatty acids were absorbed. However, the technique used for quantitation was radioactivity measurement and thus cannot reveal the exact structures of absorbed molecules.

Kanazawa et al. (1998a, 1998b) have studied the fate of dietary hydroperoxides of linoleic acid trilinoleoylglycerol in rat intestines. They found that the hydroperoxides were extensively decomposed to different aldehydes and fatty acid hydroxyls, and that hexenal and 4-hydroxynonenal were detected in liver from administration after 15h. Previously, α,β-unsaturated aldehydes were determined to be absorbed, metabolised and secreted to the urine of experimental rats after administration of these compounds via gavage tube (Grootveld 1997). More recently, the absorption of 4-hydroxy-2-hexenal was observed from the intestines of experimental rats after the

consumption of meal containing oxidised oil. 4-Hydroxy-2-hexenal was detected both in the intestines and in circulating plasma (Awada et al. 2012).

In another study, pigs were fed with oxidised sunflower oil and the levels of oxidised triacylglycerols were studied in the plasma lipoproteins (Suomela et al. 2004). Oxidised TAGs were detected both in chylomicrons and very-low-density lipoprotein (VLDL) particles, thus reflecting the oxidised dietary fats. The authors estimated the levels of several oxidised TAG species with attached oxo-, hydroxy-, and epoxy groups. Also, core aldehydes were detected in the samples. In a similar study, Suomela et al. (2005) investigated the fate of hydroperoxidised TAGs after the feeding of oxidised oils to pigs and the harvesting of small intestine epithelia. No hydroperoxidised TAGs were detected, but other oxidised TAGs were found in the epithelia.

A limited number of human experiments have been done on the absorption of specific oxidised lipids. Wilson et al. (2002) studied the fate of dietary U-13C-labelled monoepoxy and diepoxy stearic acids (as TAGs containing two oxidised FAs) and discovered that they are indeed absorbed. The absorption of monoepoxy stearic acid was greater than the diepoxy form (17% vs. 8% of dose). In another study, Wilson et al. (2002b) determined the absorption rate of monohydroxy and dihydroxy fatty acids. Both forms were found to be bioavailable, but monohydroxy fatty acids were absorbed more.

The effects of carbonyl compounds on digestive enzymes have also been studied. Synthesised lipophilic aldehydes inhibited gastric lipase and pancreatic lipases (Kotsovolou et al. 2002). Oxidised oils contain carbonyl compounds, which may inhibit the lipolysis of fats and some intact oxidised lipids may be left unabsorbed and enter the large intestine.

2.5.4 Oxidative potential of the digestive system

The gastrointestinal tract is thought to be a major site for antioxidant action (Halliwell et al. 2002). The inside of a stomach is a highly unfavourable place for any enzyme activity other than select specialised enzymes evolved for acidic environments. Antioxidative enzymes such as SODs can function only in environments where the enzymes are not deactivated *e.g.* by low pH. The buffering mechanisms in cellular compartments normally protect the enzymes, but not in the gastric environment. This limits the systemic antioxidant activity in the gut and intestines. Furthermore, the extremely low pH denatures most proteins, releasing and ionising any metals that are present in food components such as meat and blood. Copper, zinc, and ferrous ions are always present at

fluctuating concentrations in gastric juice (Powell et al. 1992). At low pH, these ions can function as a catalyst for oxidation reactions (Kanner 1977, Kanner 1994). Oxygen is present abundantly in the gut and the continuous siphon of ingested and chewed food in the stomach generates smaller and smaller lipid droplets mixed with high-oxygen-containing digestive juices (Dun et al. 1923). As the lipid droplets get smaller, their surface area increases, and this increases the probability of lipids interacting with ROS.

Lipid peroxidation in digested red muscle tissue is catalysed by an iron-redox cycle formed by ferrous ions and ascorbic acid or metmyoglobin; the oxidising effect is significantly increased in acidic environments, such as the stomach (Kanner 1994, Kanner et al. 2001, Lapidot 2005a). Gorelik et al. (2005) studied lipid peroxidation and coupled vitamin oxidation in simulated and human gastric fluids, and found that vitamin E and β-carotene were depleted in low pH in the presence of red meat homogenate. Red wine polyphenols worked synergistically with ascorbic acid and prevented lipid peroxidation and β-carotene degradation. Lapidot et al. (2005b) studied the effects of metmyoglobin and phenolic antioxidants on lipid peroxidation in simulated gastric fluid. Metmyoglobin was found to behave as both a pro-oxidant and antioxidant, depending on the pH and concentration. At high concentration, and especially when coupled with phenolic antioxidants (catechin and quercetin), metmyoglobin demonstrated antioxidative properties, decomposing linoleate hydroperoxides and keeping the hydroperoxide levels at zero for extended periods.

Aqueous infusions from *Capparis spinosa* L. and *Crithmum maritimum* L., which contained several phenolic compounds (including rutin, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside, chlorogenic acid), showed good antioxidant activity as measured by the DPPH method, the β-carotene bleaching method, and copper-induced oxidation of human LDL. However, after submission to two-step *in vitro* digestion model a significant reduction of antioxidant activity was observed (Siracusa et al. 2011).

Saliva has dual roles in the oxidative reactions in the stomach. As saliva contains several different antioxidative properties, such as uric acid and peroxidase and superoxide dismutase enzymes, but the amounts vary significantly between the fed and starved states in humans, there may not always be enough antioxidative potential to prevent oxidation (Nagler 2002). Furthermore, Gorelik et al. (2007) discovered that common saliva components such as lactoperoxidase had pro-oxidative effects on simulated gastric fluid and that thiocyanate and nitrite reduced peroxidation. Peroxidation was

estimated by FOX2 (estimates hydroperoxides) and TBARS (estimated malondialdehyde) measurements.

2.6 Methods for measuring lipid oxidation

Several types of techniques are available for the measurement of lipid oxidation. They range from specialised tandem mass spectrometric methods to general titrimetric methods and even simple sensory evaluation (Kuksis et al. 2009a, Kuksis et al. 2009b, Gray 1978). A problem with measuring lipid oxidation is that there are several pathways for the formation of oxidised lipids and the end-products of secondary oxidation reactions are numerous (Frankel 1983, 1984). A single method for complete lipid oxidation determination does not exist. General methods may be affected by unknown substances and the results should be interpreted carefully.

2.6.1 Antioxidant activity tests

Strongly related to the measurement of lipid oxidation is the measurement of different antioxidants present in the samples under investigation. Antioxidant activity and capacity tests aim to measure the potential of antioxidants in biological fluids. The tests can be divided into two main categories: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET) reactions (Huang et al. 2005). There are, however, significant problems with one-dimensional methods for evaluating multidimensional biological systems composed of lipid bilayers, colloids, emulsions, and where multitude of factors affect the activity of different antioxidants (Frankel et al. 2000). Authors of the paper suggested that, in addition to general tests, much more specific methods should be used and the results compared.

The ferric-reducing antioxidant power (FRAP) test is claimed to measure the antioxidant, or reducing, potential of biological fluids. In the method, ferric ion that is added into the samples is reduced to ferrous ion by antioxidants and the resulting absorbance change at 593 nm is measured. (Benzie et al. 1996). There are both supporting and critical studies of general antioxidant activity methods. In one study, FRAP values decreased by 37% along with actual plasma malondialdehyde (MDA) concentration (−53%) in haemodialytic patients after dialysis. The authors, however, used creatinine concentration-corrected MDA values in their analysis and only then obtained the expected negative correlation. The authors concluded that the measurement of individual antioxidants in plasma is the preferred method for assessing the antioxidant status of patients (Reddy

et al. 2010). Katalinic et al. (2007) had a significantly higher minimum level of plasma FRAP of healthy adult males than Benzie et al. (1996). The authors concluded that it is vital to also measure other biochemical variables such as plasma triglycerides and bilirubin as these can significantly affect the FRAP measurements.

The oxygen radical absorbance capacity (ORAC) is a widely used assay for the measurement of antioxidant capacity of antioxidants, "super foods", purified fractions and biological fluids. The test can, however, give varying results when compared with other similar tests. Villano et al. (2005) compared three different antioxidant activity tests: the ORAC, 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS or TEAC), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) values of wine phenolic compounds and metabolites *in vitro* and found that ORAC values had poor correlation with the other tests. The ORAC test usually measures only hydrophilic antioxidants, but the modified method has been developed for the measurement of lipophilic antioxidants present in biological fluids and foods (Prior et al. 2003).

The modified Trolox equivalent antioxidant capacity (TEAC) test was used to evaluate the efficacy of the test on various solvents for both hydrophilic and lipophilic antioxidants (van den Berg et al. 1999). The investigators found that different solvents had very different effects on the TEAC values of different antioxidants. Tetrahydrofurane (THF) seemed to be the most reliable and gave, on average, the highest TEAC values for α-tocopherol, β-carotene, quercetin and ascorbic acid. Acetone failed to show any antioxidant activity for β-carotene, proving that it is vital to standardise different methods and to be aware of the shortcomings of specific tests.

Total peroxyl radical trapping potential (TRAP) is a commonly used antioxidant activity test that aims to measure the ability of antioxidants to trap peroxyl radicals (Wayner et al. 1985). Mulholland et al. (1993) investigated whether high doses of α-tocopherol and ascorbic acid affect the TRAP values of plasma from healthy young volunteers. The supplementation failed to show any statistical difference in plasma TRAP values at the end of the supplementation (29 days) despite markedly increased plasma antioxidant concentrations.

2.6.2 Unspecific methods for measuring lipid oxidation

A recent review by Barriuso et al. (2013) covers most of the standard methods used in the determination of lipid oxidation. Official Methods and Recommended Practices of the AOCS (Firestone 2009) is a detailed method book of most of the common protocols used. Common methods

for generalised lipid oxidation measurement include peroxide value (PV) measurement by iodometric titration or by ferrous oxide xylenol orange (FOX) measurements or by Fourier transform infrared (FTIR) spectroscopy determination. Conjugated dienes (CDs) and trienes (CTs) can be measured with UV spectroscopy by measuring the absorbance at 234 nm and 268 nm, respectively, of samples dissolved in isooctane. Aldehydes are commonly determined by reaction with p-anisidine and subsequent absorbance measurement at 350 nm (AnV). Carbonyl compounds in oils and fats can also be determined by measuring absorbance at 420 nm after reaction with 2,4-dinitrophenylhydrazine (DNPH) (Endo et al. 2001). DNPH-derivatisation is useful as it stabilises the reactive carbonyls. The presence of malondialdehyde can be determined by thiobarbituric acid reactive substance (TBARS) measurements.

2.6.3 Specific methods for measuring lipid oxidation

The specific measurement of lipid oxidation aims to determine the specific constituents in oxidised lipid samples. Usually, any method used has to be very sensitive as the concentrations of the oxidised components are often low. Measurements can be done qualitatively and quantitatively, but the quantitative results are frequently not well correlated to non-specific methods and underestimate the level of oxidation. The reason for this is that the numerous oxidation products are not always at sufficient concentration to be detected and quantified individually.

2.6.3.1 Chromatographic methods

Chromatography offers the possibility to separate, identify and quantify individual oxidised molecules, and thus provides a much more detailed view of lipid oxidation. Thin layer chromatography (TLC) is not covered in this review, although it can be used in preparative steps, when purifying oxidised fractions and even in the rough identification of oxidised lipid species (Oette 1965). High performance liquid chromatography (HPLC) and more recently ultra-high performance liquid chromatography (UHPLC) are used to study non-volatile lipid oxidation products. Gas chromatography (GC), on the other hand, is used for the analysis of volatile oxidation products. GC analyses are routinely performed with the flame ionisation detector (FID), which does not give any additional structural information about the detected molecules. Possible complimentary detectors include the photo ionisation detector (PID) that is very sensitive to carbon double bonds and aromatics (Driscoll 1977). Unoxidised fatty acids are routinely analysed by GC, but only after methylation to more volatile methyl esters. Primary oxidation products, hydroperoxides, are heat sensitive, so the use of derivatising reactions such as trimethylsilylation (TMS) is preferred when GC analysis is used (Turnipseed et al. 1993). Carbonyl compounds may be derivatised to corresponding

thiazolidines to increase the sensitivity and improve chromatographic separation (Yasuhara et al. 1998). Costa et al. (1998) used trifluoroacetylation of hydroxyl groups and *tert*-butyldimethylsilylation of the carboxyl groups when analysing 3-hydroxy fatty acids in plasma. Derivatisation enabled the baseline separation of many oxidised and unoxidised fatty acids by GC analysis. The common stationary phases of GC columns used in fatty acid methyl ester analyses include mid to highly polar polyester based columns such as Carbowax and SP-2340 (Christie 1989). Analysis of sterols requires derivatisation to either TMS ethers or acetylation prior to separation with polysiloxane phase columns such as ones composed of 95% dimethylpolysiloxane and 5% phenyl groups (Laakso et al. 2005).

Non-volatile lipid oxidation products can be analysed by HPLC or UHPLC coupled with appropriate detectors. Simple UV or diode array detectors (DAD/PAD) are not suited for analysing lipids without chromophores. Nevertheless, when combined with other, more universal detectors, the UV detector and DAD can give additional information e.g. of conjugated dienes and trienes, which absorb at 234 nm and 268 nm. The evaporative light scattering detector (ELSD) is a universal detector that detects most compounds suitable for liquid chromatography. Reverse phase (RP) columns (e.g. C18) are commonly utilised for lipid analyses (Kuksis et al. 2009a, 2009b), but some methods rely on normal phase silica columns. For example hydroperoxidised fatty acids can be separated with normal phase LC, although some oxidised isomers usually overlap with each other (Gardner 1975, Rayner et al. 2004). Normal phase (NP) LC is also typically used for the separation of different lipid classes (Hamilton et al. 1988).

Oxidised lipids can also be initially separated into groups with RP LC and subsequently with NP LC to produce pure isomers. Neff et al. (1990) used preparative RP LC (methylene chloride/acetonitrile, 30:70, v/v; 5 µm, 25 x 2.14 cm silica column) for subsequent analysis with NP LC (isopropanol/hexane, 0.5:99.5, v/v; 5 µm, 25 x 0.49 cm C18 column) to first separate *mono*-, *bis*-, and *tris*-hydroperoxidised trilinoleoylglycerols and then the positional isomers of those hydroperoxides. Interestingly, UV and refractive index (RI) detectors were used for the detection. Gladovič et al. (1997) used HPLC–UV (234 nm) to analyse primary lipid oxidation products of linoleic acid and triacylglycerols. The presence of hydroperoxides was confirmed with the fast atom bombardment (FAB)–MS and NMR. The authors also performed an organoleptic evaluation of the oxidised oils, and the rancid taste correlated well with PV and hydroperoxide determinations.

2.6.3.2 Mass spectrometric methods

Mass spectrometry (MS) is a powerful detection method for the analysis of a wide range of unoxidised and oxidised lipids. Direct injection and infusion MS and different matrix-assisted laser desorption ionisation (MALDI)–MS equipment enable the simple and quick analysis of oxidised lipid samples. Hsu et al. (1998) utilised direct infusion ESI–MS/MS to analyse glycerophosphocholine (GPC) lipids as lithium adducts. Lithium hydroxide was added to the infusion solution and the cationic GPC molecules (quaternary nitrogen in the structure) readily formed positive lithium adducts, which were easily fragmented in collision-assisted dissociation (CAD), forming characteristic fragments for the identification of GPCs. Hsu et al. (1999) also utilised the same method successfully for the investigation of positional isomers of triacylglycerols. The resulting ESI–MS/MS spectra contained $[M + Li – (RnCO2H)]^+$, $[M + Li – (RnCO2Li)]^+$, and $[RnCO]^+$ ions that enabled the assignment of positional isomers of TAGs when the relative abundances of those characteristic ions were considered. The locations of double bonds were assigned by CAD–MS^2 experiments.

Duffin et al. (1991) analysed different acylglycerols (MAGs, DAGs, and TAGs) with electrospray ionisation tandem mass spectrometry. The study revealed that sodium adducts and ammonium adducts behave differently in MS/MS analyses. Ammonium adducts are much more easily fragmented and thus better suited for analysis aimed at structural elucidation. Vu et al. (2012) used direct infusion ESI tandem mass spectrometry for analysing intact oxylipin-containing *Arabidopsis thaliana* membrane lipids. The authors identified and quantified a large group of oxidised membrane lipids: phosphatidylcholines, phosphatidylethanolamines, monogalactosyldiacylglycerols, digalactosyldiacylglycerols, and phosphatidylglycerols. In addition to quadrupole equipment, they used Fourier transform ion cyclotron resonance mass spectrometry for the determination of accurate masses. Stübiger et al. (2010) used MALDI–MS to analyse major oxidised phospholipid classes such as ox-phosphatidylcholines, ox-phosphatidylethanolamines, and ox-phophatidylserines found e.g. in human plasma and other biological samples.

Coupling MS detectors to chromatography systems increases the specificity of the analysis and enables the separation of oxidised molecules with the same nominal mass. Complex lipidomic analyses have greatly advanced from modern combined techniques. GC–MS and LC–MS are nowadays routinely used in the analysis of oxidised lipids. Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) interfaces are the most used ones when coupling

LC to MS equipment (Kuksis 2009a, 2009b). TLC–MS systems were reviewed recently by Cheng et al. (2011).

Volatile lipid oxidation products were determined among other compounds by solid phase micro-extraction followed by GC–MS analysis in a study aiming to differentiate virgin and refined oils according to their botanical origins (Uriarte 2011). Wilson et al. (2002) reported the simultaneous determination of epoxy and hydroxy fatty acids as their methoxy derivatives by GC–MS. The authors used solid phase extraction (SPE) to purify oxidised fatty acid methyl esters from unoxidised fatty acid methyl esters (epoxy groups were transformed to vicinal hydroxy and methoxy groups in borontrifluoride catalysed methylation), after which the hydroxy groups of oxidised FAMEs were derivatised by tetramethylammonium to single methoxy and vicinal dimethoxy groups. Methoxy FAMEs were analysed by GC–MS (30 m x 0.25 mm i.d. x 0.25 µm HP 5MS column), which produced distinct fragment ions for positional hydroxy and epoxy isomers.

Goicoechea et al. (2008) used solid phase micro-extraction (SPME) and GC–MS to analyse several toxic aldehydes arising from oxidation n-6 fatty acid-rich oils after *in vitro* digestion. They identified several volatile oxygenated α,β-unsaturated aldehydes, such as 4-hydroxy-2-nonenal (HNE), 4-oxo-2-nonenal (ONE), and 4,5-epoxy-2-decenal (EDE), which were bioaccessible after incubation in the digestion model. Later, Goicoechea et al. (2010) continued this work by identifying more toxic aldehydes from other heated vegetable oils. In addition to those aldehydes mentioned previously, 4-hydroxy-2-hexenal, 4-oxo-2-hexenal, and 4,5-epoxy-2-heptenals were bioavailable in the digestion model.

Neff et al. (1998) analysed the autoxidation products of trilinolenin via RP HPLC–APCI–MS. They detected e.g. mono- and bishydroperoxide TAGs, mono- and diepoxidised TAGs, hydroxy TAGs, and TAGs with epoxy and hydroperoxy groups. Byrdwell et al. (1999) used RP HPLC–APCI–MS for the characterisation of non-volatile oxidation products of triolein formed after heating to frying temperatures (190°C) for 6 hours. The investigators identified several oxidation products such as epoxy-TAGs, keto-TAGs, hydroperoxy-TAGs, and dimers of TAGs. APCI is commonly used for the ionisation of neutral lipids such as TAGs (Beermann et al. 2007, Byrdwell et al. 2001a, 2001b, Holčapek 2003, Lisa et al. 2008) and ESI for the ionisation of more polar oxidised molecules (Byrdwell et al. 2004b, Sjövall et al. 1997, 2001, 2002, 2003). If possible, they should both be used simultaneously to cover larger areas of ionisable compounds (Byrdwell 2004a). The use of ESI e.g. enables the ionisation of

TAG oligomers, which are produced after the prolonged oxidation of unsaturated oils (Byrdwell et al. 2004b).

In addition to reversed phase HPLC, which commonly utilises octadecylsilyl (C18) columns, normal phase HPLC has also been used to determine the lipid profiles of oils. Kalo et al. (2006) developed a comprehensive method for analysing sterols, sterol esters, FFAs, MAGs, DAGs, and TAGs in oils with NP HPLC–ESI–MS^2. Ammonia was used to enhance the ionisation of molecules and to produce characteristic fragment ions from the parent $[M + NH_4]^+$ ions in tandem mass spectrometric analysis. Multistep binary gradient elution with hexane and hexane/methyl-*tert*-butyl ether/acetic acid was used with two silica columns (100 x 2.0 mm, 3 μm particle size) to enable separation of the analytes. More recently, Cífková et al. (2012) developed a new non-targeted identification and quantitation method for major lipid classes present in complex biological samples such as human plasma by using hydrophilic interaction (HILIC) LC–ESI–MS. The authors used positive ionisation and single internal standard (sphingosyl phosphoethanolamine (PE), d17:1/12:0) for all polar lipids.

Ammonia (post column infusion) was also used by Giuffrida et al. (2004a) to enhance the ionisation of hydroperoxy and epoxy triacylglycerols in RP HPLC–ESI–MS/MS analyses. The authors identified clear fragmentation pathways for hydroperoxidised and epoxidised TAGs, which enabled the identification of regio-isomers of oxidised TAGs. Giuffrida et al. (2004b) studied the formation of epoxides from pure TAGs, cholesterol and phytosterols under a controlled atmosphere. $^{18}O_2$ was used in the experiments to reveal how oxygen is attached and transferred during secondary oxidation reactions. The authors used HPLC–ESI–MS/MS, infusion ion-trap MS and infusion MS/MS to study the fragmentation of epoxidised compounds. Results showed that after the formation of hydroperoxides, epoxidation can proceed in the absence of molecular oxygen. In addition, they investigated how epoxidised lipids are affected by the acidic conditions of model gastric medium, and found that epoxides are readily decomposed to vicinal dihydroxides.

Reverse phase HPLC–ESI–MS has been used to identify phospholipid oxidation in oxidatively stressed cells (Spickett 2001). Combined LC–MS analyses enabled the detection of intact hydroperoxidised phosphatidyl cholines (PCs) for the first time. More recently, Suomela et al. (2011) investigated the regioisomeric structures of several oxidised TAGs with UHPLC–ESI–MS. The authors used positive ionisation ESI with ammonia supplemented via nebulising gas to produce the ammonia adducts of the oxidised TAGs. They also utilised two small particle size core shell

columns (100 x 2.1 mm, 1.7 μm) in series to improve the separation of isomers. The method enabled e.g. the separation of *sn*-2 and *sn*-1/3 isomers of hydroxy 50:1 TAG (two palmitic acids and one hydroxy oleic acid), epoxy 50:0 TAG (two palmitic acids and one epoxystearic acid) and the method also separated fully or partially some geometric isomers (the position of oxidised group within a single fatty acid) of the same positional isomer.

Hutchins et al. (2011) investigated cholesterol ester (CE) oxidation in human peripheral vascular lesions. Normal phase HPLC–MS/MS (1/5th of the LC eluent modified with 10 mM ammonium acetate in acetonitrile/water, 95:5, v/v was directed to AB Sciex API 3200 triple quadrupole mass spectrometer) was used to identify and quantify unoxidised and oxidised CEs in MRM mode. The internal standard (17:1-CE) was used for the quantitation. Reverse phase HPLC–ion trap MS/MS (Thermo Finnigan LTQ) was used to identify hydrolysed fatty acids from the cholesterol esters. Several oxidised CEs were identified, of which the most abundant were: cholesteryl hydroxyoctadecenoate (13-(*Z,E*)-HODE-CE, 9-(*E,Z*)-HODE-CE, 13-(*E,E*)-HODE-CE, 9-(*E,E*)-HODE-CE), cholesteryl epoxyoctadecenoate (12,13-(*E*)-EpOME-CE, 9,10-(*E*)-EpOMe, cholesteryl hydroperoxyoctadecenoate (13-(Z,E)-HpODE-CE, 9-(E,Z)-HpODE-CE), and cholesteryl oxo-octadecenoate (13-(*Z,E*)-oxoODE-CE, 9-(*E,Z*)-oxoODE). Of the three major unsaturated CEs, 18:2-CE, 20:4-CE and 22:6-CE were 23%, 16% and 12% oxidised, respectively, and 40% of all of the CEs were oxidised in total.

Ronsein et al. (2010) used dopant-assisted atmospheric pressure photoionisation tandem mass spectrometry coupled with HPLC to analyse cholesterol oxidation products. The use of selected reaction monitoring mode (SRM) increased the sensitivity, and MS/MS revealed specific fragmentation patterns for oxidised cholesteryl molecules. Tomono et al. (2011) used 2-hydrazino-1-methylpyridine (HMP) to derivatise cholesteryl ozonisation products prior to LC–ESI–MS/MS analysis. HMP derivatisation increased the sensitivity of the method 400 to 2000 times compared with native molecules and enabled the determination of minute amounts of oxidised cholesterol species in the plasma of experimental animals.

Feldstein et al. (2010) used LC–ESI–MS/MS in the negative ionisation mode and multiple reaction monitoring (MRM) to profile arachidonic and linoleic acid oxidation products from biopsies and plasma samples of patients with non-alcoholic fatty liver disease. The investigators used gradient elution starting from 10 min elution with methanol/water/acetic acid (85:15:0.2, by vol), then over 2 minutes to the final composition of 100:0:0.2 (by vol), which was held for 15 min (5 μm, 150 x 2

mm, Phenomenex ODS column). Several hydroxy-eicosatetraenoic acids (5-, 8-, 9-, 11-, 12-, and 15-HETEs), epoxy-eicosatetraenoic acids (11,12- and 8,9-EETs), hydroxy-octadecadienoic acids (9- and 13-HODEs), and oxo-octadecadienoic acids (9- and13-oxoODEs) were identified and quantified.

2.6.3.3 Nuclear magnetic resonance spectroscopic methods

Nuclear magnetic resonance (NMR) spectroscopy is based on the electromagnetic interactions of sample molecules nuclei such as ^{1}H, ^{13}C, ^{31}P (or other NMR active nuclei) with radio frequency (rf) pulses. Each nucleus in each molecule is slightly different in relation to its surroundings; this causes an effect known as the chemical shift. The sample, which is usually dissolved in deuterated solvent, is placed inside a very homogenous and strong magnetic field in a special thin walled glass tubes, and excited with rf pulses (Friebolin 2005).

NMR spectroscopy is increasingly used not only to identify isolated pure compounds but to profile mixtures and complex biological fluids. Metabolomics has largely been driven by advances in NMR technology. Lipid analysis with NMR has been done for many years, but more recently, via higher magnetic strengths and higher resolution equipment, NMR has started to truly reveal its potential (Guillén et al. 2001). Related to NMR spectroscopy, electron spin resonance (ESR) spectrometry has the ability to measure the concentration and type of free radicals, which is very difficult by other conventional techniques (Lund et al. 2011).

Knothe et al. (2004) used ^{1}H-NMR to quantify fatty acids in oil samples as methyl esters and as TAGs, and compared the NMR method for GC analyses. The results of both techniques were in good agreement. Standard lipid oxidation determinations and proton NMR (300 MHz) experiments were compared by Wanasundara et al. (1995). Fatty acid composition, PV, TBARS, AnV, CD, CT, and total oxidation (TOTOX, defined as PV+AnV) measurements of oxidised canola and soybean oils were conducted according to AOCS and IUPAC protocols. Changes in the aliphatic proton to diallylmethylene proton ratio and the aliphatic proton to olefinic proton ratio correlated well with TOTOX values (R^2 0.926–0.985). PV alone had slightly worse correlations (R^2 0.623–0.880). The chemical shifts of many major and minor components of unoxidised and oxidised edible oils have been reviewed by Guillén et al. (2001). Kuklev et al. (1997) synthesised and measured the proton and carbon NMR spectra of ketodienoic (9-KODE and 13-KODE) and hydroxydienoic (9-HODE and 13-HODE) fatty acid methyl esters.

Guillén et al. (2008) investigated the degradation of edible oils subjected to high temperature (190°C) oxidation by proton NMR spectroscopy (400 MHz). The authors did not detect any primary lipid oxidation products, hydroperoxides, from the oils, but secondary oxidation products were detected. The carbonyl proton signals of aldehydes were detected and several of them were identified. *Trans*-2-alkenals, *trans,trans*-2,4-alkenals, 4,5-epoxy-*trans*-alkenals, 4-hydroxy-*trans*-alkenals comprised majority of the carbonyl peaks in oxidised virgin olive, corn, and linseed oils. The lack of hydroperoxides was attributed to the high temperature used in the oxidation, which rapidly degrades heat labile lipid hydroperoxides to secondary oxidation products. Previously, hydroperoxy and hydroxyalkenals were identified from thermally oxidised (70°C) vegetable oils (Guillén et al. 2004, 2005a, 2005b). Also, Goichoechea et al. (2010) used lower temperatures (70 and 100°C) and found abundant amounts of hydroperoxidised lipids in oxidised sunflower oil samples. In addition to hydroperoxides, several other oxidised functions such as conjugated dienic systems of hydroperoxy acyl groups, aldehydes including the genotoxic and cytotoxic oxygenated α,β-unsaturated aldehydes, and mono- and diepoxides were monitored by NMR. The authors were the first to report the formation of diepoxides in the autoxidised oils. The heating of vegetable oil by microwave until the temperature reaches 190°C produced less hydroperoxides than oxidation at 70°C. Oil with the least amount of linolenic and linoleic acids was the most resistant to microwave heat induced oxidation and produced the least amounts of toxic aldehydes (Guillén et al. 2006). Claxson et al. (1994) measured lipid peroxidation products in culinary oils and fats during the normal episodes of thermal stressing (30–90 min at 180°C) by one- and two-dimensional ^{1}H NMR. The authors identified several carbonyl compounds from heated oils used in restaurants. Hydroperoxides were detected even though the high temperature strongly favoured the decomposition of hydroperoxides to secondary oxidation products.

Silwood et al. (1999) used high resolution, two-dimensional ^{1}H and ^{13}C NMR techniques to characterise lipid oxidation products from autoxidised linoleoyl and linolenoylglycerols. ^{1}H-^{1}H *J*-resolved, ^{1}H-^{1}H total correlation (TOCSY), ^{1}H-^{13}C transfer heteronuclear multiple quantum coherence (HMQC) experiments were conducted on 600 MHz equipment, while ^{1}H-^{1}H relayed coherence transfer (RCT) experiments were conducted on a 400 MHz spectrometer. The two-dimensional experiments enabled the resolution of several vinylic and aldehydic resonances of lipid oxidation products, which appear as complex overlapping patterns in conventional one-dimensional spectra.

Pajunen et al. (2008) measured one- and two-dimensional proton and carbon NMR spectra of 10 isolated hydroperoxide isomers from autoxidised methyl 9-cis,11-trans-octadecadienoate (Me 9c,11t-CLA) and methyl 10-trans,12-cis-octadecadienoate (Me 10t,12c-CLA). The hydroperoxidised FAMEs were first purified by SPE and preparative NP HPLC. Seven isomers of Me 9c,11t-CLA and six isomers from Me 10t,12c-CLA were separated by preparative HPLC, which used 250 x 10 mm, 5 µm particle size semi-preparative column, and isocratic isopropanol/heptane (0.65:100, v/v) elution. 5 mm broadband inverse probe with z-gradient was used with 500 MHz NMR. ^{1}H NMR spectra was measured with 45° excitation pulse and ^{13}C{^{1}H} NMR spectra was measured with 30° excitation pulse. Other experiments performed included ^{1}H–^{1}H correlated spectroscopy (COSY), long range COSY (LR-COSY), total correlation spectroscopy (TOCSY), gradient heteronuclear single quantum coherence (gHSQC), and gradient heteronuclear multiple bond correlation (gHMBC). The conjugated diene allylic hydroperoxides were stable during the NMR experiments, which lasted on average for 16 h. Extensive data for chemical shifts, including solvent effects, were collected and analysed. The authors also used PERCH to simulate NMR spectra and the simulations were in good agreement with the measurements.

2.6.3.4 Analysis of core aldehydes and related compounds

Core aldehydes and other aldehydes can be detected from physiological samples and oxidised oils by many methods. Analysis of underivatised core aldehydes has been done previously by HPLC–APCI–MS analyses (Byrdwell et al. 2001). Core aldehydes were detected among other lipid oxidation products such as hydroperoxidised and epoxidised TAGs. Two distinct classes of epoxides were identified: ones where the epoxy group had replaced the site of unsaturation and ones with the epoxy group attached adjacent to the double bond.

The most convenient methods for analysing aldehydes include derivatisation with 2,4-dinitrophenylhydrazine and the subsequent detection of DNPH derivatives by LC–UV and/or LC–ESI–MS because of the increased detector response and specificity. DNPH-derivatives of common core aldehydes behave very similarly as free aldehydes in chromatographic systems enabling the comparison of underivatised and derivatised samples. The acidic derivatisation reaction is strong enough to revert Schiff bases back to free aldehydes and convert them to DNPH derivatives (Kamido et al. 1995). This enables the detection of bound aldehydes from complex samples.

Kamido et al. (1995) used HPLC–ESI–MS to identify lipid ester bound aldehydes from human plasma lipoproteins after copper-catalysed peroxidation. They used DNPH to release the aldehydes

from their adducts, and to enable more sensitive detection. Several phosphatidylcholine-derived core aldehydes were identified (1-palmitoyl-2-(9-oxononanoyl)-, 1-palmitoyl-2-(8-oxooctanoyl)-, and 1-palmitoyl-2-(5-oxovaleroy1)-*sn*-glycerols and similar 1-stearoyl-2-(oxoacyl)-*sn*-glycerols). In addition, several cholesterol ester derived aldehydes were identified (9-oxononanoyl, 8-oxooctanoy1, and 5-oxovaleroyl esters of cholesterol and 7-ketocholesterol). Previously, Kamido et al. (1992) synthesised cholesteryl esters, diacylglycerols and glycerophospholipids containing aldehyde functions, and later identified several core aldehydes from the *in vitro* peroxidation products of cholesteryl esters (Kamido et al. 1993).

Kurvinen et al. (1999) synthesised several Schiff base reference compounds from 2-[9-oxo]nonanoyl-*sn*-glycerol and different amino acids, peptides and aminophospholipids. They characterised the synthesised compounds with NP HPLC–ESI–MS and RP HPLC–ESI–MS after reducing the lipid adducts with cyanoborohydride to corresponding reduced Schiff bases. The RP LC system utilised gradient elution with 0.5% ammonium hydroxide in water/methanol/hexane (12:88:0 to 0:88:12, by vol; 100 x 2.1mm, 5 µm ODS column), and was used for the identification of peptide based Schiff bases. The normal phase LC method was based on a binary gradient consisting of (A) chloroform/methanol/30% ammonium hydroxide (80:19.5:0.5, by vol) and (B) chloroform/methanol/water/30% ammonium hydroxide (60:34:5.5:05, by vol), and was eluted from 100% A to 100% B in 14 min after 3 min initial 100% A with the final hold of 100% B for 10 min (250 x 4.6 mm, 5 µm particle size silica column). The NP LC system was used for the identification of aminophospholipid- and amino acid-derived Schiff bases.

Sn-1-alkyl- and *sn*-1-acyl type glycerophosphocholine (GPC) core aldehydes have been detected in human atheromas (Kamido et al. 2002). The study revealed that certain GPC core aldehydes can induce platelet aggregation and shape changes in biologically significant concentrations. The GPC core aldehydes also inhibited the endothelium-dependent relaxation of the artery. Tissue homogenates were treated with DNPH to convert all bound and unbound core aldehydes to DNPH derivatives, which were later identified with RP HPLC–ESI–MS.

1-palmitoyl-2-(5-hydroxy-8-oxo-oct-6-enoyl)-*sn*-glycero-3-phosphocholine (HOOA-PC) was found to regulate the inflammatory functions of endothelial cells in a study investigating polar lipid components of minimally modified/oxidised LDL (Subbanagounder et al. 2002). HOOA-PC was identified and quantified from ox LDL with RP HPLC–ESI–MS and HPLC–ESI–MS/MS analyses, and the structure was confirmed with identical chromatographic and mass spectrometric

characteristics of synthetic HOOA-PC. Synthetic HOOA-PC dose-dependently activated human aortic endothelial cells to bind monocytes. HPLC–ESI–MS analysis was performed with C8 column (250 x 2 mm, 5 µm particle size), which was eluted with methanol/water containing 1 mM ammonium acetate (linear gradient from 60% to 100% methanol in 60 min).

Sjövall et al. (2003) identified and quantified a large group of TAG core aldehydes as dinitrophenylhydrazones in autoxidised sunflower oil by RP HPLC–ESI–MS. They also used UV (358 nm) and a light scattering detector for the detection and compared the responses of MS and UV detections. A linear gradient elution with 20% to 80% isopropanol in methanol was used along with a 250 x 4.6 mm, 5 µm particle size ODS column. DNPH derivatives were detected in the negative ionisation mode as $[M–H]^-$ ions. After autoxidation for 18 days (60°C), the content of hydroperoxides and core aldehydes was estimated to be approx. 5% of the total acylglycerols. The authors identified several multifunctional dihydroxy and diepoxy core aldehydes. Previously, several core aldehydes were identified after the rapid oxidation of corn oil with *tert*-butyl hydroperoxide/Fe^{2+} by HPLC–ESI–MS (Sjövall et al. 2002).

Enoiu et al. (2000) used GC–MS and LC–ESI–MS to identify aldehydes produced from the ascorbic acid/$FeSO_4$ induced autoxidation of linoleic and linolenic acids after initial separation to polar and non-polar aldehydes with TLC. The polar fraction contained toxic hydroxyaldehydes e.g. several mono- and dihydroxy substituted alkanals, alkenals, and alkadienals such as 2-hydroxy-nona-3,5-dienal. Malondialdehyde was found to be a major oxidation product of linolenic acid but not linoleic acid. The LC separation of DNPH derivatives was achieved by elution with acetonitrile/water (50% to 95% acetonitrile in 30 min) and C18 column (125 x 2 mm, 5 µm particle size).

Zhang et al. (2010) developed a fast UHPLC–UV method for the analysis of several carbonyl compounds as DNPH derivatives. The method used stepwise gradient elution with water/acetonitrile (initially 68:32 and at the end 18:82, v/v) in 2.1 x 100 mm, 1.9 µm particle size C18 column. The method was linear over a large range (98–50000 ng/mL) for most of the compounds tested.

Handelman et al. (1998) investigated the changes of bovine insulin structure after hypochlorite-induced oxidation. They estimated that 5% of the hypochlorite-modified insulin reacted to DNPH, which specifically reacts with carbonyls. After total amino acid analysis, tryptic digestion and

HPLC–ESI–MS/MS analyses, amino-terminal pheynylalanine of the insulin B-chain was revealed to be the target of carbonyl modification. The authors hypothesised that the terminal amino groups of proteins are highly vulnerable to carbonyl formation. HPLC analyses of insulin and insulin derivatives were carried out with water/acetonitrile (95:5 to 50:50 in 20 min, v/v) and 250 x 4.6 mm, 5 µm particle size C4 column.

Also, derivatising reagents other than DNPH have been used for the analysis of aldehydes. Derivatisation of cholesteryl aldehydes with fluorescent 1-pyrenebutyric hydrazine enables the use of a highly sensitive fluorescence detector when performing HPLC analysis of oxidised cholesterol species. The method is quantitative, highly sensitive (femtomole range) and specific (Mansano et al. 2010).

Macrophages have been observed to increase LDL oxidation and even internalise core aldehydes in certain experimental settings (Karten et al. 1999). 5% of CE 18:2 and 4% of CE 20:4 in LDL were converted to 9-oxononanoyl (9-ONC) and 5-oxovaleroyl (5-OVC) cholesteryl ester core aldehydes, respectively. 9-ONC and 5-OVC were first derivatised with 1,3-cyclohexanedione and concentrated with SPE, and then analysed by HPLC equipped with the fluorescent detector (366 nm excitation and 455 nm emission). A C18 column (250 x 2.1 mm, 5 µm particle size) was used and eluted with the isocratic solvent system of acetonitrile/methanol/isopropanol (68:17:15, by vol).

2.7 Summary

Unsaturated fatty acids (UFAs) compose an important and essential part of human nutrition. Unfortunately, the double bonds in the UFAs make them susceptible to oxidation by reactive oxygen species (ROS). The formation of oxidised lipids increases rapidly when protective antioxidants are exhausted. On the other hand, the nature of antioxidants can lead to problems when fortifying foods with too many antioxidants. Pro-oxidative effects of several antioxidants have been observed when used in excessive amounts. The primary oxidation products of lipids are usually hydroperoxides, which ultimately decompose to secondary oxidation products with numerous end-products. Volatile oxidation products not only degrade the sensory quality of oils and fats but in many cases have also been found to be carcinogenic. Non-volatile lipid oxidation products contain typically hydroperoxy, hydroxyl, aldehyde, oxo, and epoxy groups. These functional groups alter the chemical and physiological properties of lipids. Core aldehydes, which are formed after the

breakdown of hydroperoxidised glycerolipids, can form adducts with amino acids, peptides, proteins, phospholipids, and DNA. Ingestion of these oxidised lipids can eventually lead to serious health effects.

Lipid digestion begins in the stomach when lingual and gastric lipases start to hydrolyse triacylglycerols, which are the main components of edible oils and fats. *Sn*-1 and *sn*-3 positions and shorter chain fatty acids are preferred by the two lipases. The stomach also contains oxygen, a low pH, and possibly abundant amounts of iron ions from any consumed meat. All of these enable the formation of ROS. Smooth muscles constrict the stomach and the formation of emulsion begins. The surface area of lipid droplets increases and oxygen can more easily attack the double bonds. Lipolysis continues in the intestine when pancreatic lipase along with co-lipase hydrolyses the remaining intact triacylglycerols and previously formed diacylglycerols to *sn*-2 monoacylglycerols and free fatty acids. Lipid absorption takes place mainly in the duodenum and jejunum.

Several antioxidative enzymes are found in the mammalian system. Glutathione peroxidases (GPx) are probably the most important lipid peroxide neutralising enzymes. Gastrointestinal GPx is active in the digestive system and likely eliminates most of the ROS present. Several superoxide dismutases (SOD) exist, but their action during digestion is unknown. Extracellular SOD is the most likely candidate of the SOD family of enzymes to neutralise ROS formed in the digestive system.

Methods for studying lipid oxidation are numerous. They range from unspecific titrimetric methods to highly specialised chromatographic and mass spectrometric methods. ROS can be measured in various matrices by luminescence and fluorescence methods. General antioxidant capacity tests give some idea of the protective capacity of different antioxidants, but fail to identify any possible oxidised lipids that may be formed. Gas chromatography (GC) or high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS), on the other hand, can give very detailed information about the lipid oxidation products. Some tandem mass spectrometric methods even enable the determination of the exact placement of oxidised groups in the lipid molecules. Nuclear magnetic resonance (NMR) spectroscopy, especially proton (^{1}H) NMR, is a promising technique for the fast screening of lipid samples, as it is non-destructive and because of the large dynamic scale of the technique. Drawbacks of NMR are that a relatively large amount of sample is required for the analysis and that specific molecular structures may be difficult to identify from complex spectra.

3 AIMS OF THE CURRENT STUDIES

Previous experiments by different research groups have established deep knowledge of lipid autoxidation by different mechanisms outside the human body, while some limited studies have started to enlighten the less researched area of the *in vivo* and *in vitro* oxidation of lipids. The aims of the experiments in this thesis were defined as:

i) To study how lipid oxidation is affected by an *in vitro* digestion model

ii) To develop novel comprehensive analysis techniques for the analysis of lipid oxidation in complex biological samples such as *in vitro* digestion samples

iii) To develop faster and more sensitive methods for the analysis of lipid oxidation

iv) To study the efficacy of common antioxidants against autoxidation during digestion

v) To study the fate of toxic core aldehydes and the formation of Schiff bases during digestion

4 MATERIALS AND METHODS

4.1 Oxidation of rapeseed oil

Kultasula branded rapeseed oil (Raisio Oy, Raisio, Finland) was oxidised in several ways to produce oxidised oils for the experiments. Thermal oxidation in a convection oven was used to create mildly and highly oxidised oils. Two oxidised oils were produced by heating fresh oil at 60°C and at 100°C for 48h. Chemical oxidation was done according to Harry-O'Kuru et al. (2002) by mixing fresh rapeseed oil with formic acid and slowly adding hydrogen peroxide while constantly stirring the mixture for 15h at 70°C. Water and HCl was added and the mixture was heated for another 15h at 70°C. The resulting oxidised oil was purified with liquid-liquid extraction. Oxidised oil in ethyl acetate was transferred into a separatory funnel and washed sequentially first with saturated NaCl solution, then with saturated $NaHCO_3$ solution and finally with purified water (Millipore, Molsheim, France). The oxidised oil was recovered from the lower organic phase and dried in a rotary evaporator under vacuum. Peroxide values for the oxidised oils were determined according to AOCS protocol Cd 8-53 (Firestone 2009) and they were 100 meqO_2/kg for the oil heated at 100°C and 20 meqO_2/kg for the chemically treated oil.

Special ozonisation procedure was used for the synthesis of core aldehyde rich oils. Rapeseed oil was bubbled with freshly prepared O_3 to produce triacylglycerol ozonides, which in turn were converted to core aldehydes by reducing the ozonide groups with triphenylphosphine (TPP). Several reaction times with ozone were tested, as the oxidation proceeded from mono-ozonides to diozonides and triozonides very quickly. TLC was used to monitor the formation of ozonides. The presence of core aldehydes was confirmed with UHPLC−ESI−MS analyses after derivatisation to core aldehyde 2,4-dinitrophenyl hydrazones with DNPH.

4.2 Synthesis of oxidised reference compounds

Free fatty acids (oleic acid, linoleic acid, and α-linolenic acid) were oxidised by the method of Neff et al. (1982) to produce hydroperoxidised fatty acids. Briefly, fresh free fatty acid was dissolved in dichloromethane containing 0.1 mM methylene blue as photosensitiser. The mixture was held under 250W UV light for 15h (distance from the light 20 cm) in an ice bath. In addition to free fatty acid

hydroperoxides, also hydroperoxidised monoacylglycerols and diacylglycerols were prepared from 1(3)-monooleoyl-*sn*-glycerol, 1(3)-monolinoleoyl-*sn*-glycerol, 1(3)-monolinolenoyl-*sn*-glycerol, 1,2(2,3)-dioleoyl-*sn*-glycerol, and 1,2(2,3)-dilinoleoyl-*sn*-glycerol. The hydroxy derivatives of the hydroperoxidised oxylipids were prepared by treating the previously synthesised peroxidised lipids with triphenylphospine, which reduces the hydroperoxy groups to hydroxyl groups. The epoxy derivatives of free fatty acids were prepared by the method of Deffense (1993) by mixing 15 mg of free fatty acid in dichloromethane with 3-chloroperoxybenzoic acid.

Reference Schiff bases were prepared as previously described by Kurvinen et al. (1999). First, 2-(9-oxo)-nonanoyl-*sn*-glycerol was prepared from 2-oleyl-*sn*-glycerol by ozonolysis and TPP reduction, and purified with TLC (heptane/isopropyl ether/acetic acid, 60:40:4, by vol). Several Schiff bases were then prepared by reacting 2-(9-oxo)-nonanoyl-*sn*-glycerol with valine, glycine−glycine, and leucine−glycine−glycine. The reactions were done in the dark at 0°C, after which the resulting Schiff bases were converted to stable reduced Schiff bases by reaction with cyanoborohydride.

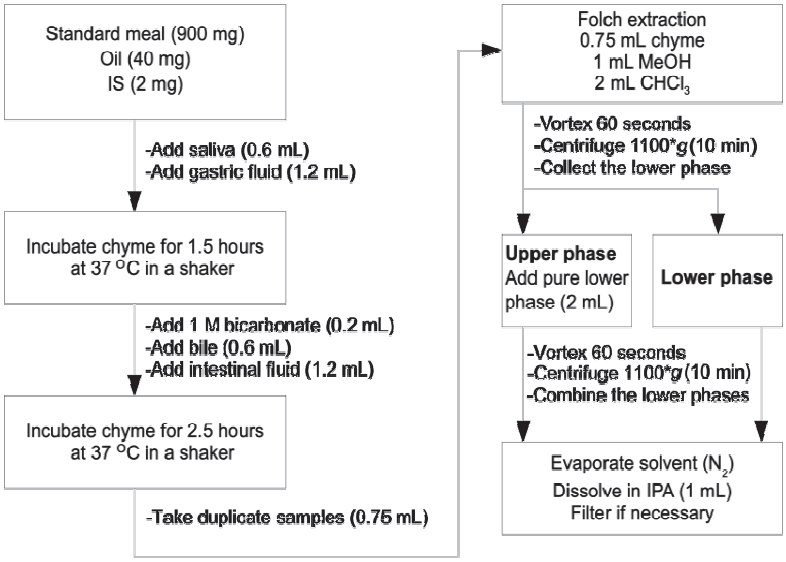
4.3 Adaptation of artificial digestion model (I−IV)

The artificial digestion model was adopted from Versantvoort et al. (2009a). The composition of the digestive juices was adjusted as in the fed state in the human stomach Versantvoort et al. (2009b). The model was down-scaled to enable work with multiple parallel samples. **Table 1** describes the composition of the model and **Scheme 1** all the different stages of digestion and extraction. Dry reagents were weighed into 25 mL volumetric bottles, purified water was added and after the mixing of the enzymes, other reagents added. Digestive juices were prepared just before the experiments and warmed to 37°C before use. Incubation was performed in an angled (45°) mixing device at 800 rpm in a 37°C heat room.

The standard meal was composed of milk protein isolate, potato starch, and fibre preparation (cereal hull mix). All lipids were extracted away from the protein isolate and fibre preparation by 48 hour chloroform/methanol (2:1, v/v) extraction in a Soxhlet apparatus. Fibre preparation was milled to fine powder prior to use.

Table 1. Composition of the digestive juices in the artificial digestion model.

Reagents	**Stock solution**	**Saliva**	**Gastric juice**	**Intestinal juice**	**Bile**
	(mg/25mL)	*(µL/25mL)*	*(µL/25mL)*	*(µL/25mL)*	*(µL/25mL)*
KCl	2240	500	460	315	210
KSCN	500	500	–	–	–
$NaH_2PO_4*2H_2O$	2889	500	150	–	–
$Na2SO_4$	1425	500	–	–	–
NaCl	4382.5	85	785	2000	1500
$NaHCO_3$	2117.5	1000	–	2000	3415
$CaCl_2*2H_2O$	555	–	900	450	500
NH4Cl	765	–	500	–	–
HCl (37%)	–	–	325	9	7.5
KH_2PO_4	200	–	–	500	–
$MgCl_2*6H_2O$	266.9	–	–	500	–
Urea	625	400	170	200	500
Glucose	1625	–	500	–	–
Glucuronic acid	50	–	500	–	–
Glucoseamine*HCl	825	–	500	–	–
		(mg/25mL)	*(mg/25mL)*	*(mg/25mL)*	*(mg/25mL)*
α-amylase		14.5	–	–	–
BSA		–	50	50	90
Uric acid		0.75	–	–	–
Mucin		1.25	150	–	–
Pepsin		–	125	–	–
Pancreatin		–	–	450	–
Lipase		–	–	75	–
Bile		–	–	–	1500



Scheme 1. The artificial digestion and extraction processes.

4.4 Sample preparation for experiments with different antioxidants (III)

Two sets of experiments were performed with antioxidants. A preliminary experiment was conducted using an UHPLC-ESI-MS method (Tarvainen et al. 2011) and a second, complimentary experiment was done with the improved lithium adduct UHPLC-ESI-MS method (Tarvainen et al. 2012). For the preliminary experiments, three different oils were incubated in the artificial digestion model with L-ascorbic acid, 6-palmitoyl-*O*-L-ascorbic acid, 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), DL-α-tocopherol, and DL-α-tocopheryl acetate in different concentrations and combinations (see **Table 2**). For the second experiment, only fresh unoxidised rapeseed oil was incubated with the mentioned antioxidants (see **Table 3**).

Table 2. Samples[a] prepared in the preliminary *in vitro* digestion study for mixing with the standard meal.

I. Fresh rapeseed oil (40 mg)[b]
II. Chemically oxidised rapeseed oil
III. Thermally oxidised rapeseed oil

Antioxidant addition	Amount of added antioxidant (% of oil)
1. No added antioxidant	–
2. FFAs and DAGs without added antioxidant[c]	–
3. FFAs and DAGs with DL-α-tocopherol[c]	0.0125
4. DL-α-tocopherol, high	1.2500
5. DL-α-tocopherol (T)	0.0125
6. DL-α-tocopheryl acetate (TA)	0.0125
7. 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT)	0.0125
8. 6-palmitoyl-*O*-L-ascorbic acid (PA)	0.0125
9. T + PA	0.0250[d]
10. T + TA	0.0250[d]
11. T + BHT	0.0250[d]
12. TA + BHT	0.0250[d]
13. TA + PA	0.0250[d]
14. PA + BHT	0.0250[d]

[a] 3 replicates of each antioxidant combination. (12 x 3 + 2) x 3 separate digestions in total.
[b] Natural vitamin E content in the rapeseed oil was 0.025%.
[c] Free fatty acids (FFAs) and diacylglycerols (DAGs) added to fresh rapeseed oil (I.) only.
[d] Combined concentration of the added antioxidants.

Table 3. Samples[a] prepared in the second *in vitro* digestion study with fresh rapeseed oil for mixing with the standard meal.

Antioxidant addition	Amount of added antioxidant (% of oil)	
1. No added antioxidant[b]	-	-
2. DL-α-tocopherol (T)	0.01	0.10
3. DL-α-tocopheryl acetate	0.01	0.10
4. 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT)	0.01	0.10
5. L-ascorbic acid (AA)	0.01	0.10
6. 6-palmitoyl-*O*-L-ascorbic acid (PA)	0.01	0.10
7. T + AA	0.01[c]	0.10[c]
8. T + BHT	0.01[c]	0.10[c]
9. T + PA	0.01[c]	0.10[c]

[a] 10 replicates of each antioxidant level. 17 x 10 digestions in total.
[b] Natural vitamin E content in the rapeseed oil was 0.025%.
[c] Combined concentration of the added antioxidants.

4.5 Extraction of samples (I−IV)

A modified Folch (1956) extraction was used to extract all of the lipophilic components from the digestion samples. 750 µL duplicate samples were taken from the digestion tubes and placed in disposable glass test tubes; 2 mL of chloroform and 1 mL of methanol was added. After thorough mixing, the tubes were centrifuged at 1000 *g* (3500 rpm) and the lower phase was collected. The pure lower phase was added (2 mL) and the extraction was repeated. The lower phases were combined and evaporated to dryness. For LC analysis, the residue was dissolved into 1 mL of isopropanol.

In the studies with core aldehydes and Schiff's bases, the upper, more hydrophilic phase was also examined. First, 2 mL of chloroform and 1 mL of methanol was added to 750 µL duplicate samples. After thorough mixing and centrifugation, the upper and lower phases were carefully collected separately into new disposable glass test tubes. Pure upper and lower phases were added into the original extract tube, mixed, and centrifuged. The upper and lower phases were again collected and combined separately. The solvents were removed by nitrogen stream and the upper phase residue was dissolved into methanol:acetonitrile (50:50, v/v) and the lower phase residue into isopropanol for UHPLC analyses or into deuterated chloroform for ^{1}H NMR analysis.

4.6 HPLC−ELSD−ESI−MS analysis of digested unoxidised and oxidised rapeseed oils (I)

A high performance liquid chromatography−evaporative light scattering detector−electrospray ionisation−mass spectrometric method (HPLC−ELSD−ESI−MS) was developed for the analysis of the digested lipid components in the chyme. The method enabled the separation of nearly all of the individual free fatty acids, monoacylglycerols, diacylglycerols, triacylglycerols and their oxidised equivalents. Previous works by Hsu et al. (2007) and Sjövall et al. (1997) were used as the basis for the combined method for analysing free fatty acids and acylglycerols. A Waters Acquity UPLC equipped with a column oven and a reversed phase Nucleodur ISIS C-18 column (250 x 4.6 mm, 5 µm particle size; Macherey-Nagel, Betlehem, PA, USA) was used as an LC inlet. The flow line was split to a Sedex LT-75 evaporative light scattering detector (Sedere Inc., Lawrencewille, NJ, USA) and to a Waters Quattro Premier tandem mass spectrometer equipped with electrospray ionisation (ESI) probe (Waters Corp., Milford, MA, USA) with an adjustable tee. Total flow-rate was 1.0 mL/min, of which 20% was directed to the mass spectrometer. A solvent gradient program was used for the separation of analytes after 10 µL full loop injections. Solvent B was increased from initial 10% to 60% in 40 min and to 95% in 85 min. Solvent A was composed of acetonitrile/H_2O/formic acid (50:50:0.1, by vol) and solvent B of isopropanol/acetone/formic acid (90:10:0.1, by vol). The ELSD was operated at 40 psi and 70°C and the column oven at 60°C.

The mass spectrometer was mass calibrated by the infusion of 0.1% TFA. The resulting water clusters $[H_3O + nH_2O]^+$ peaks (31 peaks) were used for the accurate mass calibration of each quadrupole for mass range of 50−1,800 Da with scanning and static scans. Scan speed compensation calibration was performed for scanning speeds between 300−5,000 amu/s. The mass spectrometer was operated in the positive ionisation mode and full scans of 150−1,500 *m/z* were collected. The ESI capillary voltage was set at 3.00 kV, the cone voltage at 300 V, the extractor voltage at 2 V the source temperature at 120°C, the desolvation temperature at 170°C, the desolvation gas flow at 600 L/h and the cone gas flow at 500 L/h.

The quantifications were based on the calibration curves of reference compounds made by linear fitting of the area of selected single ion mass chromatogram peaks versus concentration. Waters MassLynx 4.1 and Origin 8.4 SR software (OriginLab Co., Northampton, MA, USA) was used for the extraction and area integrations of mass chromatograms and the preparation of calibration curves. The duplicate sets of the mixtures of reference compounds were injected at 5 different

levels. 10, 5, 2.5, 1.25, 0.625 µg loads were used for free fatty acids, and one tenth of the amount of FFAs for MAGs, DAGs, and TAGs. An internal standard (12-hydroxyoctadeacnoic acid) was used to correct for any sample loss during the extraction procedure. The saturation of the MS signal limited the accuracy of quantification in higher concentrations. No reliable quantification was performed for the oxidised lipids because the reference compounds acquired by synthesis were not sufficiently pure.

4.7 GC–FID analysis of fatty acids (I)

Fatty acid methyl esters were prepared from the unoxidised rapeseed oil by borotrifluoride catalysed methylation and subsequent gas chromatographic (GC) analysis. Triheptadecanoate was used as an internal standard. A Perkin-Elmer Auto-System gas chromatograph equipped with an autosampler and flame ionisation detector (FID) was used with a J.W. Scientific DB-23 column (60 m x 0.25 mm i.d., 0.25 µm film thickness, Agilent Technologies Inc., Santa Clara, CA, USA). A gradient temperature program was used to separate the fatty acid methyl esters. The initial oven temperature of 130°C was held for 1 min, then increased to 170°C (6.5°C/min), then to 215°C (2.8°C/min) and held for 12 min; finally, the temperature was increased to 230°C (40°C/min) and held for 3 min. The detector and autosampler were held at 270°C. The injection volume was 1 µL and one analysis was done of each of the four replicate methylations.

4.8 UHPLC−ESI−MS analysis of digested unoxidised and oxidised rapeseed oils (II, III, and IV)

An ultra-high performance LC−ESI−MS method was developed to increase the speed of the analysis of free fatty acids and acylglycerols. A Waters Acquity UPLC™ was used with a reversed phase BEH C18 column (100 x 2.1 mm, 1.7 µm particle size, Waters Corp.) A solvent gradient and a column at elevated temperature were used to enhance the separation of different lipid components. Solvent A was composed of H_2O/acetonitrile/formic acid (50:50:0.1, by vol). Solvent B was composed of isopropanol/formic acid (100:0.1, v/v). The initial composition of 14% of B was increased to 85% of B in 14.0 minutes and reduced then back to 14%. The initial flow rate of 0.65 mL/min was reduced to 0.5 mL/min in 14 minutes. A column oven capable of up to 65°C temperature was used to increase mass transfer inside the column and to decrease the high back

pressure. The back pressure would have risen over the limits of the used UHPLC system (1,000 bars) if the moderately high flow rate had been used with the high isopropanol content solvent mixture at room temperature. The temperature of 60°C was selected as an optimum temperature for increased resolution of the analytes and sufficiently decreased back pressure. Maximum back pressure during optimised analysis was 965 bars, which was within the usable operating range of the UHPLC system.

All of the solvent flow from the UHPLC was directed to the ESI probe of Water Quattro Premier tandem mass spectrometer. The tuning was optimised for the simultaneous detection of FFAs, MAGs, DAGs and TAGs. The ESI capillary voltage was set at 3.0 kV and the cone voltage at 320 V. The extractor voltage was set at 8 V and RF lens voltage at 1.0 V. Ion source temperature was set at 80°C and desolvation temperature at 350°C. Desolvation gas flow was set at 500 L/h and cone gas flow at 300 L/h.

Synthesised reference compounds were used to estimate the amounts of oxidised lipids present in the samples. The mass chromatogram peaks identified as oxidised compounds were integrated and the areas were used in the quantitative calculation. 12-Hydroxyoctadecanoic acid was used as an internal standard. Multiple known and characteristic ions were used in the detection of oxidised compounds from the mass chromatograms. Because the purities of the synthesised oxidised fatty acids and acylglycerols were not sufficient, the results are only semi-quantitative.

4.9 UHPLC–ESI–MS lithium adduct analysis of digested unoxidised rapeseed oil (III)

To increase the sensitivity of the UHPLC−ESI−MS analysis, a new method utilising lithium formate as solvent additive was developed. Lithium was previously used by Hsu et al. (1998, 1999) and Cheng et al. (1998) in the analysis of GPCs and TAGs. A Waters Acquity UPLC™ was used as an LC inlet for a Waters Quattro Premier tandem mass spectrometer. MassLynx v4.1 and QuanLynx (Waters Corp.) were used for the collection and analysis of mass chromatograms and spectra. A reversed phase 2.1 x 100 mm (1.8 μm particle size) Phenomenex Kinetex C18 column (Phenomenex Inc., Torrence, CA) was used for the chromatographic separation of oxidised and unoxidised free fatty acids and acylglycerols. A column oven was utilised and set at 60°C. Solvent A was composed of acetonitrile/H_2O/formic acid (50:50:0.1, by vol) and solvent B of

acetone/formic acid (100:0.1, by vol). Both solvents also contained lithium formate (1 mM) as an ionisation enhancer. The initial gradient of 1% B was increased to 99% B in 14.00 minutes, and back to 1% B in 14.05 minutes with the constant level of 1% B until 16.50 minutes. The flow rate was 0.90 mL min^{-1} and the injection volume was 3 µL.

The ESI capillary voltage was set at 4.00 kV. Cone voltage was initially 60 V and after 8.70 minutes was 350 V. RF lens voltage was set at 0.2 V and Extractor voltage at 8 V. Ion source temperature was set at 100°C and desolvation temperature was 400°C. Desolvation gas (N_2) flow was set at 900 L/h and cone gas (N_2) flow at 400 L/h. The mass spectrometer was mass calibrated with water clusters for *m/z* 50–1,800 and the scanning speeds of 300–5,000 amu/s. Mass spectra were collected initially of ions with *m/z* 165–800 and after 8.70 minutes *m/z* 350–1,100.

4.10 UHPLC–ESI–MS analysis of amino acids and peptides (IV)

Amino acids and peptides were analysed with a Waters Acquity UPLC and Waters Quattro Premier tandem quadrupole mass spectrometer. A Phenomenex Kinetex C18 column (100 mm x 2.1 mm, 1.7 µm pore size) was used for chromatographic separations. An electrospray ionisation probe (ESI) was used to ionise the sample molecules in the positive ionisation mode and full scans were acquired between *m/z* 50–1500. The mass spectrometer was mass calibrated with water cluster calibration as explained previously. All acquisitions were done so that there was a minimum of 17 data points across the peaks. The ESI probe voltage was set at 3.0 kV, the cone voltage at 30 V, the RF lens voltage at 0.2 V and extractor voltage at 8 V. Ion source temperature was set at 100°C and desolvation temperature at 400°C. Nitrogen was used as the nebulising gas at 900 L h^{-1} and cone gas flow was set at 400 L h^{-1}.

A solvent gradient program was used to analyse the hydrophilic components in the samples. Solvent A was composed of water/TFA (100:0.1, by vol) and solvent B of acetonitrile. A linear gradient starting from 5% B was increased to 100% B in 20 minutes. The injection volume was 3 µL. Reference amino acids, dipeptides and tripeptides were used to identify the peaks.

4.11 NMR experiments

Bruker Avance 500 NMR spectrometer equipped with a BBI-5mm-Zgrad-ATM probe (Bruker BioSpin, Fällanden, Switzerland) was used to collect ^{1}H NMR spectra of the samples. All spectra were acquired with 128 scans at 25°C using standard ^{1}H NMR pulse sequence with a 30° flip angle. Free induction decays (FIDs) were collected with 64k data points and an acquisition time of 3.28 s, a spectral width being 10000.0 Hz. Tetramethylsilane (TMS) was used as an internal standard (chemical shift 0.00 ppm). Extracted samples in deuterated chloroform (600 µL) were transferred into 5 mm diameter NMR tubes (WILMAD, Emperor grade) for analysis. Spectra were processed with Topspin 1.3 and Amix Viewer 3.9.7 (Bruker BioSpin) software. For quantitative analysis, spectra were scaled and equalised to the chloroform peak.

4.12 Statistical Analysis

Data from the antioxidant experiments were analysed by the one-way analysis of variance (ANOVA) with post-hoc Tukey's HSD for statistical differences between the groups. *P*-values of less than 0.05 were considered statistically significant. IBM SPSS Statistics version 19 (IBM Corporation, New York) was used for the statistical calculations.

5 RESULTS AND DISCUSSION

Major results were acquired during the four separate studies marked in roman numerals (**I**–**IV**) in the text. Hundreds of different lipid molecules were identified from the unoxidised and oxidised oils and digested oil samples. The mass chromatograms, spectra and extensive tables of identified compounds are published in the corresponding papers (**I**–**IV**) and not repeated here, excluding some examples of chromatograms.

5.1 Applicability of the newly developed analytical methods

The new analytical methods developed during this thesis work enabled the study of lipid hydrolysis and the formation of oxygenated fragments of oxidised oils in great detail. These new methods enable not only the identification and even quantification of almost all major lipid classes present in the digestive system, but they can be adapted to other areas of research, such as biodiesel quality control, etc. Previously, several methods would have to be utilised simultaneously to obtain similar results. Also, the increased speed and sensitivity of analysis enabled screening the effects of several antioxidants against oxidation in the artificial digestion model. The use of artificial digestion model enabled the study of unhealthy oxidised oils without the ethical problems arising from the use of experimental animals or human test subjects.

5.2 Identification of oxidised molecular species in oxidised oils

Studies **I**–**IV** used oxidised oils in the digestion experiments. The oils were characterised by HPLC–ELSD–ESI–MS (study **I**), UHPLC–ESI–MS (studies **II–IV**), and ^{1}H NMR (study **IV**) analyses. Hydroperoxidised triacylglycerols, epoxy-triacylglycerols, hydroxy-triacylglycerols, oxo-triacylglycerols and oxoTAGs with two different oxidised groups were detected from the thermally oxidised oils (**I**–**III**). Hydroxy-triacylglycerols and epoxy-triacylglycerols were the major constituent of chemically oxidised oil (**I**–**III**). Minor amounts of core aldehydes were present in heat treated oils (**I**–**IV**), as expected, and abundantly in specially prepared ozonised oils (**IV**). The findings were in accordance with the previous studies of the constituents of oxidised seed oils by different LC–MS methods (Sjövall 2001, 2002a, 2002b, 2003, Frankel 1984, Byrdwell 1999, 2001, 2004).

5.3 Identification of oxidised lipolysis products (I–III)

Lipid hydrolysis and incubation in the artificial digestion model had major effect on the lipid constituents of the chyme. **Figure 1** shows the total ion current mass chromatograms of three oils before and after digestion, analysed by UHPLC–ESI–MS (from study **III** as an example). Hydroperoxidised lipids were quickly degraded to secondary oxidation products and were not detected in any samples after the three-part digestion. Epoxidised lipids, however, were detected from digested heat treated oil samples, implying that they could be available for absorption in the intestines. Oxylipids containing hydroxyl groups were the major class of oxidised triacylglycerol fragments present in the digested oxidised rapeseed oil samples, with hydroxy fatty acids and hydroxy monoacylglycerols being the major individual classes.

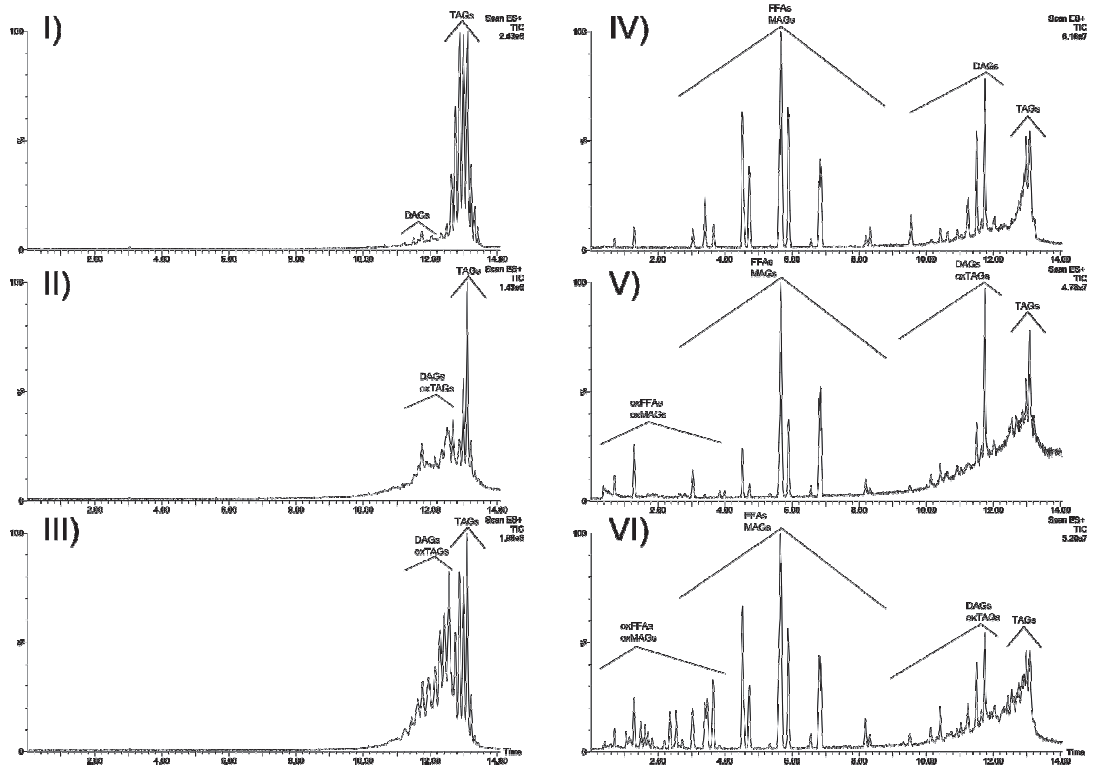


Figure 1. Undigested unoxidised rapeseed oil (**I**), undigested thermally oxidised rapeseed oil (**II**), undigested chemically oxidised rapeseed oil (**III**), digested unoxidised rapeseed oil (**IV**), digested thermally oxidised rapeseed oil (**V**), and digested chemically oxidised rapeseed oil (**VI**). Analysed with the UHPLC–ESI–MS lithium adduct method as explained in materials and methods. Detailed peak listings and identifications provided in Tarvainen et al. (2012). The effects of antioxidants on rapeseed oil oxidation in an artificial digestion model analysed by UHPLC–ESI–MS. *J. Agric. Food Chem.* 60: 3564–3579.

Significant amounts of different constitutional isomers of saturated 18 carbon monoepoxy fatty acid (*m/z* 321.3 corresponding to $[M + Na]^+$ adduct and *m/z* 297.4, corresponding to $[M - H]^-$ ion) and 18 carbon monoepoxy fatty acid with one remaining double bond (*m/z* 319.3 corresponding to $[M + Na]^+$ adduct and *m/z* 295.4 corresponding to $[M - H]^-$ ion) were identified in digested thermally oxidised oil samples. Small amounts of monoepoxy octadecadienoic acid were also detected at *m/z* 293.4 and *m/z* 317.3. On the basis of both negative and positive ionisation MS analyses, there were approximately equal amounts of the isomers of monoepoxy octadecanoic and monoepoxy octadecenoic acids present. The combined amounts of the three epoxidised free fatty acids strongly reflected the overall amounts of oxylipids in the samples.

5.4 Proportions of monoacylglycerol positional isomers in digested chyme (I)

Particularly interesting observation from the LC–MS studies of the digested oils was that the amounts of *sn*-1(3)-monoacylglycerols in relation to *sn*-2-monoacylglycerols were much higher than expected. It is thought that pancreatic lipase almost exclusively cleaves fatty acids from s*n*-1 and *sn*-3 positions and the resulting monoacylglycerol should be *sn*-2-monoacylglycerol. In our experiments, which closely mimic human digestion, the proportions of 2-oleoyl-*sn*-glycerol/1(3)-oleoyl-*sn*-glycerol, 2-linoleoyl-*sn*-glycerol/1(3)-linoleoyl-*sn*-glycerol, 2-linolenoyl-*sn*-glycerol/1(3)-linolenoyl-*sn*-glycerol, produced from unoxidised rapeseed oil, were 65%/35%, 75%/25%, and 85%/15%, respectively. The reason for high amounts of *sn*-1(3)-monoacylglycerol positional isomers remains unknown, although speculations of enzyme preference or larger than believed acyl migration during digestion could be presented.

5.5 Effects of antioxidants on rapeseed oil oxidation in artificial digestion model (III)

Several different antioxidants were screened for their effects on the artificial digestion model. L-ascorbic acid, 6-palmitoyl-*O*-L-ascorbic acid, 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), DL-α-tocopherol, and DL-α-tocopheryl acetate had different kinds of effects on thermally oxidised oil than chemically oxidised oil. A total 114 of assays, excluding blanks and reference compound analyses, were performed with the three oil preparations in a preliminary experiment.

Hydroperoxides, epoxides and hydroxyl compounds acids were given special attention in the analysis of mass spectral data.

As epoxidised free fatty acids were detected in thermally oxidised oil, it was interesting to note that only in samples with the high tocopherol addition were the concentrations of epoxidised FFAs slightly lower than in samples without any added antioxidants. In samples with DL-α-tocopherol and BHT, there were equal amounts of epoxidised FFAs compared with the digested samples without added antioxidants. The rest of the antioxidant additions seemed to increase the amounts of epoxidised FFAs two- to three-fold. Previously, epoxides were observed to decompose to vicinal diol structures in acidic conditions (Giuffrida 2004a). The difference in the fate of epoxidised lipids compared with the previous report may be attributed to the shorter exposure time to the acidic medium in our experiments and differences in the gastric models.

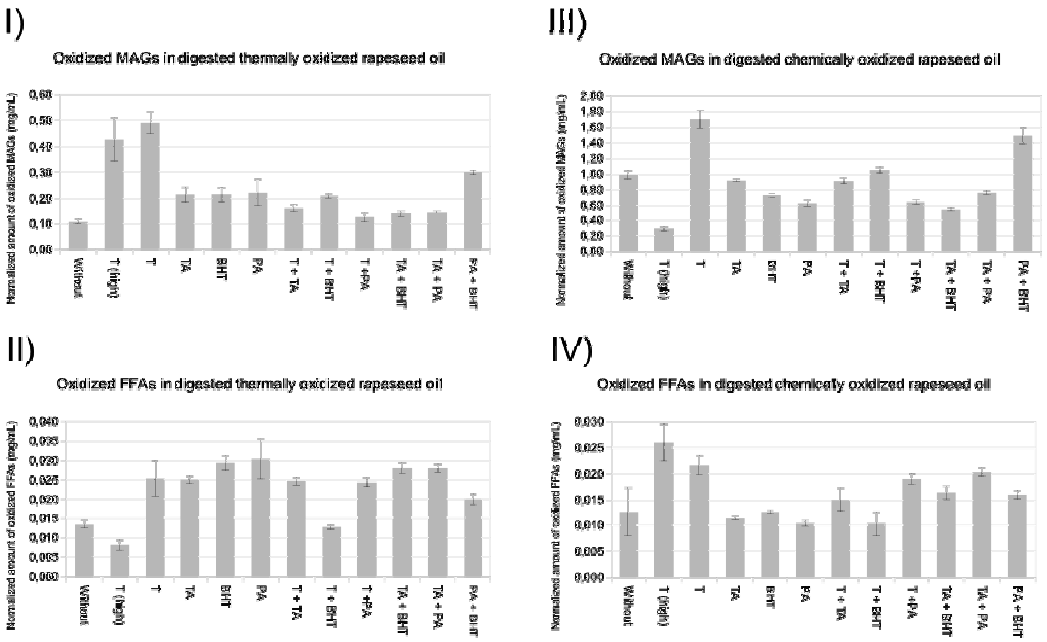


Figure 2. The normalised amount of detected oxidised MAGs in digested thermally oxidised rapeseed oil (**I**), oxidised FFAs in digested thermally oxidised rapeseed oil (**II**), oxidised MAGs in digested chemically oxidised rapeseed oil (**III**), and oxidised FFAs in chemically oxidised rapeseed oil (**IV**). Preliminary experiment.

Figure 2 shows the amounts of oxidised lipids in the digested oxidised rapeseed oils in a preliminary experiment. Normalised amounts of detected oxidised MAGs in the digested thermally oxidised rapeseed oil are shown in (**Fig. 2 I**), oxidised FFAs in digested thermally oxidised

rapeseed oil in (**Fig. 2 II**), oxidised MAGs in digested chemically oxidised rapeseed oil in (**Fig. 2 III**), and oxidised FFAs in chemically oxidised rapeseed oil in (**Fig. 2 IV**). The addition of high amounts of DL-α-tocopherol (T, high) had varying results. In the case of thermally oxidised rapeseed oil, high amounts of oxidised MAGs were detected, but little oxidised FFAs. In the case of chemically oxidised rapeseed oil, high amounts of oxidised FFAs were detected and low amounts of oxidised MAGs. High amount of antioxidants perhaps selectively stabilise some oxidised molecules found in the oxidised oils. In general, other tested antioxidants had no positive (decreasing) effect on the amounts of oxidised lipids detected. The amounts of oxidised FFAs and MAGs were on a similar level or higher than in the samples without any added antioxidant. A small addition of DL-α-tocopherol (T) seemed to increase the amount of oxidised lipids. The explanation of this phenomenon might be that primary oxidation products are rapidly decomposed to secondary oxidation products in the artificial digestion model, but the addition of antioxidants alters the decomposition pathways resulting in different end-products. Specifically, the action of tocopheryl acetate (TA) was interesting, as the acetyl group would need to be hydrolysed before any antioxidative effects can be expected. Thermally oxidised digested rapeseed oil samples containing TA had, on average, slightly more oxidised lipids than samples without any added antioxidants. On the other hand, chemically oxidised rapeseed oil samples had equal amounts of oxylipids in samples with and without TA.

No oxidised lipids were detected after the digestion of native rapeseed oil samples and samples with added FFAs and DAGs in the preliminary experiment. Because the amounts of oxidised lipids generated during the digestion of rapeseed oil were small and challenging to determine, a more sensitive lithium adduct-based detection method was developed. The lithium adduct method enabled the detection of oxidised lipids from digested fresh rapeseed oil. **Figure 3** shows the amounts of oxidised MAGs in different antioxidant addition groups. Oxidised MAGs were detected in all groups, thus questioning the efficacy of the tested antioxidants against lipid oxidation during the digestion of lipids.

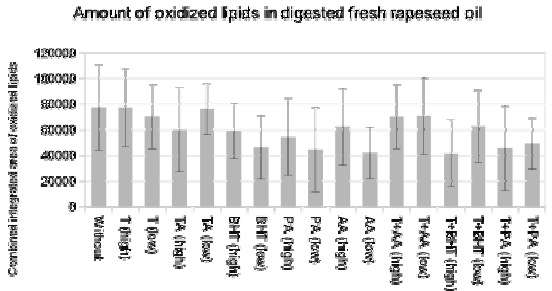


Figure 3. The amount of oxidised MAGs in digested fresh rapeseed oil in different antioxidant addition groups as analysed by UHPLC–ESI–MS.

5.6 Core aldehydes in artificial digestion model (IV)

Core aldehyde-rich oil was synthesised for studying the behaviour of carbonyl compounds in the artificial digestion model. While the undigested oxidised oil contained abundant amounts of core aldehydes, none were detected in the digested oxidised oils by UHPLC–ESI–MS analyses. However, ^{1}H NMR analyses clearly revealed that carbonyl functions were, indeed, present even in digested mildly oxidised rapeseed oil (see **Figure 4**). Incubation in the digestion model up to the stomach phase for extended periods of time did not lower the amount of oxo-groups in the samples. However, further incubation in the intestinal phase resulted in the marked reduction of carbonyl functions in the NMR spectra (we estimated that 80% of the signals were lost, see **Fig. 4 II**). There were also small changes in chemical shifts of the carbonyl signals, but this may be attributed to the different pH in the stomach phase and intestinal phase. Several control incubations were done along the samples and based on all the analyses, it appears that lipid aldehydes may be present and available for absorption in the intestinal phase of digestion.

The lost core aldehydes in the UHPLC–ESI–MS analyses and reduced carbonyl signals in proton NMR experiments of digested oxidised oils indicate that protein-lipid and amino acid-lipid interactions were taking place. Schiff base and Michael addition products were expected and reference Schiff bases were synthesised from amino acids and small peptides. The purification of these adduct molecules was not completely achieved, but they served as reference for the screening of similar compounds in the digested chyme. However, specific Schiff bases were not detected by UHPLC–ESI–MS analyses. This raises the question of where the carbonyl signals were coming from in the proton NMR analyses, as no aldehydes were detectable by MS analyses. The simplest explanation for this may be that the huge amount of free amino groups present in the sample

mixtures resulted in a too wide distribution of aldehyde-adducts to have sufficient concentration for MS analyses. Other explanations may be that the digested chyme contained such short chain aldehydes that the UHPLC–ESI–MS analysis method was not able to detect them.

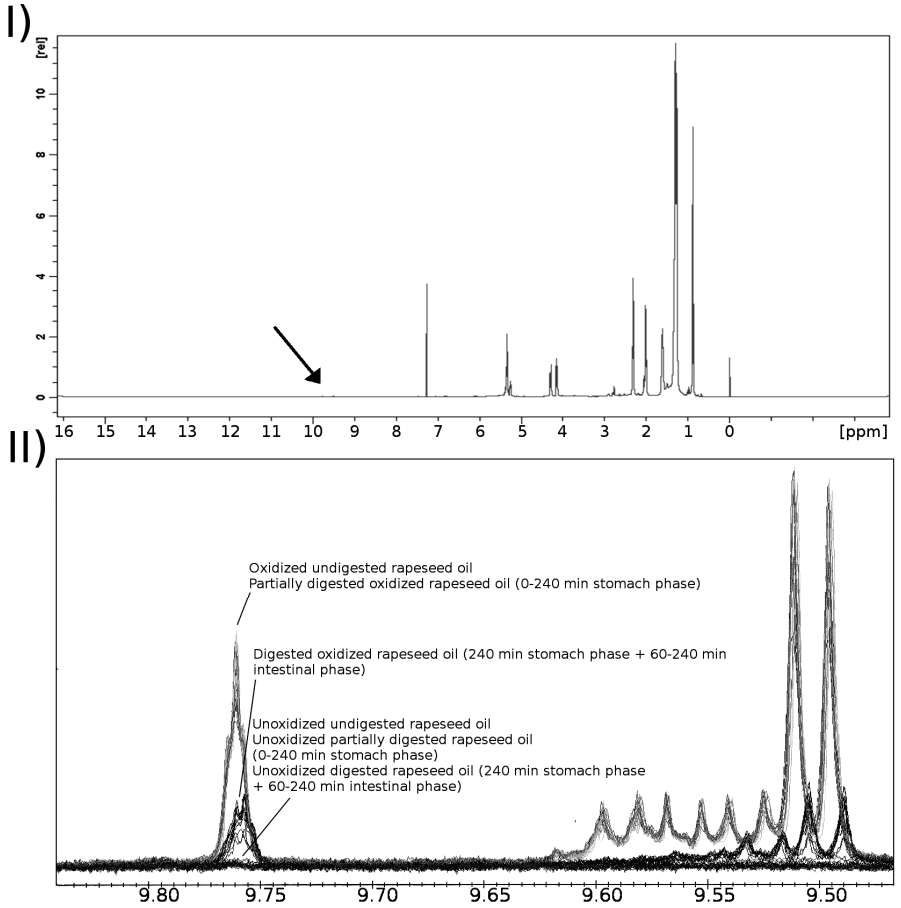


Figure 4. The ^{1}H NMR spectrum of mildly oxidised rapeseed oil containing small amounts of core aldehydes (**I**). The enlargement of the carbonyl signal region of several overlaid samples. The spectra in panel (**II**) were scaled and equalised by the chloroform peak to enable comparison.

6 SIGNIFICANCE OF THE STUDIES

New analytical methods were created during these studies in order to investigate all lipid components present in the digested samples. The new methods enable the separation of all the major lipid classes and individual lipid molecules, including the oxidised lipids, of oils subjected to partial or full lipolysis. The increased analytical speed and sensitivity enable the lipidomic profiling of hundreds of samples in a relatively short time, thus enabling new kinds of experiments.

Hundreds of compounds were identified and many quantified from the undigested and digested fresh and differently oxidised rapeseed oils, resulting in deeper knowledge regarding the oxidation and behaviour of oxidised lipids in the artificial digestion model. The results help in the evaluation of the safety of (oxidised) highly unsaturated oils.

Significant findings were done on the efficacy or rather the lack of efficacy of various common antioxidants against lipid oxidation during digestion. The findings merit more detailed research on the benefits and possible negative aspects of antioxidant fortification in foods and supplementation.

The results regarding core aldehydes were particularly interesting, as many carbonyl compounds are known to be toxic to humans. Mildly oxidised oils can significantly contribute to the oxidative burden of the digestive system, and potentially lead to health problems if consumed for extended periods of time. More detailed studies should be conducted on the effects of lipid based carbonyl compounds in human health.

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