Autoxidation of conjugated linoleic acid methyl ester in the presence of α-tocopherol: the hydroperoxide pathway

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ACADEMIC DISSERTATION

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Abstract

The autoxidation of conjugated linoleic acid (CLA) is poorly understood in spite of increasing interest in the beneficial biological properties of CLA and growing consumption of CLA-rich foods. In this thesis, the autoxidation reactions of the two major CLA isomers, 9-*cis*,11-*trans*-octadecadienoic acid and 10-*trans*,12-*cis*-octadecadienoic acid, are investigated. The results contribute to an understanding of the early stages of the autoxidation of CLA methyl ester, and provide for the first time a means of producing and separating intact CLA methyl ester hydroperoxides as well as basic knowledge on lipid hydroperoxides and their hydroxy derivatives.

Conjugated diene allylic monohydroperoxides were discovered as primary autoxidation products formed during autoxidation of CLA methyl esters in the presence and absence of α-tocopherol. This established that one of the autoxidation pathways of CLA methyl ester is the hydroperoxide pathway.

Hydroperoxides were produced from the two major CLA methyl esters by taking advantage of the effect of α-tocopherol to promote hydroperoxide formation. The hydroperoxides were analysed and separated first as methyl hydroxyoctadecadienoates and then as intact hydroperoxides by HPLC. The isolated products were characterized by UV, GC-MS, and NMR techniques. In the presence of a high amount of α-tocopherol, the autoxidation of CLA methyl ester yields six kinetically-controlled conjugated diene monohydroperoxides and is diastereoselective in favour of one particular geometric isomer as a pair of enantiomers. The primary autoxidation products produced from the two major CLA isomers include new positional isomers of conjugated diene monohydroperoxides, the 8-, 10-, 12-, and 14-hydroperoxyoctadecadienoates. Furthermore, two of these new positional isomers have an unusual structure for a *cis,trans* lipid hydroperoxide where the allylic methine carbon is adjacent to the *cis* instead of the usual *trans* double bond.

The ^{1}H and ^{13}C NMR spectra of nine isomeric methyl hydroxyoctadecadienoates and of ten isomeric methyl hydroperoxyoctadecadienoates including the unusual *cis,trans* hydroperoxides, *i.e.* Me 8-OOH-9c,11*t* and Me 14-OOH-10*t*,12*c*, were fully assigned with the aid of 2D NMR spectroscopy. The assigned NMR data enabled determination of the effects of the hydroxyl and hydroperoxyl groups on the carbon chemical shifts of CLA isomers, identification of diagnostic signals, and determination of chemical shift differences of the olefinic resonances that may help with the assignment of structure to as yet unknown lipid hydroperoxides either as hydroxy derivatives or as intact hydroperoxides.

A mechanism for the hydroperoxide pathway of CLA autoxidation in the presence of a high amount of α-tocopherol was proposed based on the characterized primary products, their relative distribution, and theoretical calculations. This is an important step forward in CLA research, where exact mechanisms for the autoxidation of CLA have not been presented before. Knowledge of these hydroperoxide formation steps is of crucial importance for understanding the subsequent steps and the different pathways of the autoxidation of CLA. Moreover, a deeper understanding of the autoxidation mechanisms is required for ensuring the safety of CLA-rich foods. Knowledge of CLA oxidation and how it differs from the oxidation of nonconjugated polyunsaturated fatty acids may also be the key to understanding the biological mechanisms of CLA activity.

Preface

Fatty acid oxidation is one of the most fundamental reactions in lipid chemistry. The autoxidation of monoene and nonconjugated diene fatty acids has been extensively studied. The autoxidation of conjugated fatty acids such as conjugated linoleic acid (CLA) is, however, poorly understood despite the large scientific interest in the potential health effects of CLA isomers. This study, which was initiated by Professor Anu Hopia, was aimed at filling this gap in our knowledge about the autoxidation of CLA, and it has offered me a challenging and attractive research topic.

The experimental work for this thesis was carried out at the laboratory of organic chemistry and at the food chemistry division at the University of Helsinki. I wish to express my thanks to Professor Anu Hopia and Professor Tapio Hase for introducing me to the world of lipid chemistry and giving me the opportunity to participate in this study. I am grateful to Professor Anu Hopia for her guidance, enthusiasm, trust, and ongoing encouragement, and to Professor Tapio Hase for his guidance and for providing the excellent research facilities of the laboratory of organic chemistry at my disposal. My gratitude also goes to Professor Vieno Piironen for providing the excellent research facilities of food chemistry division at my disposal and for creating a friendly and good working atmosphere.

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Finally, my dearest thanks belong to my family, to my parents Pirkko and Erkki Hämäläinen, to my sister Mervi Korpela and her family, and to my parents-in-law Mirja and Heikki Pajunen for their love and support during the years of this study, to my husband Lasse for his love, trust, and encouragement, and to my children Elias and Lauri for being the true blessings of my life.

Taina Pajunen
February 2009, Espoo

"Research is to see what everybody else has seen,
and to think what nobody else has thought."

Albert Szent-Gyoergi

To Elias and Lauri

List of original publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Hämäläinen T.I., Sundberg S., Mäkinen M., Kaltia S., Hase T., and Hopia A., Hydroperoxide formation during autoxidation of conjugated linoleic acid methyl ester, *Eur. J. Lipid Sci. Technol*., 2001, **103**, 588-593.

II Hämäläinen T.I., Sundberg S., Hase T., and Hopia A., Stereochemistry of the hydroperoxides formed during autoxidation of CLA methyl ester in the presence of α-tocopherol, *Lipids*, 2002, **37**, 533-540.

III Pajunen T.I., Johansson M.P., Hase T., and Hopia A., Autoxidation of conjugated linoleic acid methyl ester in the presence of α-tocopherol: the hydroperoxide pathway, *Lipids*, 2008, **43**, 599-610.

IV Pajunen T.I., Koskela H., Hase T., and Hopia A., NMR properties of conjugated linoleic acid (CLA) methyl ester hydroperoxides, *Chem. Phys. Lipids*, 2008, **154**, 105-114.

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Contribution of the author to the studies in articles I to IV

The author planned the study along with the other authors and performed the main part of the NMR experiments for article **I**. The author planned the studies for articles **II** to **IV**. She performed all the experiments for article **II**, all the experiments except the theoretical calculations for **III** and all experiments except the PERCH simulations and part of the NMR experiments for **IV**. In addition, she had the main responsibility for interpreting the results, and writing the articles, and she was the corresponding author in articles **I** to **IV**.

Contents

Abbreviations

BDE	bond dissociation energy
CLA	conjugated linoleic acid
COSY	correlated spectroscopy
ESR	electron spin resonance
FFA	furan fatty acid
GC	gas chromatography
gHMBC	gradient heteronuclear multiple bond correlation
gHSQC	gradient heteronuclear single quantum coherence
HPLC	high-performance liquid chromatography
HPSEC	high-performance size-exclusion chromatography
In•	initiator free radical
InH	initiator
L•	lipid carbon-centred radical
LH	lipid
LO•	lipid alkoxyl radical
LOO•	lipid peroxyl radical
LOOH	lipid hydroperoxide
M^{n+}	transition metal ion at the lower oxidation state
$M^{(n+1)+}$	transition metal ion at the higher oxidation state
Me 9*c*,11*t*-CLA	methyl 9-*cis*,11-*trans*-octadecadienoate
Me 9*t*,11*t*-CLA	methyl 9-*trans*,11-*trans*-octadecadienoate
Me 10*t*,12*c*-CLA	methyl 10-*trans*,12-*cis*-octadecadienoate
MS	mass spectrometry
NMR	nuclear magnetic resonance
NP-HPLC	normal phase high-performance liquid chromatography
NRP	nonradical product
PV	peroxide value
SPE	solid phase extraction
TLC	thin-layer chromatography
TMP	tocopherol-mediated peroxidation
TocO•	α-tocopheroxyl radical
TocOH	α-tocopherol; 2,5,7,8-tetramethyl-2-(4',8',12'-trimethyltridecyl)chroman-6-ol
TOCSY	total correlation spectroscopy
T-OOL	quinolide peroxide

Racemic CLA methyl ester hydroperoxides (and their hydroxy derivatives) are designated by the position of the hydroperoxyl (hydroxyl) group and configuration of the double bonds, *e.g.* Me 8-OOH-9*c*,11*t* stands for methyl 8-(*R*,*S*)-hydroperoxy-9-*cis*,11-*trans*-octadecadienoate. The methyl linoleate and methyl oleate hydroperoxides are abbreviated in a similar manner.

1 Introduction

Conjugated linoleic acid (CLA) is a generic name for a group of positional and geometric isomers of octadecadienoic acid with a 1,3-diene structure. The most abundant CLA isomers in nature are 9-*cis*,11-*trans*-octadecadienoic acid (9*c*,11*t*-CLA) and 10-*trans*,12-*cis*-octadecadienoic acid (10*t*,12*c*-CLA). CLA is present mainly in the fats of ruminant milk and meat, where 9*c*,11*t*-CLA predominates (Parodi 1977; Chin et al. 1992; Fritsche et al. 1999; Griinari & Bauman 1999). Hence, the proposed trivial name for the 9*c*,11*t*-CLA is rumenic acid (Kramer et al. 1998).

Scientific interest in CLA has risen exponentially since the discovery in the 1980's that an isomeric mixture of CLA is anti-carcinogenic in a number of rodent models (Ha et al. 1987; Pariza 1999; Pariza 2004). Today a number of beneficial physiological properties are attributed to CLA such as anti-cancer, anti-atherosclerosis, anti-inflammatory, and anti-obesity effects (reviewed in Scimeca 1999; Gnädig et al. 2001; Pariza et al. 2001; Evans ME et al. 2002; Belury 2002; Terpstra 2004; Wahle et al. 2004; Zulet et al. 2005; Kelley et al. 2007; Li et al. 2008). These beneficial effects seem to be structure-specific. Thus far, the biological activity has been detected mainly with two CLA isomers, 9*c*,11*t*-CLA and 10*t*,12*c*-CLA. Despite the interest in CLA, the mechanisms of CLA action remain unclear. They seem, however, to include induction of fatty acid oxidation. Therefore, knowledge of CLA oxidation and how it differs from the oxidation of nonconjugated polyunsaturated fatty acids may be the key to understanding the biological mechanisms of CLA activity.

Due to its potential health effects, enrichment of food with CLA (Griinari & Bauman 1999; Nurmela & Griinari 1999; Mir et al. 2004; Hennessy et al. 2007; Adamczak et al. 2008) and the design of new health products based on CLA are of increasing interest. In food technology, one of the major concerns is the autoxidation of lipids (Simic 1981). Lipid autoxidation not only produces unwanted rancid flavours in foods but may also reduce their nutritional quality and safety. Therefore, detailed information on the autoxidation of CLA that could enable the control of its oxidation in food or in food supplements is of great importance.

Despite the wide interest in CLA and the large number of scientific articles published recently (a regularly updated listing of the scientific literature on CLA is available at http://www.wisc.edu/fri/clarefs.htm), surprisingly little is known about the autoxidation of CLA. In particular, the early steps are poorly understood. The primary autoxidation products, for example, remain to be characterized. Moreover, studies on CLA autoxidation are performed, despite the structure-specificity, mostly with mixtures of CLA isomers and under widely different conditions (Pajunen & Kamal-Eldin 2008, Table 4.1). Thus, it is difficult, if not impossible, to interpret the data and to correlate the results with a single isomer or to compare the data from different studies directly.

The early literature, reviewed in Swern 1961, suggests that the autoxidation of CLA produces mainly polymeric peroxides. More recently, furan fatty acids have been identified as secondary autoxidation products of CLA (Yurawecz et al. 1995). The mechanisms for the formation of the oligomers and the identified secondary products

remain more or less a matter of speculation, due to the lack of knowledge of the early autoxidation steps and of the preceding autoxidation products. Furthermore, analyses of oligomeric product mixtures can be anticipated to be challenging due to heterogeneity of the oligomers (Muizebelt & Nielen 1996; Luna et al. 2007) and therefore characterization of the primary products may prove helpful in elucidation not only of the formation mechanisms of the oligomers but also of their structures. Moreover, if sufficient quantities of pure primary products could be produced, their further reactions may be investigated and their biological activity evaluated in a quantitative manner.

The literature review section of this thesis describes hydroperoxide formation during autoxidation of monoene and nonconjugated diene fatty acids, and the current understanding of the autoxidation of conjugated diene fatty acids. In addition, the influence of α-tocopherol on the autoxidation of fatty acids is reviewed briefly. The experimental section of the thesis presents evidence for hydroperoxide formation during the autoxidation of CLA methyl esters, discusses the analysis and the characterization of CLA methyl ester hydroperoxides from the autoxidation reactions of the two major CLA methyl esters in the presence of α-tocopherol, firstly as hydroxy derivatives and secondly as intact hydroperoxides, and proposes a mechanism for the hydroperoxide pathway of these CLA isomers. The last section of this thesis consists of four original articles in which the results of the present study were published.

2 Review of the literature

2.1 Autoxidation

The spontaneous reaction of molecular oxygen with radicals is commonly referred to as autoxidation (Porter & Wujek 1988). In a wider sense, the term autoxidation in organic chemistry is applied to any slow atmospheric oxidation of a C-H to C-OOH (March 1992). Autoxidation of lipids has been extensively studied for centuries. In the 18th century, the studies of Lavoisier on low-temperature oxidation of oils and fats played a critical role in the birth of the science of organic chemistry (Porter et al. 1995). In biology and in food chemistry, autoxidation of lipids is referred to as lipid peroxidation and lipid rancidization.

Atmospheric molecular oxygen is a ground state triplet and thus, molecular oxygen, also known as triplet oxygen ($^3O_2$), is biradical in character. The direct addition of triplet oxygen to an organic molecule that exists usually at a singlet ground state would result, according to Wigner's spin-conservation rule, in a product in its triplet state. Since ground-state molecules do not usually have sufficient energy to yield a product in its exited state, an energy barrier called the spin barrier prevents the direct addition of molecular oxygen in a single step to an organic molecule (Chan 1987). This spin barrier can be, however, circumvented by mechanisms involving transition metals, free radicals, or singlet oxygen ($^1O_2$). In the following section the fundamentals of lipid autoxidation that occurs through a free-radical chain mechanism are reviewed.

The free radical nature of lipid autoxidation was established by Bolland, Bateman and colleagues at the British Rubber Producers' Research Association (Schneider et al. 2008). They defined the three well-recognized stages of the process as initiation, propagation, and termination.

Initiation The key event in initiation is the conversion of a valence-saturated lipid (LH) in one or more steps into a carbon-centred radical L• (**Table 1**, eq 1). This may occur through homolytic cleavage of a C-H bond by heat or light, by single electron transfer from a reducing agent, or by hydrogen atom abstraction by an initiator free radical (In•). Initiation that occurs by hydrogen atom abstraction is hard to define because a very small concentration of radicals is needed for the process. Moreover, since many different radicals may abstract a hydrogen atom from the lipid, it is probable that more than one initiation reaction is operating (Chan 1987; Frankel 1998a). Molecular oxygen, however, is not reactive enough to abstract a hydrogen atom from the lipid (March 1992).

Two main paradigms for initiation in lipid autoxidation have been advanced (Schneider et al. 2008). The reactions of transition metals (eqs 2 to 4) or of chemical initiators present in the lipid sample are thought to generate a pool of radicals which initiate new autoxidation chains. In the classical paradigm, the metal-catalysed reactions of lipid hydroperoxides (LOOH) (eqs 3 and 4) expand the pool of radicals. Hydroperoxides are thus, according to this paradigm, the key intermediates in autocatalytic radical generation. In the alternative paradigm, lipid peroxyl radicals (LOO•) cross-react with

Table 1 Three stages and several free-radical reactions of lipid autoxidation.

Stage	Reaction	Eq
initiation	$LH + In\bullet \rightarrow L\bullet + InH$	1
	$LH + M^{n+1} \rightarrow L\bullet + H^{+} + M^{n+}$	2
	$LOOH + M^{n+1} \rightarrow LOO\bullet + H^{+} + M^{n+}$	3
	$LOOH + M^{n+} \rightarrow LO\bullet + HO^{-} + M^{n+1}$	4
propagation	$L\bullet + {}^{3}O_2 \rightarrow LOO\bullet$	5
	$LOO\bullet + LH \rightarrow LOOH + L\bullet$	6
termination	$LOO\bullet + LOO\bullet \rightarrow [LOO\text{-}OOL] \rightarrow NRP$	7
	$LOO\bullet + L\bullet \rightarrow LOOL$	8
	$L\bullet + L\bullet \rightarrow L\text{-}L$	9

lipid hydroperoxides giving dimer fatty acids, and these dimers, instead of the hydroperoxides, are centrally responsible for autocatalytic radical generation. Experimental evidence supports not only the lack of autocatalytic activity of hydroperoxides in the absence of added transition metals (Morita & Fujimaki 1973; Morita & Tokita 1990, 1993, 2006) but also the formation of peroxide-linked dimers as significant products even at the early stages of lipid autoxidation (Miyashita et al. 1982). The exact structures of the autocatalytic dimer fatty acids remain to be determined. Recently, however, Morita & Tokita (2006, 2008) reported that in methyl linoleate autoxidation the non-hydroperoxide peroxides that are responsible for the autocatalytic activity consist of two peroxide-linked linoleate moieties with two hydroperoxyl groups. Such dimer fatty acids would be prone to breakage of the cross-molecular peroxide bond and would produce, as depicted in **Fig. 1**, free radicals and aldehydes (Schneider et al. 2008). This illustrates the expected importance of the dimer fatty acids not only in the autocatalytic radical supply but also in the formation of volatile secondary autoxidation products.

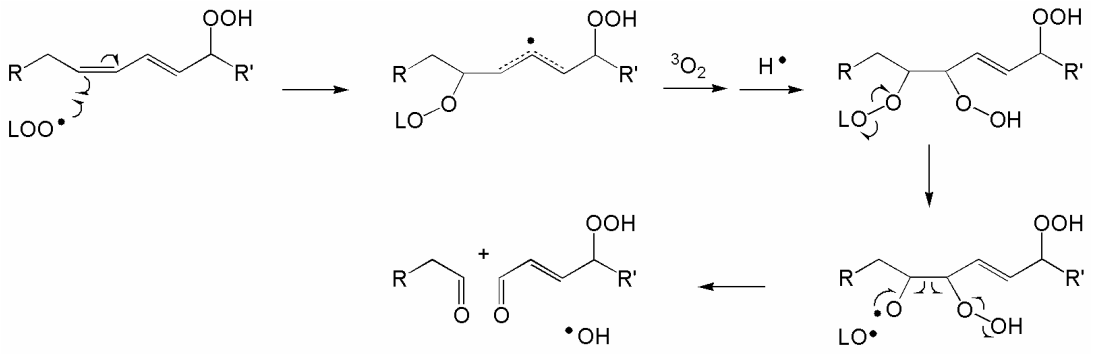


Figure 1 Proposed formation and decomposition of a dimer fatty acid dihydroperoxide at the initiation stage of lipid autoxidation (modified from Schneider et al. 2008).

In lipid autoxidation, the rate of initiation by hydrogen atom abstraction depends on the electronic, polar and steric properties of the initiator free radical and the lipid, on the stability of the radicals formed, and on the activation energy of the reaction. The activation energy of the reaction has been shown to correlate well with the bond dissociation energy (BDE) of the C-H bond that undergoes cleavage (Chan 1987). This dependency explains the high susceptibility of polyunsaturated fatty acids to autoxidation; the bisallylic hydrogen atom has a relatively low BDE of 75 kcal/mol (Gardner 1989) and may thus be easily abstracted. Consequently, methyl linoleate autoxidizes readily at room temperature while methyl oleate, for which the BDE of the allylic C-H bond is approximately 10 kcal/mol greater than that of the bisallylic C-H bond, autoxidizes at a reasonable rate only at elevated temperatures (Gardner 1989; Porter et al. 1995).

Propagation Once formed, the L• radical starts propagation by reacting with triplet oxygen (eq 5). This reaction, which is essentially a radical-radical coupling reaction, produces a lipid peroxyl radical and proceeds under normal oxygen pressure at or near the diffusion-controlled rate of approximately 10^9 $M^{-1}s^{-1}$ (Ingold 1969; Maillard et al. 1983). As a result, the peroxyl radical is the main chain-carrying species in the autoxidation reaction mixture provided that oxygen is present in sufficient concentration. Subsequently, the peroxyl radical abstracts a hydrogen atom from the lipid in a reaction classified as an atom transfer reaction (eq 6). This hydrogen atom transfer occurs in a selective manner, preferring the most weakly bound hydrogen atom, as demonstrated by the large kinetic deuterium isotope effect (Howard et al. 1968; Ingold 1969). Furthermore, this reaction is the rate-limiting step in the autoxidation sequence and it produces a lipid hydroperoxide and a new L• radical. The L• radical sets off a new cycle of the propagation steps and thus, every initiation event may set off several propagation cycles.

In lipid autoxidation, the propagation steps may be more complicated than the simple addition and transfer reactions listed in **Table 1**. In addition to these listed steps, lipid autoxidation may propagate by fragmentation of the peroxyl radical to give an oxygen and a carbon radical, by rearrangement of the peroxyl radical, or by cyclization of the peroxyl radical (Porter et al. 1995). Mechanisms that involve these five reaction types have been formulated to explain primary product formation in the autoxidation of nonconjugated polyunsaturated fatty acids. Autoxidation mechanisms of monoene and nonconjugated diene fatty acids are discussed in section 2.1.1.

Termination The most important feature in termination is the formation of nonradical end products that results to termination of the propagation chain. Propagation chains are terminated as illustrated in **Table 1** by radical-radical coupling reactions. Because the formation of peroxyl radicals is fast and that of hydroperoxides slow, the termination reactions involving L• (eqs 8 and 9) are unimportant under normal reaction conditions, and the only termination reaction that is significant or likely to be the most important is the radical-radical coupling between two peroxyl radicals (eq 7). This self-reaction yields a tetroxide, which decomposes in an irreversible manner to stable end products. The decomposition (**Fig. 2**) has been proposed to occur through a cyclic six-membered transition state which gives rise to a secondary alcohol, a ketone, and oxygen (Russell 1957). The exact mechanism, however, has not been fully resolved (Gardner 1987) and it may depend on the nature of a particular peroxyl radical and the experimental conditions (Simic 1981).

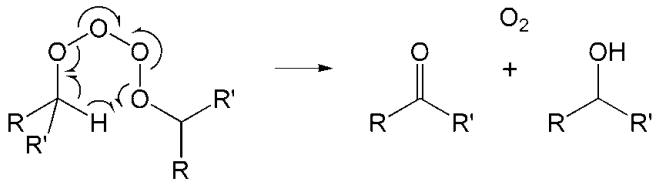


Figure 2 Decomposition of a tetroxide by the Russell mechanism.

Termination may also occur by radical disproportionation. For example, the disproportionation of two secondary alkoxyl radicals yields a ketone and an alcohol. The significance of this reaction may be anticipated to be greater in metal-catalysed autoxidations because alkoxyl radicals are formed through homolytic cleavage of hydroperoxides. Autoxidation is defined as a chain reaction if the chain termination steps are slower than the chain propagation steps (Denisov & Khudyakov 1987).

2.1.1 Autoxidation of monoene and nonconjugated diene fatty acids

Autoxidation of monoene and nonconjugated diene fatty acids has been extensively studied (reviewed in Swern 1961; Frankel 1985, 1998b; Porter 1986; Chan 1987; Chan & Coxon 1987; Gardner 1987, 1989; Porter et al. 1995). The overlapping stages of the autoxidation of polyunsaturated lipids are depicted in **Fig. 3**. This figure illustrates the kinetic behaviour that typifies the involvement of an autocatalytic free-radical chain mechanism: the reaction starts with a slow induction period during which the radical concentration builds up and is followed by a rapid propagation stage. The fatty acid concentration will rapidly decrease as hydroperoxides are formed. The hydroperoxide concentration will go through a maximum when hydroperoxide formation is surpassed by hydroperoxide decomposition. Decomposition of hydroperoxides leads to the formation of volatile and non-volatile secondary autoxidation products. This section of the literature review focuses on hydroperoxide formation during autoxidation of monoene and nonconjugated diene fatty acids for which methyl oleate and methyl linoleate serve as model compounds.

Hydroperoxides were identified as oxidation products of methyl oleate and methyl linoleate in the work of Farmer and co-workers (Farmer et al. 1943; Bolland & Koch 1945). These studies led to the development of an autoxidation mechanism for monounsaturated and nonconjugated polyunsaturated fatty acids, also known as Farmer's hydroperoxide theory, which replaced the previous cyclic peroxide and ethylene oxide theories (reviewed in Swern 1961). According to the hydroperoxide theory, the autoxidation of monoene and nonconjugated diene fatty acids is a free-radical chain reaction that leads to hydroperoxides in which the hydroperoxide group is attached to an allylic carbon atom, and it may be accompanied by a shift of a double bond. Contrary to earlier thinking, the initial reaction with oxygen and the unsaturated centre is according to Farmer's hydroperoxide theory substitution rather than addition (Gunstone 2003).

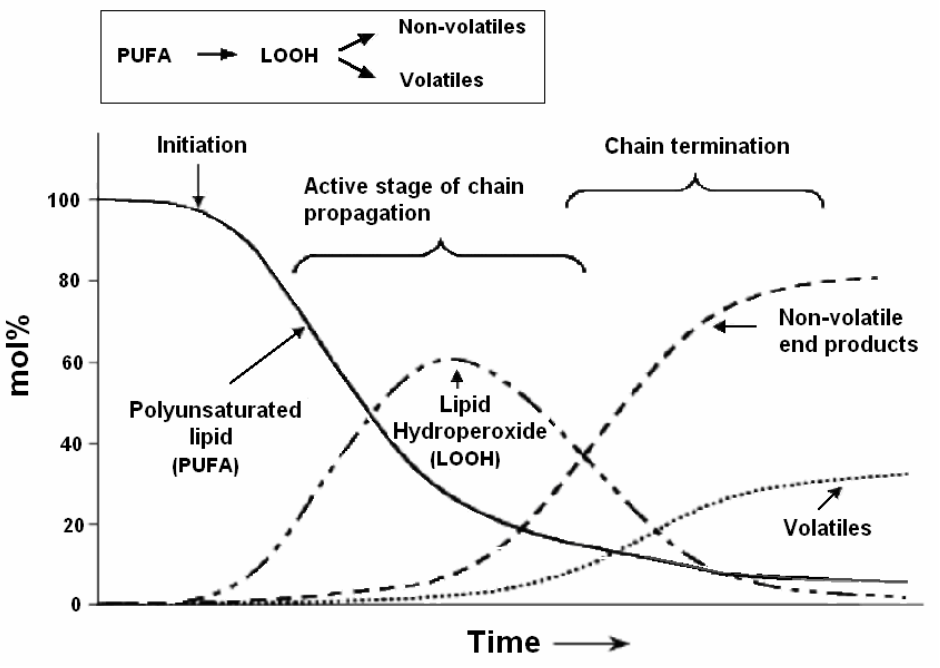


Figure 3 Autoxidation of a polyunsaturated lipid as a function of time showing the various stages in the reaction (redrawn from Gardner 1987).

Hydroperoxide formation during autoxidation of monoene fatty acids Autoxidation of methyl oleate produces eight geometric isomers of 8-, 9-, 10-, and 11-hydroperoxyoctadecenoates as pairs of enantiomers (Chan & Levett 1977b; Frankel et al. 1977, 1984). Quantification of the relative proportions of the four positional isomers as corresponding hydroxystearates by HPLC (Chan & Levett 1977b) and as trimethyl silyl ethers of the hydroxystearates by GC-MS (Frankel et al. 1977) showed a small preference for formation of 8- and 11-hydroperoxide isomers.

Mechanistically the autoxidation of methyl oleate has presented a challenge. Frankel et al. (1984) constructed a mechanism for methyl oleate autoxidation (**Fig. 4**) based on the autoxidation of the simple model compound 3-*cis*-hexene (Frankel et al. 1982). Frankel (1998b) suggests that the conformations of methyl oleate may establish the conformations of the initially-formed allyl radicals, depending on the temperature of the oxidation. Hence, initiation by hydrogen atom abstraction from the three most likely conformations of methyl oleate produces four distinct allyl radicals. Subsequent propagation steps of these radicals explain the formation of six of the eight methyl oleate-derived hydroperoxides. For the formation of the two remaining isomers, *trans* 8- and 11-hydroperoxides, the mechanism proposes that the first-formed allyl radicals lose their defined stereochemistry, particularly at elevated temperatures, by direct isomerization and that this isomerization is followed by the propagation steps. This mechanistic proposal has been critically viewed. First, direct isomerization of the allyl radicals is considered unlikely because the rearrangement of the allyl radical from *cis* to *trans* is thermodynamically unfavourable (Gardner 1989) and because under normal oxygen pressures the allyl radials are trapped by molecular oxygen at or near the diffusion

controlled rate (Ingold 1969; Maillard et al. 1983). Secondly, this mechanism fails to give an adequate explanation for the observed isomeric distribution or its dependency on the concentration of hydrogen atom donors added to the autoxidation mixture (Porter et al. 1994a).

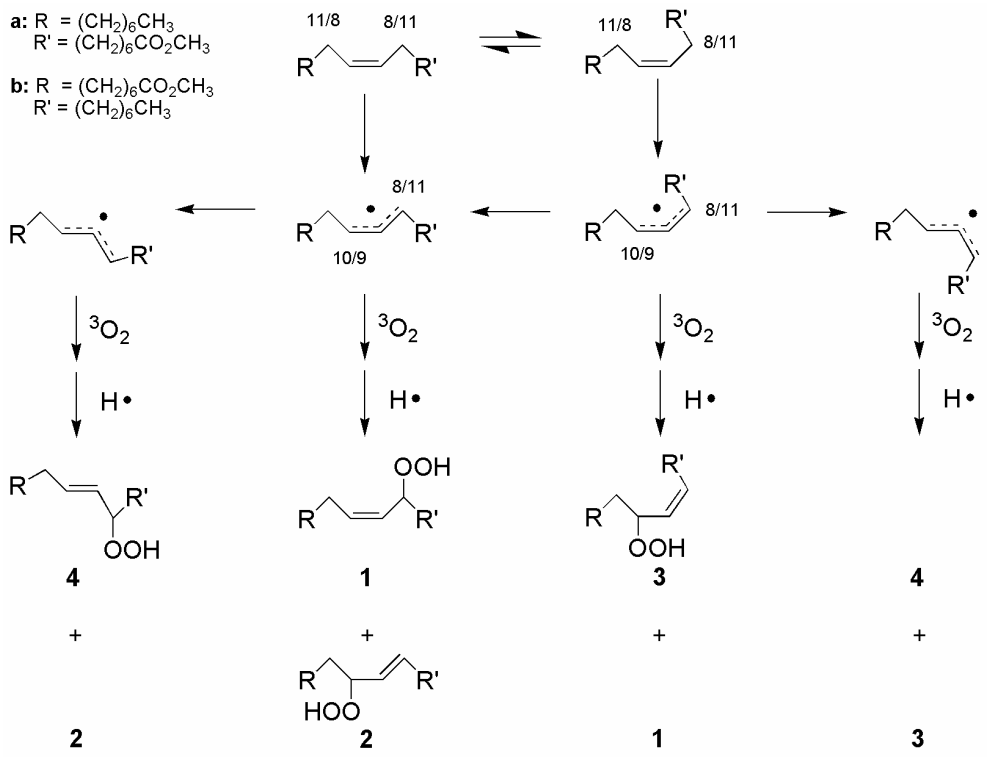


Figure 4 Proposed mechanism for methyl oleate autoxidation (modified from Frankel et al. 1984 and Frankel 1998b); **1** Me 8-OOH-9*c*/Me 11-OOH-9*c*; **2** Me 10-OOH-8*t*/Me 9-OOH-10*t*; **3** Me 10-OOH-8*c*/Me 9-OOH-10*c*; **4** Me 8-OOH-9*t*/Me 11-OOH-9*t*.

An alternative mechanism for the formation of the six major hydroperoxides during methyl oleate autoxidation (**Fig. 5**) was presented by Porter et al. (1994a). This mechanism explains not only the formation of the isolated products but also their isomeric distribution and its dependency on the concentration of hydrogen atom donors present in the autoxidation mixture. Two allyl radicals arise initially from the extended conformation of methyl oleate. The propagation steps of these first-formed allyl radicals yield four kinetically-controlled hydroperoxides. The *trans* 8- and 11-hydroperoxides are thought to be formed as thermodynamically-controlled hydroperoxides that arise from the allylperoxyl radical rearrangement followed by hydrogen atom abstraction. It should be noted that the formation of methyl 9-hydroperoxy-10-*cis*-octadecenoate and methyl 10-hydroperoxy-8-*cis*-octadecenoate (not drawn), two tentatively assigned minor products (*i.e.* giving altogether eight isomers), may also be explained by the allylperoxyl radical rearrangement.

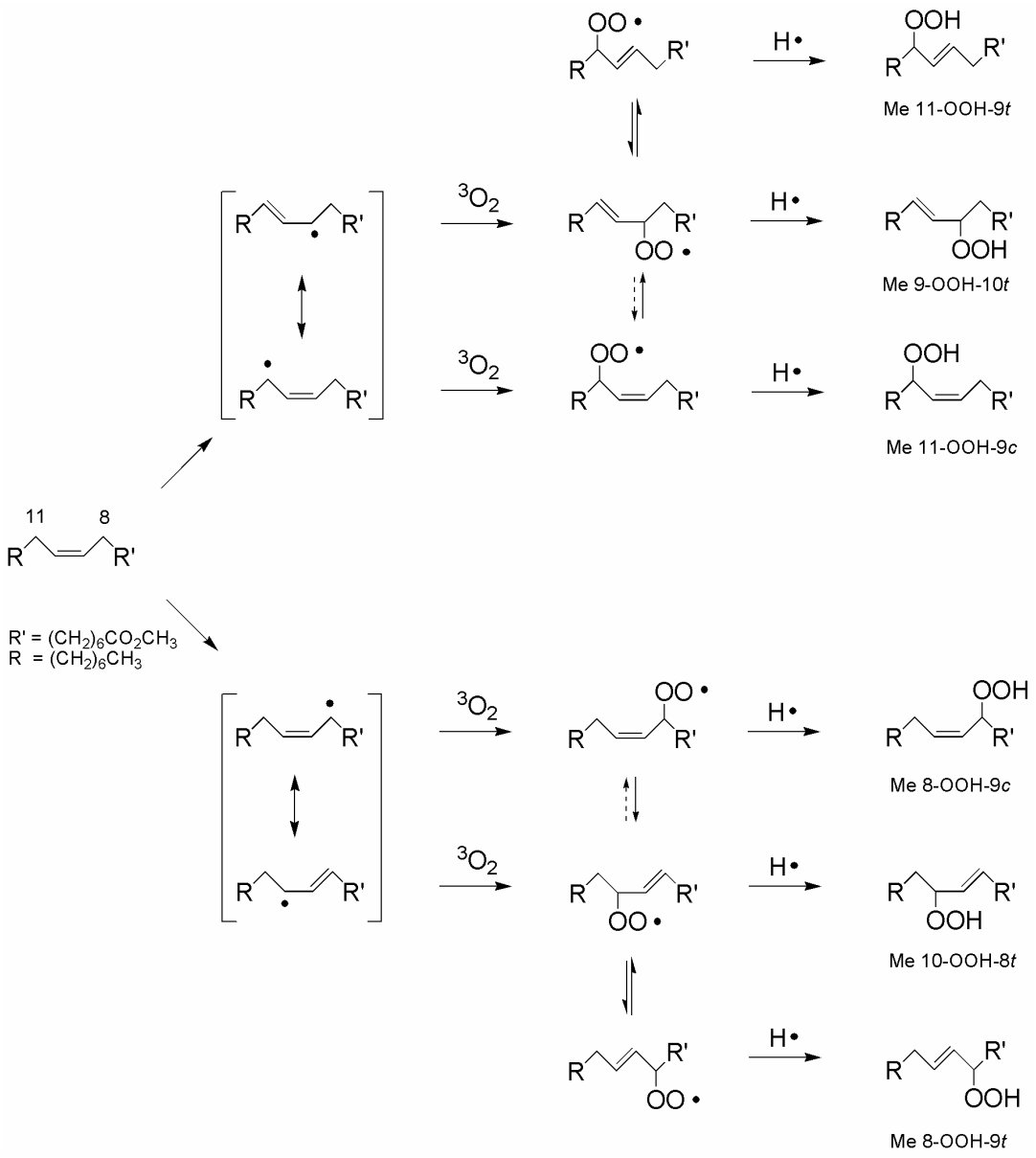


Figure 5 Hydroperoxide formation during autoxidation of methyl oleate (modified from Porter et al. 1995).

The mechanism of the allylperoxyl radical rearrangement has long been debated. Three pathways proposed for this rearrangement, also referred to as 1,3-rearrangement or [2,3] allylperoxyl rearrangement, are illustrated in **Fig. 6** (reviewed in Porter et al. 1995). The first stepwise process involves a dioxolanyl radical intermediate, the second proceeds through a concerted mechanism involving a five-membered ring peroxide transition state and the third stepwise process consists of β-fragmentation and oxygen readdition. Experimental studies conducted by Porter's group suggest that the most plausible mechanism for allylperoxyl radical rearrangement is β-fragmentation of the peroxyl radical, leading to a caged pair consisting of molecular oxygen and an allyl radical,

followed by rearrangement to the isomerized peroxyl radical (Porter & Wujek 1987; Porter et al. 1990, 1994a,b; Mills et al. 1992; Boyd et al. 1993; Lowe & Porter 1997; Tallman et al. 2004). This allyl radical-triplet dioxygen complex formation provides a rationale for the observed high stereoselectivity of the rearrangement and is supported by recent independent computational study by Olivella and Sole (2003). The rate constant for the β-fragmentation is dependent on the geometry of the allylperoxyl radical and the allyl radical generated in the process. Porter et al. (1994a) determined the rate constants for the β-fragmentation of the methyl oleate-derived allylperoxyl radicals by computer simulation and established that *cis* or *trans* allylperoxyls rearrange to give *trans* allyl products, but that *trans* allylperoxyls do not rearrange to *cis* allylperoxyls to any significant extent (Porter et al. 1994a).

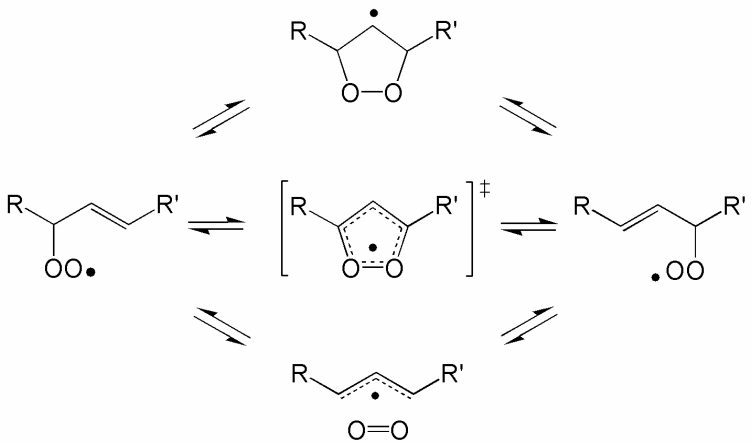


Figure 6 Three suggested mechanisms for the allylperoxyl rearrangement (Porter et al. 1995).

The isomeric distribution of the methyl oleate hydroperoxides may be understood based on the regioselectivity of oxygen addition, and the competition between hydrogen atom abstraction by and β-fragmentation of the intermediate peroxyl radicals. Porter et al. (1994a) showed based on kinetic evidence that a *cisoid* terminus of the allyl radical reacts more readily with oxygen than a *transoid* terminus. This is in line with ESR data on allyl radicals (Bascetta et al. 1982, 1983). Furthermore, they demonstrated that as the hydrogen atom concentration of the autoxidation medium is increased, increased amounts of kinetic hydroperoxides and decreased amounts of allylperoxyl radical rearrangement products were obtained.

In the late 1980's, Gardner (1989) suggested another mechanism for the autoxidation of methyl oleate that combines the idea of the contribution of the conformations of methyl oleate to the product distribution in Frankel's mechanism with the idea of allylperoxyl radical rearrangement in Porter's mechanism. No studies have been reported that would provide estimations of contributions of different conformations of methyl oleate to the product distribution. In light of experimental data and the mechanism proposed by Porter et al. (1994a), however, it seems likely that the extended conformation of methyl oleate is the most important contributor to the isomeric distribution of the methyl oleate hydroperoxides and that the other conformations may be disregarded.

Hydroperoxide formation during autoxidation of nonconjugated diene fatty acids

Autoxidation of methyl linoleate yields four isomeric conjugated diene allylic hydroperoxides as pairs of enantiomers: 9- and 13-hydroperoxides with *trans,trans* and *cis,trans* diene stereochemistry, where the *trans* double bond is adjacent to the hydroperoxyl group bearing methine carbon. Together these products account for more than 97% of the oxygen consumed in methyl linoleate autoxidation at low conversion (Chan & Levett 1977a; Porter et al. 1981). Moreover, these four hydroperoxides are the main products also formed from the other three geometric isomers of methyl linoleate, *i.e.* 9-*cis*,12-*trans*-; 9-*trans*,12-*cis*-; and 9-*trans*,12-*trans*-octadecadienoic acid methyl ester (Porter & Wujek 1984). Isomeric distribution of the four hydroperoxides depends on the extent of oxidation, concentration of the fatty ester, and temperature (Porter 1986). The sum of total products formed from oxygen addition at C-9 is the same as products formed from oxygen addition at C-13. Higher initial concentrations of methyl linoleate give more *cis,trans* products, whereas higher autoxidation temperatures give rise to *trans,trans* products. The product distribution is also affected by the presence of a hydrogen atom donor. For example, in the absence of 1,4-cyclohexadiene or with a low concentration of this hydrogen atom donor, the product distribution from the various isomeric methyl linoleates was equivalent or nearly so. With a high 1,4-cyclohexadiene concentration, the product mixtures became non-equivalent and reflected the stereochemistry of the particular diene precursor (Porter & Wujek 1984). The product distribution is, however, independent of oxygen pressure between 10 and 1000 mm Hg (Porter 1986).

In the two mechanisms that have most often been presented to explain hydroperoxide formation in the autoxidation of methyl linoleate (*vide infra*), the precursor fatty ester is in the extended conformation. The first step is the abstraction of one of the bisallylic hydrogen atoms, which produces one resonance-stabilized pentadienyl radical. Experimental evidence suggests that the first-formed pentadienyl radical has a W- or *cis,cis*-conformation (Bascetta et al. 1983). The formation of the other possible configurations, the Z- and U-conformations (**Fig. 7**), is expected to be highly unlikely because of the additional strain involved in having three or four *cisoid* interactions (Porter & Wujek 1988).

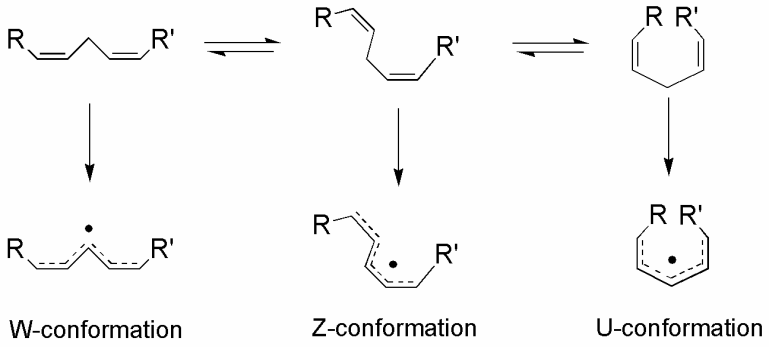


Figure 7 W-, Z-, and U-conformations of pentadienyl radicals derived from methyl linoleate.

In the mechanism proposed by Frankel (**Fig. 8**) the *cis,trans* isomers arise from the initial pentadienyl radical by oxygenation at either end of the pentadienyl radical followed by hydrogen atom transfer, while the *trans,trans* isomers are formed through direct isomerization of the pentadienyl radical followed by the propagation steps (Frankel 1998b). As in the case of methyl oleate, the suggestion of direct isomerization of the initial carbon radical has received criticism. Moreover, experiments with α-tocopherol have provided evidence against direct pentadienyl radical isomerization. α-Tocopherol, which acts in the autoxidation reaction at the level of the peroxyl radicals and not of the carbon radicals, has been shown to direct autoxidation toward the *cis,trans* products (Porter et al. 1980; Peers et al. 1981). If the loss of stereochemistry in the product hydroperoxides occurred by pentadienyl radical isomerization, then α-tocopherol would have no effect on the product composition (Porter 1986). This mechanism also fails to account for the effects of hydrogen atom donors and the initial fatty ester concentration on product composition (Porter 1986).

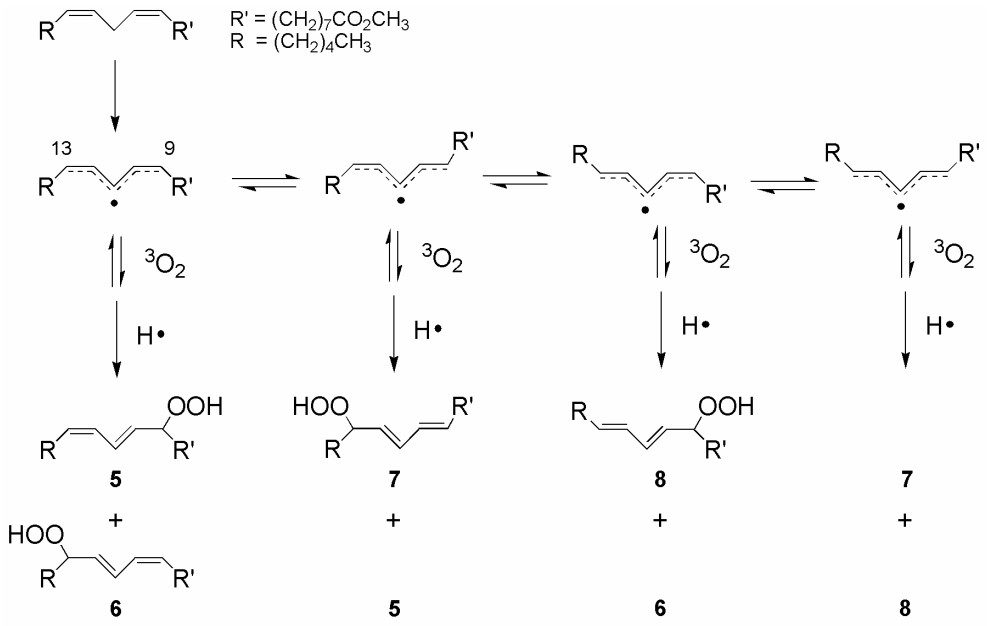


Figure 8 Proposed mechanism for autoxidation of methyl linoleate (modified from Frankel 1998b); **5** Me 9-OOH-10*t*,12*c*; **6** Me 13-OOH-9*c*,11*t*; **7** Me 13-OOH-9*t*,11*t*; **8** Me 9-OOH-10*t*,12*t*.

The mechanism proposed for the autoxidation of methyl linoleate (and its geometric isomers) by Porter and Wujek (1984) is depicted in **Fig. 9**. After the initial pentadienyl radical formation, oxygen addition yields two *cis,trans* conjugated diene peroxyl radicals. Subsequent hydrogen atom abstraction by these peroxyl radicals generates the two kinetically-controlled *cis,trans* hydroperoxides (**5** and **6**). Alternatively, the peroxyl radicals may react by β-fragmentation. Stereochemically non-productive β-fragmentation gives the initial pentadienyl radical, whereas the β-fragmentation that is preceded by bond rotation yields a pentadienyl radical that differs from the initial pentadienyl radical. The propagation steps of these new pentadienyl radicals generate the two thermodynamically-controlled *trans,trans* conjugated diene hydroperoxides (**7** and **8**).

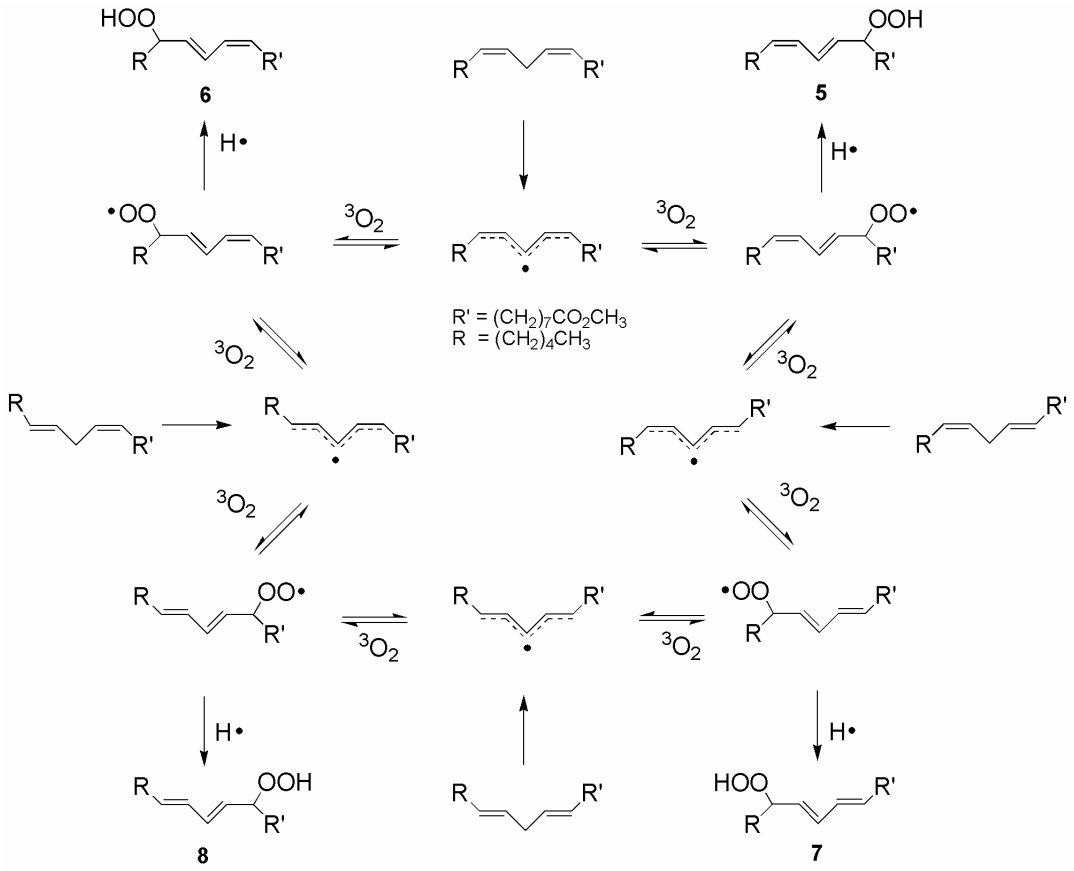


Figure 9 Hydroperoxide formation during autoxidation of methyl linoleate and its geometric isomers (Porter & Wujek 1984) **5** Me 9-OOH-10*t*,12*c*; **6** Me 13-OOH-9*c*,11*t*; **7** Me 13-OOH-9*t*,11*t*; **8** Me 9-OOH-10*t*,12*t*.

The isomeric distribution of the methyl linoleate hydroperoxides depends, according to Porter's mechanism, on the rates of partitioning of oxygen to the reactive sites of the pentadienyl radical, and on the rates of hydrogen atom transfer to and β-fragmentation of the intermediate peroxyl radicals (Pratt et al. 2003).

Oxygen addition to intermediate pentadienyl radicals is expected to occur preferentially at centres having the highest spin density. Theoretical calculations (Pratt et al. 2003) and ESR data (Bascetta et al. 1983) suggest that significant unpaired electron spin density is present at the central carbon atom of the pentadienyl radical derived from methyl linoleate. The bisallylic hydroperoxide, methyl 11-hydoperoxy-9-*cis*,12-*cis*-octadecadienoate, that arises from the addition of oxygen at this position was identified only recently by Brash (2000) when the autoxidation of methyl linoleate was performed in the presence of a high concentration of α-tocopherol. Tallman et al. (2001) report that this nonconjugated hydroperoxide is the major product formed from autoxidation of methyl or cholesteryl linoleate at high α-tocopherol concentrations and at low conversion. More recently, Tallman et al. (2004) demonstrated that the autoxidations of *cis*,*cis*; *cis*,*trans*; and *trans*,*trans* nonconjugated dienes and their corresponding octadecadienoates give rise to kinetically-controlled hydroperoxides in the presence of a high concentration of α-

tocopherol with the bisallylic hydroperoxides being the major isomers (**Fig. 10**). In symmetrical pentadienyl radicals, the two termini of the radical react at equal rates with oxygen. In contrast to expectations based on spin density, in unsymmetrical pentadienyl radicals generated from *cis,trans* dienes, the *transoid* terminus reacts faster with molecular oxygen than the *cisoid* terminus based on the kinetic product distribution (Tallmann et al. 2004). Tallman et al. (2004) suggest that this unexpected finding result from rearrangement of the major peroxyl radical product, the bisallylic peroxyl radical, preferentially into a conjugated diene peroxyl radical that is the same as the product from the attack of oxygen on the *transoid* terminus.

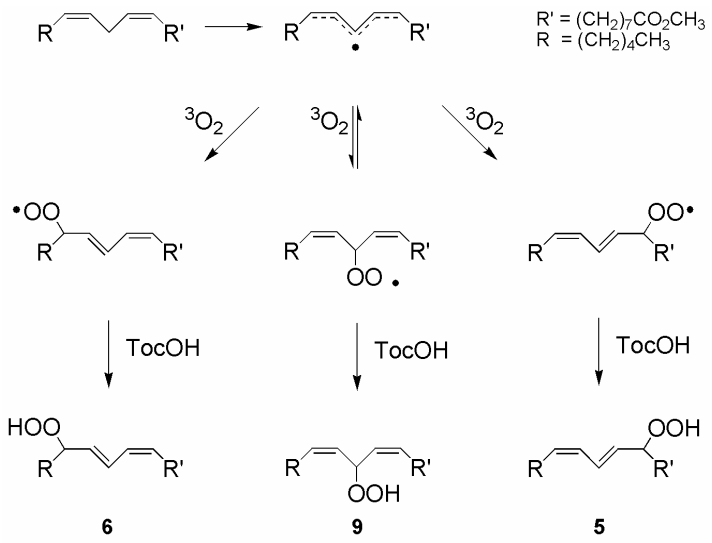


Figure 10 Mechanism of kinetically-controlled methyl linoleate autoxidation (Tallman et al. 2004) **5** Me 9-OOH-10*t*,12*c*; **6** Me 13-OOH-9*c*,11*t*; **9** Me 11-OOH-9*c*,12*c*.

The addition of molecular oxygen to pentadienyl radicals is reversible. The reversibility of oxygen addition has been established by oxygen scrambling in studies on hydroperoxide rearrangements with isotopically labeled hydroperoxides or molecular oxygen and has led to the conclusion that the rearrangement of conjugated diene hydroperoxides proceeds via β-fragmentation (Chan et al. 1978, 1979; Porter et al. 1980). As in the autoxidation of methyl oleate, the rate of the β-fragmentation depends on the geometry of the pentadienyl radical generated in the process. The β-fragmentation of a conjugated diene peroxyl radical leading to a *cisoid* terminus occurs ($k_ß$~30 $^{s-1}$; Porter & Wujek 1984; Tallman et al. 2001) more than twenty times slower than that leading to a *transoid* terminus ($k_ß$=620 $^{s-1}$; Tallman et al. 2004). Moreover, unsymmetrical pentadienyl radicals with partial s-*cis* orientation are not re-formed from conjugated diene peroxyl radicals by β-fragmentation because s-*cis* diene peroxyl radicals isomerize into s-*trans* diene peroxyl radicals (Frankel et al. 1982; Porter 1986). The β-fragmentation of the nonconjugated bisallylic peroxyl radicals is several orders of magnitude faster than that of the conjugated peroxyl radicals, and it occurs irrespective of the mechanism, with a rate constant between 2.2 to 2.8 x 10^6 s^{-1} (Tallman et al. 2004). Tallman et al. (2004) suggest that the β-fragmentation of a nonconjugated bisallylic peroxyl radical occurs, in a similar manner to methyl oleate rearrangement, through an allyl radical-dioxygen complex.

Several minor hydroperoxide products have been identified from the autoxidation of methyl linoleate. These products include nonconjugated diene hydroperoxides (<1%) that arise by abstraction of an allylic hydrogen atom at the C-8 or C-14 of the linoleate precursor (Schieberle & Grosch 1981; Haslbeck et al. 1983), *i.e.* the two double bonds behave as isolated identities, as in methyl oleate autoxidation. Porter & Wujek (1984) have reported that detectable amounts of conjugated diene hydroperoxides other than the four main products were formed when linoleate and the three other geometric isomers of methyl 9,12-octadecadienoate were oxidized in the presence of 1.5-3.0 M cyclohexadiene. These minor conjugated hydroperoxides were less than 5 mol% of the total hydroperoxides. More recently, Tokita et al. (1999) have detected methyl 9-hydroperoxy-10-*cis*,12-*trans*-octadecadienoate and methyl 13-hydroperoxy-9-*trans*,11-*cis*-octadecadienoate (0.4% of each) from methyl linoleate autoxidation by HPLC, and Tokita & Morita (2000) have identified methyl 9-hydroperoxy-10-*cis*,12-*cis*-octadecadienoate (0.11%) and methyl 13-hydroperoxy-9-*cis*,11-*cis*-octadecadienoate (0.23%) as corresponding oxo-derivatives. These isomers are similar to those characterized from the autoxidation of *cis,cis*-, *cis,trans*- and *trans,trans*-hepta-2,5-diene model compounds (Frankel et al. 1982). The formation of these isomers, if the direct isomerization of the pentadienyl radical is excluded, may be explained as being derived from a pentadienyl radical in Z-or U-conformation (Frankel et al. 1982; Porter 1986).

2.1.2 Autoxidation of conjugated diene fatty acids

The literature on the autoxidation of conjugated fatty acids is minimal. **Fig. 11** depicts the pathways A to C suggested for the autoxidation of CLA. Early studies, reviewed in Swern 1961, reported that the autoxidation of CLA mainly produces relatively low molecular weight polymeric peroxides (pathway A). More recently, CLA has been proposed, based on identification of secondary oxidation products, to autoxidize through two distinct routes (pathway B, Yurawecz et al. 1997) or to yield products identical to those formed during singlet oxygen oxidation of CLA (pathway C, Eulitz et al. 1999; Yurawecz et al. 2003). In the following section the different pathways are reviewed. It is noteworthy that the primary autoxidation products in all of these pathways are unknown. Moreover, no hydroperoxide products have been identified or proposed. In fact, the primary products are expected not to be similar to methyl linoleate hydroperoxides (Yurawecz et al. 1997).

Polymerization (Pathway A) The early literature reports that the autoxidation of conjugated fatty acids differs from that of the nonconjugated fatty acids and produces in an autocatalytic manner relatively low molecular weight polymeric peroxides (Allen et al. 1949; Kern et al. 1955, 1956; Privett 1959). The data supports oxygen-carbon rather than carbon-carbon polymerization, and indicates that appreciable amounts of isolated *trans*-unsaturation resides in the polymer fraction. Empirical kinetic reassessment of the early data by Brimberg & Kamal-Eldin (2003) suggests that oligomers having an average of three monomers would be kinetically favoured at the beginning of the oxidation and that the extent of oligomerization increases with increased temperature. Recently, Luna et al. (2007) confirmed the formation of polymeric products during autoxidation of Me 9*c*,11*t*-CLA and Me 10*t*,12*c*-CLA. In their study, oxidized monomers, dimers and polymers were quantified concomitantly by high-performance size-exclusion chromatography (HPSEC). The polymers were the first and major compounds formed, and monomers were found

only in negligible amounts. The structures of both the polymeric and the monomeric compounds, however, remain to be determined.

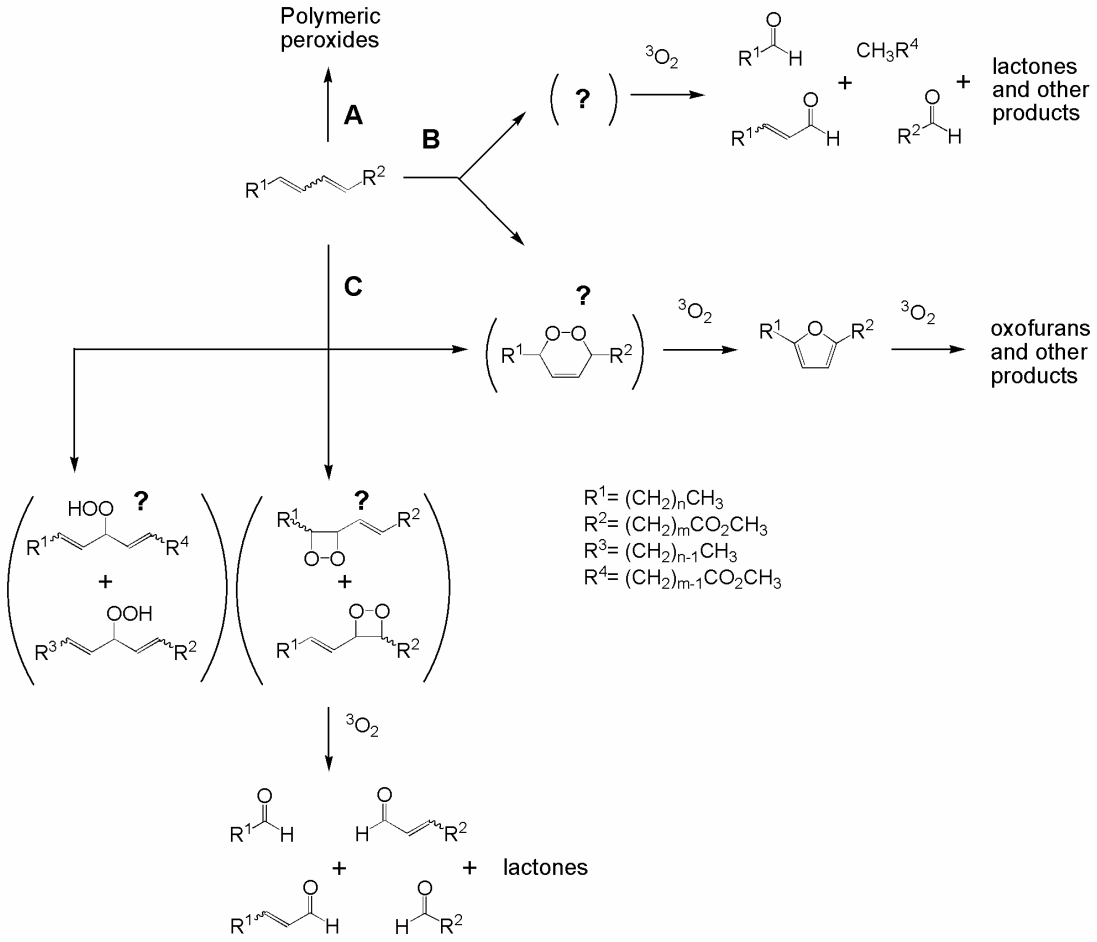


Figure 11 Proposed pathways A to C of the autoxidation of CLA (A Swern 1961 and references therein; B Yurawecz et al. 1997; C Eulitz et al. 1999, Yurawecz et al. 2003); ? = proposed or unknown compound(s).

Unsaturated fatty acids are constituents of alkyd paints and the studies on oxidative crosslinking of fatty acids in these paints, as reviewed in van Gorkum 2005, provide insight into the autoxidative polymerization mechanism of nonconjugated and conjugated fatty acids. For example, some important mechanistic conclusions have been drawn based on MS analysis of the oxidative crosslinking products of ethyl linoleate and methyl 9-*trans*,11-*trans*-octadecadienoate (Me 9*t*,11*t*-CLA) formed in the presence of a Co/Ca/Zr drier (Muizebelt & Nielen 1996). While the oxidative crosslinking of ethyl linoleate yielded dimers to tetramers, that of Me 9*t*,11*t*-CLA yielded dimers to hexamers. Within each group of dimers to hexamers, the Me 9*t*,11*t*-CLA oligomers varied in molecular weight, suggesting structural heterogeneity, *e.g*. oligomers with ether, peroxy, and carbon-carbon crosslinks. It was concluded that in nonconjugated dienes, crosslinking occurred through termination reactions and in conjugated dienes through radical addition to the

double bond. Moreover, because the Me 9*t*,11*t*-CLA dimer peaks were all doubled in the mass spectra with a mass difference of 2 Da as compared with ethyl linoleate dimer peaks, it was thought that while ethyl linoleate produces dimers by recombination of radicals, dimerization in Me 9*t*,11*t*-CLA occurs through addition of radicals to the double bond with subsequent termination by disproportionation. When the oxidative crosslinking reactions were performed in the presence of Co or Co/Ca/Zr catalysts and reactive diluents, further differences were observed between ethyl linoleate and conjugated *cis,trans* fatty esters (Muizebelt et al. 2000). The oxygen distribution in the conjugated fatty ester oligomers was much narrower than in the ethyl linoleate oligomers; about two oxygen atoms per monomeric unit were incorporated in all conjugated fatty ester oligomers. This suggested that the carbon-centred radical formed from the conjugated fatty esters reacted rapidly with oxygen before adding to another fatty ester and thus, that the oligomers had a polyperoxide character.

Formation of secondary/monomeric oxidation products (pathways B, C) Four furan fatty acids (FFAs), 8,11-epoxy-8,10-octadecadienoic acid ($F_{8,11}$), 9,12-epoxy-9,11-octadecadienoic acid ($F_{9,12}$), 10,13-epoxy-10,12-octadecadienoic acid ($F_{10,13}$) and 11,14-epoxy-11,13-octadecadienoic acid ($F_{11,14}$), have been identified as secondary autoxidation products of a mixture of CLA isomers in water-methanol solution at 50 °C by GC-MS and/or GC with flame-ionization detection (Yurawecz et al. 1995). The mechanism for the formation of these FFAs and the structures of their precursor compounds are unknown. The FFAs are suggested to arise from cyclic peroxides or possibly from dioxo fatty acids (Yurawecz et al. 1995; Eulitz et al. 1999). The former route seems plausible, because Bascetta et al. (1984) have reported that both thermal dehydration of a 1,2-dioxine, the major photo-oxidation product of Me 9*t*,11*t*-CLA, and the treatment of this cyclic peroxide with ferrous ion in aqueous tetrahydrofuran result in an FFA.

Besides FFAs, secondary oxidation products such as alkanals (hexanal, heptanal, and octanal), alkenals (2*t*-heptenal, 2*t*-octenal, 2*t*-nonenal, and 2*t*-decenal), alkanoates (heptanoate, octanoate, nonanoate, and decanoate), oxoalkanoates (7-oxoheptanoate, 8-oxooctanoate, 9-oxononanoate, 10-oxodecanoate, and 11-oxoundecanoate), and four α,β-unsaturated lactones (Yurawecz et al. 1995, 1997; Sehat et al. 1998; Eulitz et al. 1999) have been detected in CLA autoxidation mixtures. The autoxidation of methyl $F_{9,12}$ as a neat oil at 50 °C produced several secondary products including 5-hexyl-2-furaldehyde, methyl 8-oxooctanoate, methyl 13-oxo-9,12-epoxytrideca-9,11-dienoate, methyl 8-oxo-9,12-epoxy-9,11-octadecadienoate, and methyl 13-oxo-9,12-epoxy-9,11-octadecadienoate (Sehat et al. 1998).

It has been suggested that the autoxidation of CLA yields products identical to those produced by singlet oxygen oxidation of CLA (Eulitz et al. 1999). The major primary oxidation products of methylene blue-sensitized oxidation of methyl 8-*trans*,10-*trans*-octadecadienoate (Gunstone & Wijesundera 1979) in methanol and of Me 9*t*,11*t*-CLA (Bascetta et al. 1984) in tetrachloromethane/methanol (95/5, v/v) have been identified as 1,2-dioxines. In addition, based on photo-oxidation studies performed with 1,3-dienes (Manring & Foote 1983; Clennan & L'Esperance 1985a,b; O'Shea & Foote 1988), it may be anticipated that the singlet oxygen oxidation of CLA yields nonconjugated diene monohydroperoxides and 1,2-dioxetanes as minor primary photo-oxidation products. Thus far, none of these monomeric products have been isolated or characterized from CLA

autoxidation. The nonconjugated diene hydroperoxides have been suggested to form through a concerted ene-reaction and the cyclic peroxides through 1,2- and 1,4-addition of molecular oxygen. These mechanisms seem, however, highly unlikely because the direct addition of triplet oxygen to the double bonds of CLA violates the Wigner's spin-conservation rule. Brimberg and Kamal-Eldin (2003) have proposed, based on kinetic data, that monomeric products are formed through splitting of the oligomers. This mechanism seems plausible but remains to be confirmed.

Kinetics and mechanism of CLA autoxidation As a thin film, 10*t*,12*c*-CLA oxidizes faster than 9*c*,11*t*-CLA at 40-80 °C when residual unoxidized substrate is monitored (Minemoto et al. 2003; Tsuzuki et al. 2004). In addition, it has been established that the stability of CLA isomers decreases in the order *trans,trans*>*cis,trans*>*cis,cis*, which shows the stabilizing effect of a *trans* double bond (Yang et al. 2000), as is seen for other fatty acids such as elaidic (18:1, 9*t*) and oleic acids (Lanser et al. 1986). The literature on the susceptibility of CLA to autoxidation compared to other polyunsaturated fatty acids, however, is highly controversial; some studies suggest that CLA is less oxidizable than linoleic acid (Allen et al. 1949; Fukumi & Ikeda 1970; Ha et al. 1990), some others find an opposite trend (van den Berg et al. 1995; Zhang & Chen 1997; Chen et al. 1997; Chen et al. 2001; Yang et al. 2000; Tsuzuki et al. 2004) and yet others find similar susceptibility (Holman & Elmer 1947; Suzuki et al. 2001). This controversy may perhaps be partly explained by the lack of studies performed with pure CLA isomers, the widely different conditions under which the oxidations are performed, and by problems in identification of adequate methods to monitor CLA autoxidation. Tsuzuki et al. (2004) have, for example, reported that 9*c*,11*t*-CLA oxidizes at the same rate as linolenic acid (18:3; 9*c*,12*c*,15*c*) when monitored by substrate consumption, whereas when monitored by oxygen consumption it oxidizes at a similar rate to linoleic acid, and linolenic acid at a comparable rate to α-eleostearic acid (18:3; 9*c*,11*t*,13*t*). Moreover, Van den Berg et al. (1995) and Suzuki et al. (2004) have demonstrated that peroxide value (PV) measurements produce incorrect results in kinetic studies when compared with result obtained by following substrate consumption.

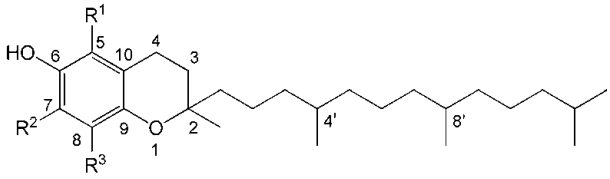
The problems in finding an adequate method for monitoring the autoxidation of CLA stem most likely from the fact that CLA autoxidizes mainly through a different mechanism and produces different and as yet uncharacterized oxidation products compared to monounsaturated and nonconjugated polyunsaturated fatty acids. It is well established by PV and oxygen consumption measurements (Allen et al. 1949), by MS (Muizebelt et al. 2000), and by HPSEC (Luna et al. 2007) that the autoxidation profile of CLA differs from that of its nonconjugated counterpart. Peroxide products accumulate more slowly and only in small amounts compared to linoleic acid (Allen et al. 1949; Suzuki et al. 2004). CLA absorb less oxygen per mole of oxidized substrate than linoleic acid (Allen et al. 1949; Tsuzuki et al. 2004) and produces mainly polymeric products (Luna et al. 2007). Because of these differences between the autoxidation of conjugated and nonconjugated diene fatty acids, the result should be interpreted with care. PV, for example, is generally taken as indication of hydroperoxide formation. PV measurement is, however, an indirect method and cannot be used to distinguish between hydroperoxides (ROOH) and dialkyl peroxides (ROOR, for example, cyclic or oligomeric peroxides). Therefore if PV measurements are taken as an indication of hydroperoxide formation, it most certainly leads to incorrect results if the autoxidation primarily yields oligomeric polyperoxides.

Nevertheless, some mechanistic conclusions about the autoxidation of conjugated dienes have been drawn from the kinetic studies. Kinetic evidence supports the idea that the autoxidation of CLA occurs by an autocatalytic free-radical chain reaction (Kern et al. 1955; Minemoto et al. 2003) similarly to autoxidation of nonconjugated fatty acids. The role of conjugated fatty acids in their own autocatalytic oxidation, however, seems to differ from that of their nonconjugated counterparts (Allen & Kummerow 1951; Kern et al. 1955). For example, the rate of the reaction is one-half order with respect to the catalytic product in the case of CLA, while it is first order in the case of linoleic acid. Comparing the oxidation kinetics of 9*c*,11*t*-CLA and 10*t*,12*c*-CLA at different temperatures, Minemoto et al. (2003) observed that the oxidation of 9*c*,11*t*-CLA followed an autocatalytic rate expression through the entire oxidation process, while the oxidation of 10*t*,12*c*-CLA followed the autocatalytic rate expression only during the first half of the oxidation, which then was followed by first order kinetics.

2.2 Role of α-tocopherol in fatty acid autoxidation

Tocopherols constitute a series of related benzopyranols or methyl tocols. They are composed of a hydroxychroman ring with a phytyl side chain attached at C-2. Four structurally related tocopherols, which differ from each other in the number and position of the methyl groups in the aromatic ring, are depicted in **Table 2**.

Table 2 Structures and CA registry numbers of tocopherols.



Tocopherol	R^1	R^2	R^3	CA registry number
α-tocopherol	CH_3	CH_3	CH_3	[59-02-9]
β-tocopherol	CH_3	H	CH_3	[16698-53-4]
γ-tocopherol	H	CH_3	CH_3	[54-28-4]
δ-tocopherol	H	H	CH_3	[199-13-1]

As illustrated in **Fig. 12**, in the crystal form, the heterocyclic ring in the hydroxychromans has a half-chair conformation with the ring puckering being to some extent limited by a 1,3-steric interaction between the pseudoaxial hydrogen at C-4, and the pseudoaxial substituent at C-2 (Doba et al. 1983; Burton et al. 1985). All naturally occurring tocopherols have a 2*R*,4'*R*,8'*R* configuration and have been referred to as 2D,4'D,8'D, *d*-tocopherols or (+)-tocopherols (Kamal-Eldin & Appelqvist 1996).

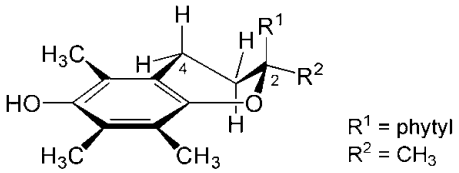


Figure 12 The half-chair conformation of a hydroxychroman (Burton et al. 1985).

Reactions that contribute to the antioxidant properties of α-tocopherol Antioxidants are, in a broad sense, compounds that when present in small quantities protect organic molecules, including those in living organisms, against oxidative degradation (Frankel 1998c). Antioxidants can be divided into two broad classes, referred to as preventive and chain-breaking antioxidants. Preventive antioxidants decrease the rate of autoxidation by reducing the rate of chain initiation reactions, whereas the chain-breaking antioxidants slow the reaction by interfering with one or more of the propagation steps.

The chemistry and antioxidant properties of tocopherols have been extensively studied (reviewed in Niki 1987; Kamal-Eldin & Appelqvist 1996; Frankel 1998c; Kamal-Eldin et al. 2008). The antioxidant activity of tocopherols is determined by the rate constant k_{inh} and the stoichiometric number (Niki 1987). The rate constant k_{inh} is the rate constant for the transfer of a phenolic hydrogen atom to the lipid peroxyl radical, which is the rate determining step (**Table 3**, eq 10). In methyl linoleate autoxidation, the rate constant k_{inh} for α-tocopherol is 3.8 x 10^6 $M^{-1}s^{-1}$ (Tallman et al. 2001), which is *ca.* 60,000 times faster than the self-propagation reaction of methyl linoleate (k=62 $M^{-1}s^{-1}$, Roschek et al. 2006). Kinetic studies suggest that the stoichiometric value for α-tocopherol is two (Burton & Ingold 1981) *i.e.* one α-tocopherol molecule traps two peroxyl radicals. Hence, the tocopheroxyl radical (*i.e.* the chroman-6-oxyl radical, TocO•) formed in the rate determining step reacts with another lipid peroxyl radical and produces a quinolide peroxide (*i.e.* a α-tocopheroxyl radical and peroxyl radical adduct; T-OOL; **Table 3**, eqs 11 to 13).

α-Tocopherol is one of the best chain-breaking phenolic antioxidants known (Burton et al. 1980, 1985; Burton & Ingold 1981). Kinetic studies have established that α-tocopherol is, *in vitro*, not only the most active of the four tocopherols but also a much better antioxidant than simple phenols or otherwise similar phenols that lack the fused 6-membered heterocyclic ring, including one of the major commercial antioxidants 2,6-di-*tert*-butyl-4-methylphenol (Burton & Ingold 1981; Burton et al. 1985). The high activity of α-tocopherol compared to other tocopherols and simple phenols stems from the structural features of α-tocopherol, such as the extent of methylation of the benzene ring and the orientation of the *p*-type lone pair on the para oxygen with respect to the aromatic plane, that stabilize the resulting tocopheroxyl radical (Burton & Ingold 1981, 1986; Burton et al. 1983). The fully methylated aromatic ring of α-tocopherol makes it not only more lipophilic than other tocopherols but also, in comparison, provides maximum inductive effect. All tocopherols, in turn, are activated over simple phenols by the stereoelectronic effects of the para oxygen. The fused heterocyclic ring forces the lone pair

at the para oxygen into a position that enables resonance stabilization by orbital overlap (mesomeric effects). The phytyl tail is not important for the antioxidant activity of tocopherols. However, the length of the tail is crucial for positioning the molecule at the site where protection is needed in biological systems (Burton & Ingold 1986).

Table 3 The reactions responsible for the anti- and pro-oxidant properties of α-tocopherol (see structures in **Fig. 13**; Tavadyan et al. 2007).

Property	Reaction	Eq
antioxidant	TocOH + LOO• → TocO• + LOOH	10
	TocO• + LOO• → *o*-T-OOL	11
	TocO• + LOO• → *o*-T-OOL'	12
	TocO• + LOO• → *p*-T-OOL	13
	TocO• + TocO• → TocO-TocO	14
pro-oxidant	TocOH + LOOH → TocO• + LO• + H_2O	15
	TocO• + LH → TocOH + L•	16
	TocO• + LOOH → TocOH + LOO•	17
	o-T-OOL → LO• + *o*-TE•	18
	o-T-OOL' → LO• + *o*-TE'•	19
	p-T-OOL → LO• + TQO•	20

Reactions that contribute to the pro-oxidant properties of α-tocopherol Ideal chain-breaking antioxidants react with peroxyl radicals and generate stable and unreactive reaction products. In reality this is not achieved. Although α-tocopherol is an effective antioxidant, the α-tocopherol molecule itself, tocopheroxyl radical, and other radicals formed in the autoxidation of α-tocopherol (**Fig. 13**) may participate in side reactions that generate free radicals to initiate new chains (chain branching) and/or propagate the chain reaction, and thus it may also be classified as pro-oxidative. The degree of such reactions depends on factors such as the structure and the concentration of the antioxidant and temperature (Kamal-Eldin & Appelqvist 1996).

Several mechanisms have been proposed to account for the pro-oxidant properties of α-tocopherol (reviewed in Kamal-Eldin & Appelqvist 1996). A recent study constructed a kinetic model of the peroxidation reactions of methyl linoleate in the presence of α-tocopherol (Tavadyan et al. 2007) that provided a good prediction of the experimental data. The initial model of the reaction mechanism included 53 individual steps and was analysed by a numerical value method to reveal the roles *i.e.* the kinetic significance of the individual steps and chemical species of the reaction. This numerical value analysis, not surprisingly, attributed the antioxidant properties of α-tocopherol to the transfer of the phenolic hydrogen to the lipid peroxyl radical and to reactions of α-tocopheroxyl radicals with the lipid peroxyl radicals and with each other (**Table 3**, eqs 10 to 14). In addition, it revealed that the three types of reactions that are responsible for the pro-oxidant properties are the autoinitiation reaction (eq 15), tocopherol-mediated peroxidation (TMP) reactions (eqs 16 and 17), and homolytic decomposition of the quinolide peroxides (eqs 18 to 20). Autoinitiation refers to a reaction where the α-tocopherol molecule itself catalyses the decomposition of hydroperoxides, producing highly reactive alkoxyl radicals. TMP

reactions are chain transfer reactions in which the tocopheroxyl radical abstracts a hydrogen atom from the unoxidized lipid or lipid hydroperoxide, generating a carbon-centred radical or a lipid peroxyl radical. The decomposition of quinolide peroxides (T-OOLs) may also be catalysed by tocopherol, producing highly reactive alkoxyl radicals.

Summary Autoxidation of lipids in the presence of α-tocopherol is a cascade of different reactions, and the significance of a particular reaction varies during the course of the reaction due to some intermediates building up and some others diminishing. Based on numerical value method analysis (Tavadyan et al. 2007), the anti- and pro-oxidant properties of α-tocopherol are attributed only to a small number dominant steps. These steps have not very high relative value contributors but together they account for the descriptive and predictive abilities of the kinetic model of methyl linoleate peroxidation.

Effects of α-tocopherol on formation of lipid hydroperoxides α-Tocopherol, as an antioxidant, inhibits the formation of lipid hydroperoxides. The inhibitory activity of α-tocopherol (and other tocopherols) against lipid oxidation is associated with an optimal concentration of the antioxidant, after which stabilization decreases (Kamal-Eldin et al. 2008). The optimum concentration of α-tocopherol, for example, for inhibiting the formation of hydroperoxides in the oxidation of purified soybean oil at 55 °C was 100 ppm (Jung & Min 1990).

Several studies suggest that tocopherols at higher than the optimal concentration accelerate the formation of hydroperoxides, especially at the early stage of oxidation (Peers & Coxon 1983; Jung & Min 1990; Yanishlieva & Marinova 1992; Bowry & Stocker 1993; Huang et al. 1995; Bowry & Ingold 1999). This effect has been described as a pro-oxidant effect but is better described as loss of antioxidant efficacy at post-optimal concentrations, because it is plausible that not only the concentration of α-tocopherol but also of several other chemical species that may participate in the initiation of the oxidation process are above a certain optimal concentration (Fuster et al. 1998; Evans JC et al. 2002; Kamal-Eldin et al. 2008). For example, the pro-oxidant property was enhanced, *i.e.* the rate of methyl linoleate hydroperoxide formation increased, during the induction period due to the presence of initial levels of hydroperoxides (Kamal-Eldin et al. 2002).

α-Tocopherol affects the relative distribution of hydroperoxide isomers formed during lipid autoxidation. In methyl linoleate autoxidation, α-tocopherol inhibits the formation of the thermodynamic *trans,trans* conjugated diene hydroperoxides and thus favours the formation of the kinetic *cis,trans* products in a concentration-dependent manner (Porter et al. 1980; Peers et al. 1981; Weenen & Porter 1982; Mäkinen et al. 2000). At high concentrations of α-tocopherol (5%, 0.1 M) these effects dominate almost totally and the mixture of products is greatly simplified; only *cis,trans* isomers are formed and the quantities of the positional isomers are equalized (Peers et al. 1981). This effect of α-tocopherol has been used to advantage in the production of pure *cis,trans* hydroperoxides from methyl linoleate, methyl linolenate, and methyl arachidonate (Peers et al. 1981; Peers & Coxon 1983). As discussed above in section 2.1.1, the product distribution and the ability of α-tocopherol to direct the isomeric distribution of hydroperoxides has been rationalised based on the reversibility of oxygen addition. For example, in methyl linoleate autoxidation (**Fig. 9**) the *trans,trans* products are formed from the initial peroxyl radicals through the β-fragmentation pathway. As a good hydrogen atom donor, α-tocopherol

increases the ratio of the *cis*,*trans* products by trapping the initially-formed peroxyl radicals before they undergo rearrangement (Porter 1980).

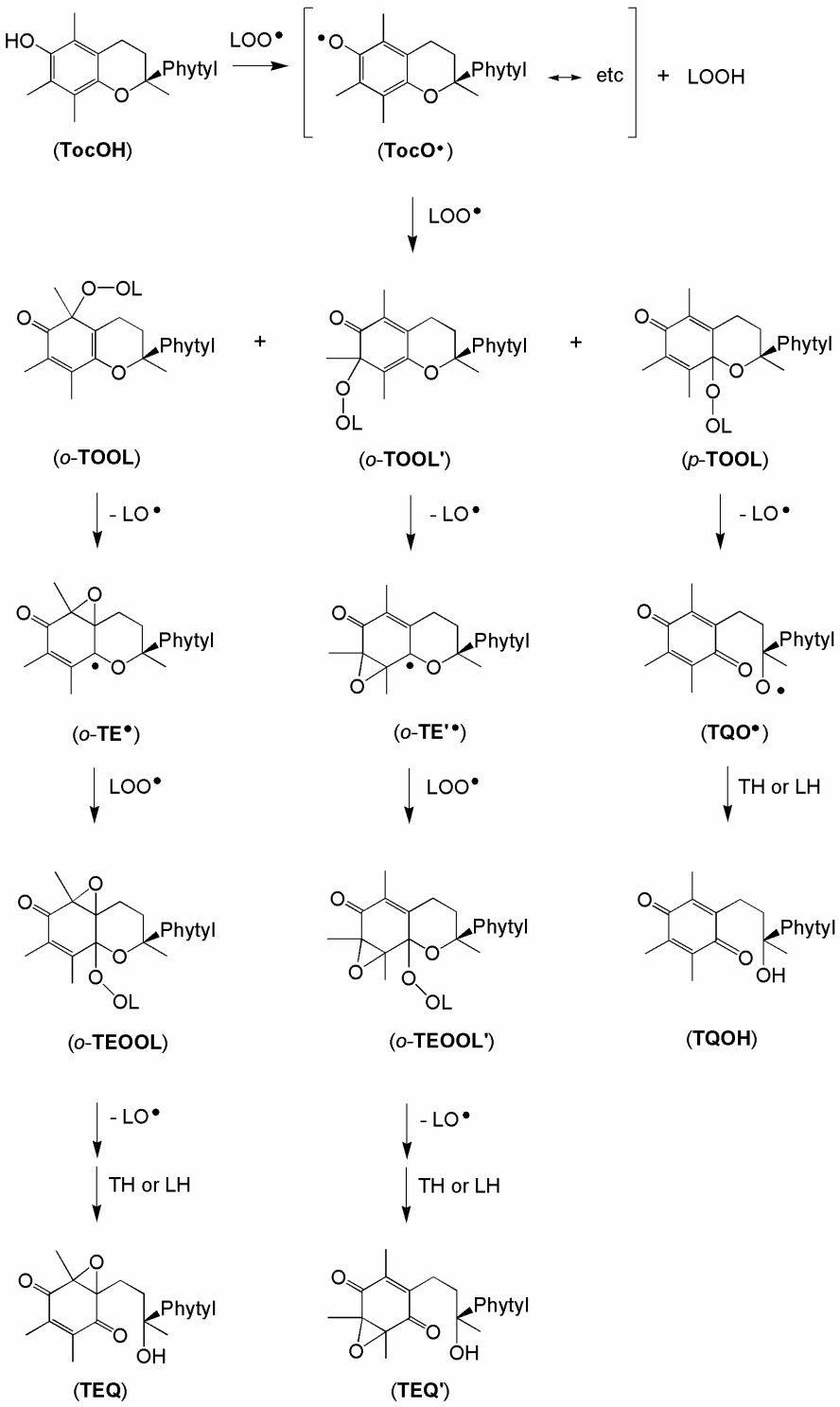


Figure 13 The autoxidation reactions of α-tocopherol (Modified from Tavadyan et al. 2007).

3 The aims of the present study

The present study was a part of a multidisciplinary study on CLA oxidation initiated by Professor Anu Hopia (Food Chemistry Division) at the University of Helsinki. Participating laboratories were the Food Chemistry Division, the Laboratory of Analytical Chemistry, and the Laboratory of Organic Chemistry. The author of this thesis focused on investigating the largely unknown early stages of CLA autoxidation.

The main objectives of the individual studies were

I to test the hypothesis that hydroperoxides are one type of primary product for the autoxidation of CLA methyl esters.

II to separate and to characterize the Me 9*c*,11*t*-CLA hydroperoxides as their hydroxy derivatives by combining HPLC, UV, GC-MS, and 1D and 2D NMR techniques, and to propose a mechanism for their formation.

III to confirm the mechanistic proposal by investigating the autoxidation of Me 10*t*,12*c*-CLA, and to draw conclusions about the autoxidation mechanism based on theoretical calculations.

IV to produce and to separate conjugated diene monohydroperoxides from the two major CLA methyl esters, and to study their NMR properties.

4 Results and discussion

Autoxidation of CLA, as discussed above in section 2.1.2, has been suggested to proceed through several pathways. The primary autoxidation products of these pathways are, however, undetermined. Consequently, the early stages of CLA autoxidation and the autoxidation mechanisms are largely unknown. Therefore, the identification of the primary autoxidation products is of crucial importance for deeper understanding of the autoxidation of CLA. In particular, the mechanism(s) of formation and the structures of the oligomeric products, which are the main products of CLA autoxidation (Kern et al. 1955, 1956; Privett 1959; Luna et al. 2007), remain more or less speculative, without a clear knowledge of the primary species involved in their initiation.

4.1 Evidence for hydroperoxide formation (I)

Conjugated diene allylic monohydroperoxides were discovered, contrary to earlier assumptions (Yurawecz et al. 1997), to be the primary autoxidation products of CLA methyl esters. A flow chart (**Fig. 14**) illustrates the strategy employed for testing the hypothesis regarding hydroperoxide formation during autoxidation of a mixture of CLA methyl esters in the presence and absence of α-tocopherol. The results, with the exception of the synthesis of the hydroxystearate standards (see section 6.2) and the GC-MS analysis of the trimethylsilyl ethers of these hydroxystearates, are reported in **I**. The conclusive evidence for the hydroperoxide formation was provided by NMR spectroscopy. The hydroperoxy protons appeared as partly overlapping singlets at 7.98-7.81 ppm in $CDCl_3$ and at 10.48-10.40 ppm in $(CD_3)_2CO$. These assignments were further confirmed by a D_2O-test, chemoselective reduction of the hydroperoxyl group to a hydroxyl group by sodium borohydride, and by comparison of the ^1H NMR spectrum of the CLA methyl ester hydroperoxides with that of methyl linoleate hydroperoxides. Furthermore, based on thin-layer chromatography (TLC) analysis, UV absorption at 234 nm, distribution of the olefinic proton resonances, the integral ratio of olefinic protons to hydroperoxy protons, and the ^1H-^1H correlation experiment (COSY), it was evident that the main CLA methyl ester hydroperoxides had a conjugated diene allyl monohydroperoxy structure.

It is necessary to explain why these primary hydroperoxides were not recognized by early investigators. Conjugated fatty acids are generally less studied than their nonconjugated counterparts because they are rare and have not been readily available until recently. The complexity of the CLA methyl ester hydroperoxide mixture (*vide infra*) compared to mixtures produced from methyl linoleate might have been one of the obstacles to discovery of the hydroperoxides. Not only is there less hydroperoxide formation but also the number of hydroperoxide isomers is greater. Since CLA autoxidation studies are, even today, often performed with mixtures of CLA isomers, the primary product mixture is even more complex and as a result more difficult to analyse. Moreover, when the autoxidation of CLA is performed in the absence of a good hydrogen atom donor, the analysis of the hydroperoxide mixture is particularly challenging because under such conditions autoxidative polymerization (Kern et al. 1955, 1956; Privett 1959;

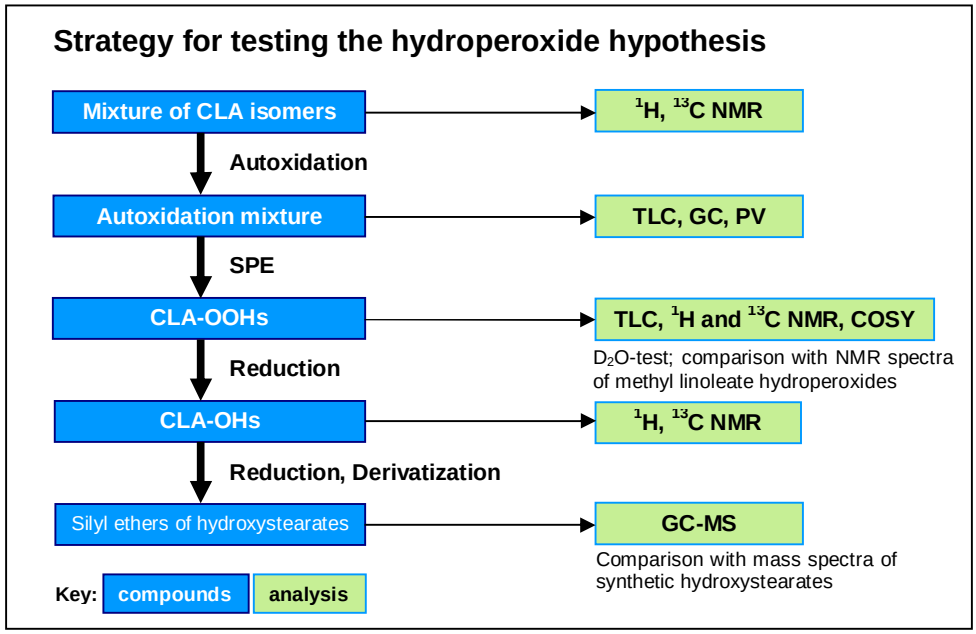


Figure 14 The strategy for testing the hypothesis for hydroperoxide formation during autoxidation of CLA methyl esters in the presence and absence of α-tocopherol.

Luna et al. 2007) prevails over hydroperoxide formation. Knowledge of the hydroperoxide formation steps is, however, essential for clarification of the early stages of CLA autoxidation. In addition, knowledge of the CLA hydroperoxide structures may aid in the prediction of possible oligomeric structures that are currently unknown and the design of experiments that could lead to their characterization.

4.2 Structure of the conjugated linoleic acid methyl ester hydroperoxides (II to IV)

Structures of nine isomeric methyl hydroxyoctadecadienoates and of ten methyl hydroperoxyoctadecadienoates were characterized as pairs of enantiomers. The hydroperoxides differed in the position of the hydroperoxyl group and in the stereochemistry of the 1,3-diene structure. Four of the six different positional isomers are new positional isomers of conjugated diene lipid hydroperoxides, the 8-, 10-, 12- and 14-hydroperoxyoctadecadienoates. Six of the hydroperoxides had a *trans,trans* geometry and four a *cis,trans* geometry. Interestingly, two of the *cis,trans* isomers had an unusual structure for lipid hydroperoxides where the double bond adjacent to the methine carbon bears a *cis* geometry.

Hydroperoxides were produced from Me 9*c*,11*t*-CLA (**II**, **IV**) and Me 10*t*,12*c*-CLA (**III**, **IV**) by autoxidizing the esters in the presence of 20% α-tocopherol under atmospheric oxygen at 40 °C in the dark. Under these conditions, CLA autoxidation is directed from

autoxidative polymerization towards hydroperoxide formation. The high amount of α-tocopherol was therefore necessary for the production of sufficient amounts of hydroperoxides for the characterization. In addition, the high amount of α-tocopherol was expected, as observed in the autoxidation of methyl linoleate (Peers et al. 1981), to simplify the hydroperoxide mixture such that it consisted of only the kinetic products, thereby simplifying the separation of the isomers as well. The extent of oxidation was kept low; in the autoxidation of Me 9*c*,11*t*-CLA the extent of oxidation was 13% based on substrate consumption followed by GC, and the yield of hydroperoxides, which was estimated based on TLC and PV measurement, was 9% (**II**).

The CLA methyl ester hydroperoxides were isolated by flash column chromatography or by solid phase extraction (SPE). The hydroperoxides were subsequently reduced to the corresponding hydroxyoctadecadienoates (**II**, **III**) or studied as intact hydroperoxides (**III**, **IV**). The structures of the individual hydroxy and hydroperoxy isomers were determined by combining HPLC separation, UV, GC-MS, and 1D and 2D NMR techniques.

4.2.1 Methyl hydroxyoctadecadienoates (II, III)

Characterization of the structures of the CLA methyl ester hydroperoxides and determination of their isomeric distribution were the primary objectives for study **II**. This knowledge is required for the development of a mechanism for the formation of these primary oxidation products. The CLA methyl ester hydroperoxides were characterized initially as their corresponding hydroxy derivatives because it was anticipated, based on the established similarity of the structures of CLA methyl ester hydroperoxides to methyl linoleate hydroperoxides (**I**), that this approach would have several advantages: the hydroxy derivatives are more stable than the hydroperoxides; the possible isomerization reactions under HPLC conditions are excluded; and the HPLC separation of the different isomers might improve, as has been reported in the case of methyl linoleate (Chan et al. 1975; Chan & Levett 1977a).

HPLC separation The semi-preparative NP-HPLC separation of seven methyl hydroxyoctadecadienoates from the autoxidation of Me 9*c*,11*t*-CLA is depicted in **II**, Fig. 1, and five isomers from that of Me 10*t*,12*c*-CLA in **III**, Fig. 1. The elution order of the hydroxyoctadecadienoates was, with one exception, as anticipated: the closer the hydroxyl group was to the methyl tail, the earlier the positional isomer eluted, and when the position of the hydroxyl group was the same, the *cis,trans* isomers always eluted before the *trans,trans* isomers. The exception was Me 13-OH-9*c*,11*t*, which appeared before the 14-hydroxy isomer. The separation of the hydroxy derivatives from Me 9*c*,11*t*-CLA autoxidation was satisfactory. In contrast, separation of the hydroxy derivatives from Me 10*t*,12*c*-CLA autoxidation proved challenging and was, despite all efforts, only partial. For this reason, Me 14-OOH-10*t*,12*c*, the minor component of the hydroperoxide mixture, was characterized only as an intact hydroperoxide.

Characterization The position of the hydroxyl group in the methyl hydroxyoctadecadienoates was determined by analysing the compounds as their corresponding silylated hydroxystearates by GC-MS. The GC-MS method (Frankel et al. 1977) was first tested with trimethylsilyl ethers of synthetic methyl 8-, 9-, and 13-

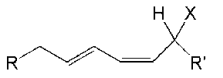
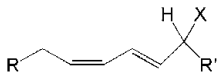
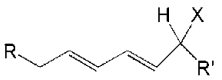
hydroxystearates (section 6.2) and commercially available methyl 12-hydroxystearate. These hydroxystearates were expected to correspond to the main positional hydroperoxide isomers formed during autoxidation of Me 9*c*,11*t*-CLA. The trimethyl silyl ethers of the hydroxystearates gave two characteristic fragment ions in their mass spectra and thus the positional isomers were unambiguously assigned (**II**, Table 1, and **III**, p. 603).

The geometry of the double bonds in the methyl hydroxyoctadecadienoates and the position of the 1,3-diene structure within the fatty ester chain were determined by NMR spectroscopy as described in **II** and **III**.

NMR properties Full assignments of NMR spectra of hydoxy derivatives of lipid hydroperoxides are rare (reviewed in Hämäläinen & Kamal-Eldin 2005). The fully assigned ^{13}C NMR spectra of the nine isomeric methyl hydroxyoctadecadienoates are listed in **II**, Table 2 (six isomers) and in **III**, Table 1 (three isomers). The assignments of the ^{1}H and ^{13}C NMR spectra of these hydroxydiene fatty esters by 2D NMR experiments are discussed in detail in **II** and in Hämäläinen & Kamal-Eldin 2005.

The chemical shifts of the allylic resonances in the ^{1}H and ^{13}C NMR spectra of the methyl hydroxyoctadecadienoates are of particular interest because they reveal the stereochemistry of the adjacent double bond. As illustrated in **Table 4**, the unusual *cis,trans* isomer where the *cis* double bond is adjacent to the allylic methine carbon may be distinguished from the common *cis,trans* isomers where the *trans* double bond is adjacent to the allylic methine carbon based on allylic methine proton and carbon resonances. The allylic methine proton is *ca.* 0.4 ppm less shielded when adjacent to a *cis* than to a *trans* double bond. The carbon resonance shows a more pronounced opposite trend: the methine carbon is *ca*. 5 ppm more shielded when adjacent to a *cis* than to a *trans* double bond. The vicinal coupling constant between the allylic methine and the olefinic protons seems to have some diagnostic value; this scalar coupling is *ca.* 1 Hz stronger in the unusual *cis,trans* hydroperoxide than in the more common *cis,trans* and *trans,trans* isomers. These findings agree with the ^{1}H and ^{13}C NMR data on hydroxy derivatives of *cis* and *trans* monoene allylic hydroperoxides from methyl oleate autoxidation (Garwood et al. 1977; Frankel et al. 1984; Kim et al. 2000).

Table 4 The range of observed allylic proton and carbon resonances of methyl hydroxyoctadecadienoates and of methyl hydroperoxyoctadecadienoates in $CDCl_3$. The vicinal coupling constants between the allylic methine and the olefinic protons are given in parenthesis (n=number of compounds) (**II** to **IV**).

		 n=1, X = OH; n=2, X = OOH		 n=2, X = OH, OOH		 n=6, X = OH, OOH	
	Allylic position	δ_H (*J*/Hz)	δ_C	δ_H(*J*/Hz)	δ_C	δ_H(*J*/Hz)	δ_C
X=OH	methylene	2.05	32.85	2.17-2.18	27.68-27.76	2.07-2.08	32.32-32.66
	methine	4.56 (8.3)	67.94	4.15-4.16 (6.9)	72.90-72.91	4.10-4.11 (7.0)	72.85-72.91
X=OOH	methylene	2.11	32.79-32.87	2.19	27.71-27.82	2.09-2.10	32.33-32.67
	methine	4.84-4.85 (9.2)	81.38-81.44	4.37-4.38 (7.7)	86.78-86.83	4.32-4.33 (8.3)	86.77-86.84

The geometry of the 1,3-diene structure is also reflected in the olefinic signals. The olefinic carbon resonances in the *trans,trans* isomers range from 129.38 to 135.70 ppm, those in the *cis,trans* isomers, where the methine carbon is adjacent to the *trans* double bonds, from 127.67 to 135.97 ppm, and those in the unusual *cis,trans* isomer from 130.62 to 137.53 ppm.

^{13}C NMR data on pure lipids may be utilized, as has been demonstrated with CLA isomers (Davis et al. 1999a,b; Lie Ken Jie 2001) and with triacylglycerols (Gunstone 1993; Lie Ken Jie & Mustafa 1997 and references therein), for developing both qualitative and quantitative NMR methods for analyses of mixtures of lipids. The ^{13}C NMR data on the methyl hydroxyoctadecadienoates presented in this thesis (**II**, **III**) may thus be helpful when studying mixtures of lipid hydroperoxides as their hydroxy derivatives. Furthermore, these ^{13}C NMR data allow the determination of the effects of allylic hydroxyl group substitution on the ^{13}C chemical shifts of CLA methyl esters (**Table 5**). In these spectra, the carbon carrying the hydroxyl group was shifted on average by 40.15 ppm to higher frequency. This is *ca*. 2 ppm less than what is observed for hydroxylated saturated fatty acids (Tulloch 1978). The effects on the olefinic carbon resonances varied depending on the position of the hydroxyl group and on the geometry of the 1,3-diene system. The direction of the shift, however, was the same and the average effects on the olefinic α, β, γ, and δ carbons were +1.27, +0.62, -0.92, and +3.11 ppm, respectively. The effects of the hydroxyl group on the saturated carbons could be calculated only for a few hydroxy derivatives where the hydroxyl group is positioned between the diene system and the methyl tail. The average effects on α, β, γ, and δ methylene carbons were +7.91, -3.83, -0.01 and -0.06 ppm, respectively. These effects on saturated carbons are similar to those reported for hydroxylation of saturated fatty acids (Tulloch 1978).

Table 5 Incremental ^{13}C chemical shifts (in ppm) due to substitution of a hydroxyl group at the allylic position in the C_{18} conjugated diene fatty acid esters in $CDCl_3$.[a,b]

Hydroxyoctadecadienoate			olefinic carbons				methylene carbons			
		CH(OH)	α	β	γ	δ	α	β	γ	δ
cis,*trans**	Me 8-OH-9c,11t	40.27	1.41	1.88	-0.60	2.74	nd	nd	nd	nd
cis,*trans*	Me 9-OH-10*t*,12*c*	40.03	1.20	0.19	-0.95	2.98	7.90	nd	nd	nd
	Me 13-OH-9*c*,11*t*	39.98	1.18	0.13	-0.90	2.86	7.92	-3.82	0.01	-0.05
trans,*trans*	Me 8-OH-9*t*,11*t*	40.24	1.34	0.69	-1.13	3.27	nd	nd	nd	nd
	Me 13-OH-9*t*,11*t*	40.23	1.29	0.40	-0.84	3.23	7.92	-3.83	-0.02	-0.07
	Me 9-OH-10*t*,12*t*[c]	nd	1.30	0.57	-0.96	3.21	nd	nd	nd	nd
	Me 10-OH-11*t*,13*t*[c]	nd	1.28	0.57	-0.97	3.22	nd	nd	nd	nd
	Me 12-OH-8*t*,10*t*[c]	nd	1.20	0.57	-1.00	3.26	nd	nd	nd	nd
	Me 14-OH-10t,12*t*[c]	nd	1.24	0.58	-0.97	3.22	nd	nd	nd	nd

[a]The α carbon refers to the carbon adjacent to the methine carbon CH(OH). [b]The values are calculated by comparison with the ^{13}C NMR spectrum of a CLA methyl ester with the appropriate double bond position and geometry. The ^{13}C NMR spectra of Me 9*c*,11*t*-CLA and Me 10*t*,12*c*-CLA were run with samples containing 100 mg of CLA methyl ester in $CDCl_3$ (0.8 mL). The ^{13}C NMR data for the other CLA isomers were obtained from the literature (Lie Ken et al. 1997, 1999; Davis et al. 1999a,b). [c]Chemical shifts compared to NMR data of CLA as an acid instead of an ester. nd=not determined. *Hydroxyl group bearing methine carbon adjacent to the *cis* double bond.

4.2.2 Methyl hydroperoxyoctadecadienoates (III, IV)

Production of pure CLA methyl ester hydroperoxides was of interest because NMR data on intact lipid hydroperoxides is scarce (reviewed in Hämäläinen & Kamal-Eldin 2005, pp. 91-95) and because these hydroperoxides may be utilised in further studies aimed at clarification of CLA autoxidation reactions, *e.g.* rearrangements, secondary autoxidation reactions, and oligomer formation, and for evaluating their biological significance. To this end CLA methyl ester hydroperoxides were produced as described above, isolated by SPE and separated by semi-preparative NP-HPLC.

HPLC separation The semi-preparative NP-HPLC separations of hydroperoxides produced from Me 9*c*,11*t*-CLA and from Me 10*t*,12*c*-CLA are depicted in **IV**, Fig. 1. The HPLC separation of the hydroperoxides was unexpectedly superior to the HPLC separation of the hydroxy derivatives, particularly in the case of Me 10*t*,12*c*-CLA hydroperoxides. Not only was the resolution improved but the total eluation time was significantly shorter; it decreased with most of the tested eluents by approximately half. The elution order of the hydroperoxides from both CLA methyl esters corresponded to that of their hydroxy derivatives.

Characterization The position of the hydroperoxyl group was confirmed when necessary by GC-MS with the same method as for the methyl hydroxyoctadecadienoates, and the stereochemistry of the double bonds as well as the position of the 1,3-diene structure within the fatty ester chain were determined by NMR Spectroscopy (**IV**).

NMR properties The ^{13}C NMR data for the ten isomeric methyl hydroperoxyoctadecadienoates are listed in **IV**, Table 1. The assignments of the ^{1}H and ^{13}C NMR spectra of methyl hydroperoxyoctadecadienoates by 2D NMR follow closely those of the corresponding hydroxy derivatives (*vide supra*).

The most notable differences between the ^{1}H NMR spectra of the hydroperoxides and the hydroxy derivatives are the presence of a hydroperoxy instead of a hydroxy proton signal, and the shift of the allylic methine proton resonance of the hydroperoxide by *ca*. 0.2 ppm to higher frequency from that of the corresponding alcohol. The hydroperoxy protons of the methyl hydroperoxyoctadecadienoates appear at 7.69-7.87 ppm in $CDCl_3$, at 10.38-10.42 ppm in $(CD_3)_2CO$ and at 7.24-7.41 ppm in C_6D_6 (**IV**). Strikingly, in the ^{13}C NMR spectra the methine carbon resonances are less shielded, by *ca*. 14 ppm, in the hydroperoxides than in the corresponding hydroxy derivatives. This applies to all three types of geometric isomers (**Table 4**).

As in methyl hydroxyoctadecadienoates, the allylic carbon and proton resonances of the methyl hydroperoxyoctadecadienoates have diagnostic value (**Table 4**). In the unusual *cis,trans* isomers the allylic methine proton shifts by *ca.* 0.5 ppm to higher frequency and the methine carbons by *ca.* 5 ppm to lower frequency compared to the corresponding signals in the more common *cis,trans* and *trans,trans* isomers. Moreover, the vicinal coupling constant between allylic methine and olefinic protons is *ca.* 1 Hz larger in the unusual *cis,trans* isomers than in the other geometric isomers. These findings agree with the ^{1}H and ^{13}C NMR data on *cis* and *trans* monoene allylic hydroperoxides from the autoxidation of methyl oleate (Garwood et al. 1977; Frankel et al. 1984; Porter et al.

1994a). The olefinic carbon resonances range in the *trans,trans* methyl hydroperoxyoctadecadienoates from 128.74 to 136.96 ppm, those in the *cis,trans* isomers from 127.40 to 134.22 ppm and those in the unusual *cis,trans* isomers from 125.03 to 138.57 ppm.

The ^{13}C NMR data on ten pure CLA methyl ester hydroperoxides enabled the determination of the effects of the hydroperoxyl group on the carbon chemical shifts of CLA isomers (**IV**, Table 2) and the determination of chemical shift differences of olefinic resonances (**IV**, p. 113) that may help assignment of structure to as yet unknown lipid hydroperoxides produced, for example, from other geometric and positional isomers of CLA methyl esters. The effect of the hydroperoxyl group on the methine carbon (average value +53.93 ppm) is significantly larger than the effect of the hydroxyl group (average value +40.15 ppm). This, as discussed above, is clearly manifested in the chemical shifts of the allylic methine carbon resonances.

Solvent effects on the ^{1}H and ^{13}C resonances of the hydroperoxides with the three types of diene geometry were investigated by running the spectra in turn in the relatively nonpolar solvent $CDCl_3$, in the polar solvent $(CD_3)_2CO$, and in the magnetically anisotropic solvent C_6D_6. Information on solvent effects on the chemical shifts of hydroperoxides (**IV**, Table 3) may be utilized for choosing the appropriate solvent for an investigation of mixtures of lipid hydroperoxides by NMR spectroscopy. It is noteworthy that the tentative assignment of the two main hydroperoxides produced in the autoxidation of a mixture of CLA methyl esters in the presence of α-tocopherol (**I**) proved correct. This demonstrates nicely the potential utility of ^{13}C NMR spectroscopy in studying mixtures of hydroperoxides. It must be emphasized, however, that the groundwork for the assignment of a NMR spectrum of a mixture of products is laid by the full assignments of NMR spectra of pure products.

4.3 Mechanism of autoxidation of conjugated linoleic acid methyl ester in the presence of α-tocopherol (I to IV)

The mechanism proposed for the hydroperoxide pathway of CLA autoxidation in the presence of a high amount of α-tocopherol is depicted in **III**, Scheme 2. A good mechanism explains not only the formation of all the products but also accounts for their relative proportions and the roles of any added reagents. The discovery of hydroperoxides in study **I** suggested that the autoxidation of CLA follows, at least in part, Farmer's hydroperoxide theory (Swern 1961), which was originally developed for monoene and nonconjugated polyene fatty acids, and that the reaction is an autocatalytic free-radical chain reaction. The free-radical nature of the autoxidation of CLA is in line with kinetic evidence (Kern et al. 1955; Minemoto et al. 2003). A mechanism that shows partial analogy to both methyl oleate and methyl linoleate autoxidation was presented (**I**, Fig. 3). It suggested the formation of a complex mixture of hydroperoxides. In study **II**, pure Me 9*c*,11*t*-CLA was autoxidized in the presence of α-tocopherol and the hydroperoxides were isolated and characterized and their relative distribution determined. This enabled us to propose a mechanism for the hydroperoxide pathway of CLA autoxidation in the presence of a high amount of α-tocopherol (**II**, Scheme 1). The proposed mechanism was tested by

autoxidizing Me 10*t*,12*c*-CLA under the same reaction conditions (**III**). The mechanism accounted correctly for the structure of the hydroperoxides produced and their isomeric distribution. However, the details of the mechanism were revised based on theoretical calculations that provided further insight into the early stages of CLA autoxidation. This revised mechanism explains the formation of the hydroperoxides as kinetically-controlled products and provides an improved explanation for the observed isomeric distribution.

4.3.1 Formation of the hydroperoxides (II, III)

The numbering of the compounds in this section corresponds to that in **III** (Scheme 2) in order to allow comparison of the discussions.

In the presence of a high amount of α-tocopherol, the autoxidation of the two major CLA methyl esters yields six conjugated diene allylic monohydroperoxides as kinetically-controlled products (**Fig. 15**). Five of these hydroperoxides (**7**, **9**, **13**, **18**, and **21**) are formed from the first formed pentadienyl radicals (**2A**, **2B** and **3A**/**3B**) by peroxidation and hydrogen atom transfer reactions. The most plausible routes for the formation of the sixth isomer (**20**) involve rearrangement and β-fragmentation reactions of a bisallylic peroxyl radical (*vide infra*). In the following section, the proposed mechanism for the hydroperoxide pathway of CLA autoxidation that accounts for the theoretical calculations performed in study **III** is reviewed briefly. The mechanistic proposal is discussed in more detail in **III**, pp. 604-608.

Hydrogen atom abstraction In study **II**, the most extended conformation (**Fig. 16**, conformation A) of Me 9*c*,11*t*-CLA was expected, in analogy to methyl linoleate autoxidation, to produce the most stable pentadienyl radicals and the initial hydrogen atom abstraction step was drawn accordingly. This assumption was proven correct by theoretical calculations (**III**). However, these calculations revealed that, in addition to conformation A, another conformation of the fatty ester, conformation B, plays a significant role in the formation of the products and their isomeric distribution. Therefore, the initial hydrogen atom abstraction leads not to the formation of two pentadienyl radicals, as expected in study **II**, but to three, as depicted in **Fig. 15**. When conformations A and B are the only conformations of the precursor lipid considered significant, two of these radicals (**2A**, **2B**) arise from the abstraction of a hydrogen atom from the allylic position adjacent to the *trans* double bond and one from the abstraction of a hydrogen atom from the allylic position adjacent to the *cis* double bond (**3A**/**3B**).

Peroxidation and hydrogen atom transfer The subsequent propagation steps for the initial pentadienyl radicals are presented in **Fig. 17**. Three of the conjugated diene allylic monohydroperoxides (**7**, **9**, and **13**) are formed directly from radicals **2A** and **2B** and two from **3A/3B** (**18** and **21**). The sixth conjugated diene hydroperoxide (**20**) is formed most likely from the bisallylic peroxyl radical (**15**). This may occur by rearrangement of **15'** into a conjugated peroxyl radical (**17**) followed by a hydrogen atom transfer reaction or by β-fragmentation of **15'** into a pentadienyl radical (**22**) followed by the propagation steps (*Note:* the later route is not included in **III**, Scheme 2).

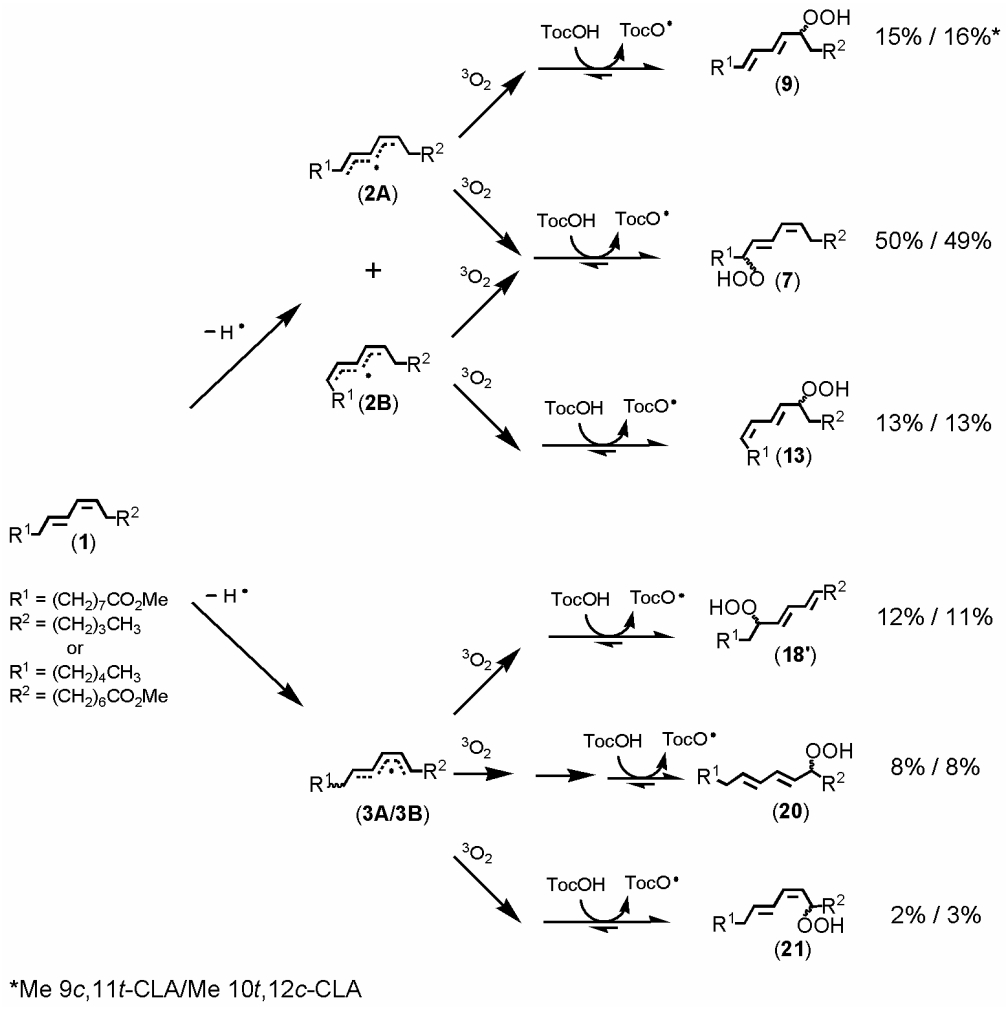


Figure 15 Proposed formation of the hydroperoxides and their isomeric distribution produced during autoxidation reactions of the two major CLA methyl esters in the presence of a high amount of α-tocopherol (**II**, **III**; Numbering of the compounds corresponds to that in **III**, Scheme 2).

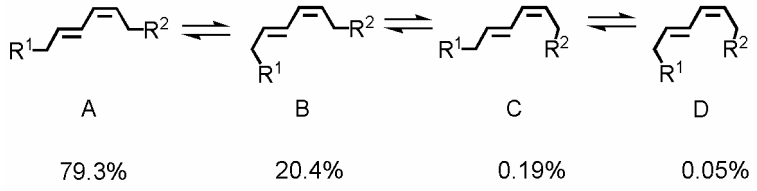


Figure 16 Boltzmann distribution of the four conformations A to D of Me 10*t*,12*c*-CLA (**III**).

The peroxidation reaction is reversible, based on oxygen scrambling studies with methyl linoleate hydroperoxides by Chan et al. (1978, 1979). In **Fig. 17**, the peroxidation reactions leading to conjugated peroxyl radicals are, however, drawn in one way only to emphasize the effect of α-tocopherol, *i.e.* creation of nearly kinetic conditions, and because the reverse reactions, β-fragmentations to the initial pentadienyl radicals, can be expected to be slow ($k_ß$=30 to 620 s^{-1}; Porter & Wujek 1984; Tallman et al. 2001, 2004) compared to the transfer of the phenolic hydrogen atom from α-tocopherol to the peroxyl radicals (k=3.2 x10^6 to 3.8 x10^6 $M^{-1}s^{-1}$; Burton & Ingold 1981; Burton et al. 1983, 1985; Tallman et al. 2001, 2004), particularly in the presence of a high amount of α-tocopherol. Stereochemically unproductive β-fragmentation of the bisallylic peroxyl radicals **5** and **10** ($k_ß$=1.9x 10^6 s^{-1}; Tallman et al. 2001) into the initial pentadienyl radicals **2A** and **2B** may, however, compete with the hydrogen atom transfer step. Pentadienyl radical **3A/3B** is not based on analogy with 3*c*,5*t*-heptadiene (Frankel et al. 1982; Porter 1986) re-formed from the bisallylic peroxyl radical **15**, but the β-fragmentation of the extended conformation **15'** is plausible and it leads to pentadienyl radical **22**, which differs from the initial pentadienyl radical **3A/3B**.

Although the β-fragmentation pathway presented in study **II**, Scheme 1 for the formation of hydroperoxide **13** is plausible, it is now apparent that this is not a significant pathway because under the reaction conditions the β-fragmentation step is slow ($k_ß$~30 s^{-1}; Porter & Wujek 1984; Tallman et al. 2001, 2004) and cannot compete efficiently with the hydrogen atom transfer reaction. The formation of Me 13-OOH-9*t*,11*t* (<1%; the seventh isolated isomer from autoxidation of Me 9*c*,11*t*-CLA) shows that the conditions are not, however, purely kinetic and the β-fragmentation of the conjugated peroxyl radicals occurs to a small extent.

Fig. 17 proposes that the bisallylic peroxyl radicals rearrange via an allyl radical-dioxygen complex to conjugated peroxyl radicals. Further research is needed to establish the true nature of this rearrangement. Interestingly, in agreement with the work of Tallman et al. (2004) on methyl linoleate autoxidation, the proposed allylperoxyl rearrangements of the bisallylic peroxyl radicals appear to occur more rapidly across the *trans* double bond than the *cis* double bond and thus explain the observed regioselectivity of the oxygen addition to unsymmetrical pentadienyl radicals.

As mentioned above, the hydrogen atom transfer step in the presence of α-tocopherol is fast. Although the reverse reaction is very slow (k=5.0 x10^{-1} $M^{-1}s^{-1}$; Mukai et al. 1993), it is included in **Fig. 17** to occur to some extent because the current literature attributes the pro-oxidant properties of α-tocopherol partly to this autoinitiation reaction, particularly at a high concentration of α-tocopherol (Tavadyan 2007; Kamal-Eldin et al. 2008).

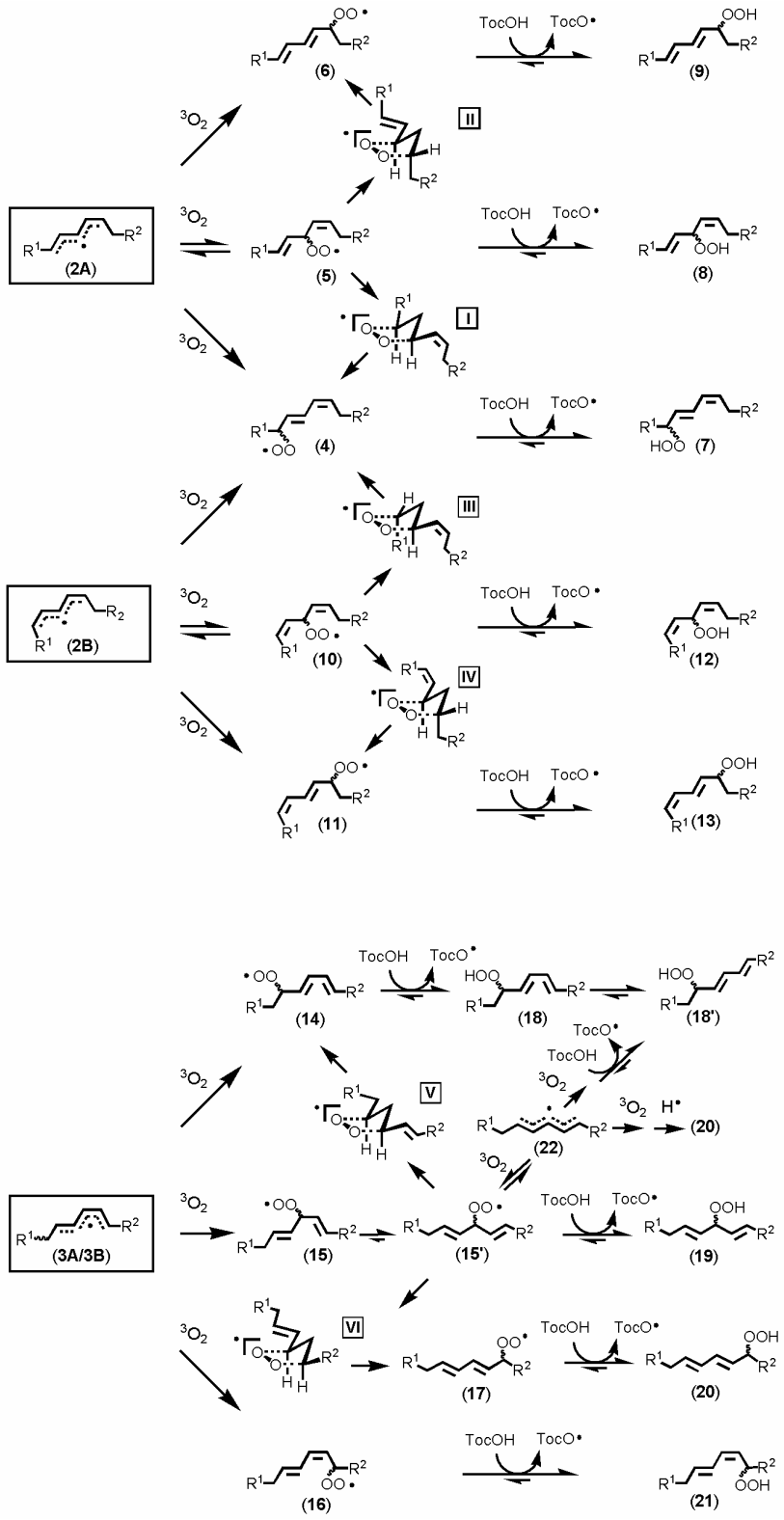


Figure 17 Proposed propagation reactions of radicals **2A**, **2B** and **3A/3B** (*Note:* The numbering of the compounds corresponds to that in **III**, Scheme 2).

4.3.2 Isomeric distribution (II, III)

In the presence of α-tocopherol the autoxidation reactions of the two major CLA methyl esters are diastereoselective in that one geometric hydroperoxide is predominantly formed, at the expense of other geometric isomers. Roughly half of the hydroperoxides arise from pentadienyl radical **2A**, a quarter from **2B** and the rest from **3A/3B**, based upon the ratios in which the hydroperoxides are formed and on what is known about the reactivity of the intermediate pentadienyl radicals **2A** and **2B** with oxygen (**II**; **III**; Pratt et al. 2003; Tallman et al. 2004). The major isomer (**7**) is formed from two of the three initially-formed pentadienyl radicals; roughly three quarters of **2A** and half of **2B** produce the major hydroperoxide product.

The origin of the diastereoselectivity lies in the regioselectivity of the initial hydrogen atom abstraction and of peroxidation, and in the fact that the major hydroperoxide product is formed from the two initial pentadienyl radicals (**2A** and **2B**; **Fig. 17**) that are formed in excess. Based on the hydroperoxide product distribution, the formation of pentadienyl radicals **2A**+**2B** is preferred over radical **3A**/**3B** in the autoxidation of the two major CLA isomers, suggesting that the abstraction of a hydrogen atom in these isomers is easier from the allylic position adjacent to a *trans* than it is from a position adjacent to a *cis* double bond. This observed regioselectivity may be explained in thermodynamic terms: invoking the Hammond postulate (Hammond 1955) we can make an assumption that the geometry of the transition state resembles that of the pentadienyl radical. Since radical **2A** is more stable than radical **3A** (radial **2B** is more stable than **3B**), the transition state leading to radical **2A** (**2B**) is more stable than that leading to radical **3A** (**3B**). The energy barrier $\Delta G^{\ddagger}$ is therefore smaller for the formation of radical **2A** (**2B**) than it is for the formation of radical **3A** (**3B**), and thus radical **2A** (**2B**) is formed faster than **3A** (**3B**).

The regioselectivity and reversibility of oxygen addition to pentadienyl radicals has been established by kinetic and ESR studies by Chan et al. (1978, 1979) and Porter and Wujek (1984). Oxygen addition to unsymmetrical pentadienyl radicals, such as **2A**, occurs faster at the *transoid* end than at the *cisoid* end. Hence, the reaction of pentadienyl radical **2A**, which is formed in excess over the other pentadienyl radicals, with oxygen favours peroxyl radical **4**, and thereafter hydroperoxide **7**. Moreover, this hydroperoxide arises not only from **2A** but also from **2B**. Symmetrical pentadienyl radical **2B** produces hydroperoxides **7** and **13** in an approximately 1 to 1 ratio.

4.3.3 Role of α-tocopherol (I to IV)

Literature reports that the autoxidation of CLA yields mainly low molecular weight polymeric peroxides (Kern et al. 1955, 1956; Privett 1959). This autoxidative polymerization occurs most likely though addition of the peroxyl radicals to the double bonds of the precursor lipid. However, the present study demonstrated that when the autoxidation is performed in the presence of a high amount of α-tocopherol, hydroperoxides are formed as major products. This effect of α-tocopherol may be rationalised easily: α-tocopherol directs the autoxidation of CLA from autoxidative polymerization towards hydroperoxide formation by trapping the first-formed conjugated

diene peroxyl radicals before they undergo reaction by the addition mechanism (or rearrange by the β-fragmentation pathway).

The present study aimed at characterization of the hydroperoxide products and therefore one of the goals was to produce as much hydroperoxides as possible. A preliminary study (unpublished results) in which CLA methyl esters were autoxidized in the presence of 5, 10, 15, 20 and 25% α-tocopherol showed that this goal was best achieved by autoxidizing CLA methyl esters with as much as 20% α-tocopherol. Hence, the ability of a high concentration of α-tocopherol to promote hydroperoxide formation was exploited in studies **I** to **IV**. It is difficult, however, to estimate the importance of the reactions that account for the pro-oxidant properties of α-tocopherol, because it is plausible, based on the recent studies on the initiation process of methyl linoleate (Morita & Tokita 2006, 2008), that unstable oligomeric peroxides are the main source of autocatalytic radicals also in the autoxidation of CLA methyl esters.

The hydroperoxide region in the ^{1}H NMR spectrum of a mixture of CLA methyl ester hydroperoxides was slightly more complex when the sample was produced by autoxidation of CLA methyl esters in the absence rather than in the presence of α-tocopherol (**I**). This suggests that α-tocopherol affects the isomeric distribution and/or number of hydroperoxide products. Such a simplifying effect can be easily explained. It is safe to assume, based on similarity to methyl linoleate hydroperoxides, that the CLA hydroperoxide may undergo rearrangement through the β-fragmentation pathway. Consequently, the autoxidation of Me 10*t*,12*c*-CLA would produce, in the absence of α-tocopherol, at least three additional conjugated diene allylic monohydroperoxides, namely Me 9-OOH-10*t*,12*t*, Me 10-OOH-11*t*,13*c*, and Me 14-OOH-10*c*,12*t* (in the autoxidation of Me 9*c*,10*t*-CLA these additional isomers are Me 13-OOH-9*t*,11*t*, Me 12-OOH-8*c*,10*t*, and Me 8-OOH-9*t*,11*c*). Furthermore, it may be anticipated that the autoxidation is also diastereoselective in the absence of α-tocopherol because the main isomer is the only hydroperoxide that would arise from two of the three initial pentadienyl radicals. However, the diastereoselectivity would not be as pronounced as it is in the presence of α-tocopherol. It is clear that further research is needed to establish the effect of α-tocopherol on CLA hydroperoxide formation. Characterization of products formed in the absence of α-tocopherol can be anticipated, however, to be challenging because of the small amount hydroperoxides and the complexity of the hydroperoxide mixture.

4.3.4 Hydroperoxide formation of conjugated methyl linoleate in comparison with that of methyl oleate and methyl linoleate

In the absence of a good hydrogen atom donor, hydroperoxide formation is only a minor pathway in the autoxidation of the two major CLA methyl esters, whereas in the autoxidation of methyl oleate (Chan & Levett 1977b; Frankel et al. 1977, 1984) and methyl linoleate (Chan & Levett 1977a; Porter et al. 1981) it is the major pathway. The autoxidation of CLA methyl ester may be, however, directed from autoxidative polymerization towards hydroperoxide formation using α-tocopherol.

The mechanism proposed for the hydroperoxide pathway of the two major CLA methyl esters shows similarities to both methyl oleate and methyl linoleate autoxidation.

The first step is an abstraction of one of the allylic hydrogen atoms from the fatty ester. As in methyl oleate, there are four allylic hydrogen atoms available for abstraction. However, differing from methyl oleate the two allylic positions are not equivalent, since one is allylic to a *trans* and the other to a *cis* double bond. Moreover, the hydrogen atom abstraction leads to the formation of pentadienyl radicals, where the lone electron is delocalized not over three but five carbon atoms, as in the autoxidation of methyl linoleate. The numbers of carbon radicals produced in the initiation step are two, one, and three in the autoxidation of methyl oleate (Porter et al. 1994a), methyl linoleate (Porter & Wujek 1984) and the two major CLA methyl esters (**III**), respectively.

Under kinetic conditions, the autoxidation of methyl linoleate yields two and that of the two major CLA methyl esters six conjugated diene hydroperoxides. Moreover, while methyl linoleate autoxidation produces two positional isomers of conjugated diene hydroperoxides both with *cis,trans* geometry in equal amounts, the autoxidation of the CLA methyl esters produce four positional isomers with three different geometries, including an unusual type of *cis,trans* hydroperoxide with the *cis* double bond adjacent to the methine carbon atom, in unequal proportions. Furthermore, the kinetic methyl linoleate hydroperoxides are included in the mixtures of hydroperoxides produced from the two major CLA methyl esters. The complexity of the hydroperoxide mixture produced during autoxidation of the CLA methyl esters compared to that produced from methyl linoleate results from the formation of three initial pentadienyl radicals instead of only one.

4.4 Autoxidation pathways of conjugated linoleic acid methyl ester

The reviewed literature and the results of the present study show that the overall process of CLA autoxidation is extremely complex and has many elementary reactions occurring simultaneously. As discussed in the literature review (section 2.1.2, **Fig. 11**) three pathways (A to C) with unknown primary products have been suggested for the autoxidation of CLA. In the following section these pathways are discussed in light of the results of the present study.

The hydroperoxide pathway (not included in **Fig. 11**) The present study has established that one of the reaction pathways of CLA autoxidation in the presence and absence of α-tocopherol is the hydroperoxide pathway. This brings new insight to the oxidation pathways of CLA as illustrated in **Fig. 18**. In the absence of a good hydrogen atom donor, autoxidative polymerization has been reported to be the major pathway in the autoxidation of CLA (Kern et al. 1955, 1956; Privett 1959; Luna et al. 2007), and under such conditions hydroperoxide formation is thus only a minor pathway. The present study has, however, demonstrated that the autoxidation of CLA can be directed from autoxidative polymerization towards hydroperoxide formation by performing the reaction in the presence of a high amount of α-tocopherol, and that this fact can be used to advantage in the production of hydroperoxides from CLA isomers. Because the autoxidation reactions of the two major CLA methyl esters in the presence of α-tocopherol produce hydroperoxides that are both identical and similar to methyl linoleate hydroperoxides, we can expect that the secondary autoxidation products of these

hydroperoxides are the same as or are homologues of the ones formed in the decomposition of methyl linoleate hydroperoxides (**Fig. 18**, Pathway 1).

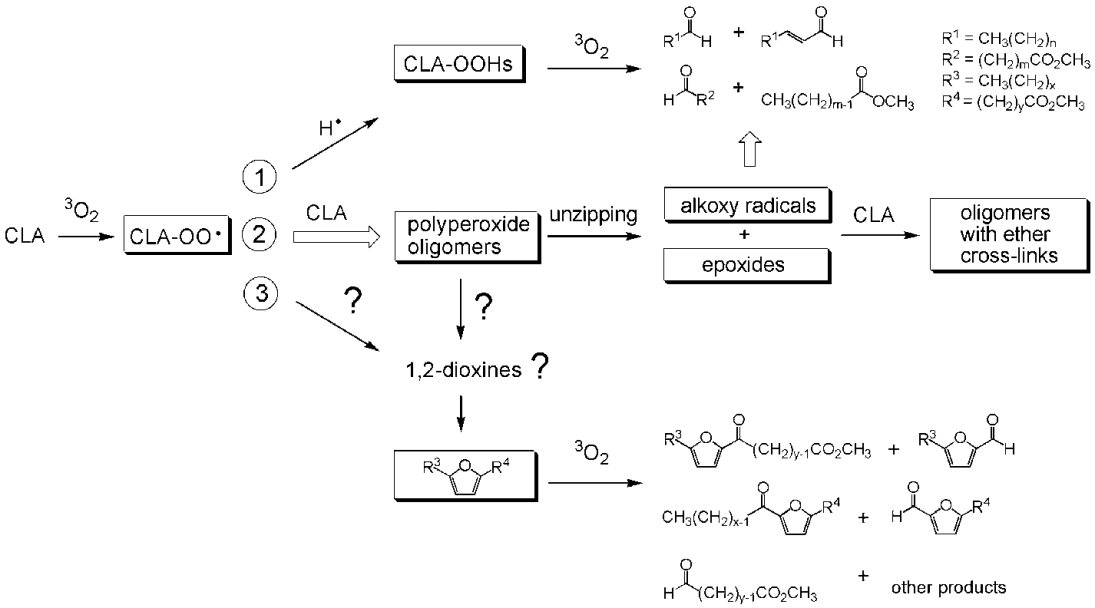


Figure 18 Overview of the autoxidation pathways of CLA methyl ester.

Autoxidative polymerization (**Fig. 11**, Pathway A) Knowledge of the hydroperoxide formation steps and characterization of the hydroperoxides produced shed new light on the autoxidative polymerization reactions (**Fig. 18**, Pathway 2). Hydroperoxide formation confirms the presence of peroxyl radicals in the autoxidation mixture. These peroxyl radicals may abstract one of the allylic hydrogen atoms from CLA methyl ester and form hydroperoxides (abstraction mechanism) or alternatively add to the double bonds of the CLA methyl ester and form dimeric allyl radicals that react with oxygen and form dimeric peroxyl radicals for the next cycle of addition to a double bond (addition mechanism) (**Fig. 19**). Mayo (1968) has suggested that in many alkene oxidations, the abstraction and addition mechanisms occur simultaneously and that those alkenes having conjugated unsaturation preferentially undergo oxidation by the latter mechanism. The tendency of conjugated dienes, including CLA methyl ester, to favour the addition reaction is easy to understand because the addition mechanism produces resonance-stabilized allyl radicals. This tendency is also in line with reports on oligomer formation in the initial phase of CLA oxidation (Muizebelt et al. 2000; Luna et al. 2007). The propagative addition is apparently less favoured in the oxidation of nonconjugated alkenes, such as methyl linoleate, in which the resulting radical intermediates will not be resonance stabilized. Using knowledge of the structures of the peroxyl radicals preceding the CLA hydroperoxides in the reaction chains, we can predict the structures of the dimeric, trimeric and higher oligomers that arise from the addition mechanism as illustrated in **Fig. 19**. It must be emphasized that the formation of these products remains to be confirmed. However, in agreement with the early literature (reviewed in Swern 1961) and the alkyd paint study of Muizebelt et al. (2000), the addition mechanism would produce oligomers

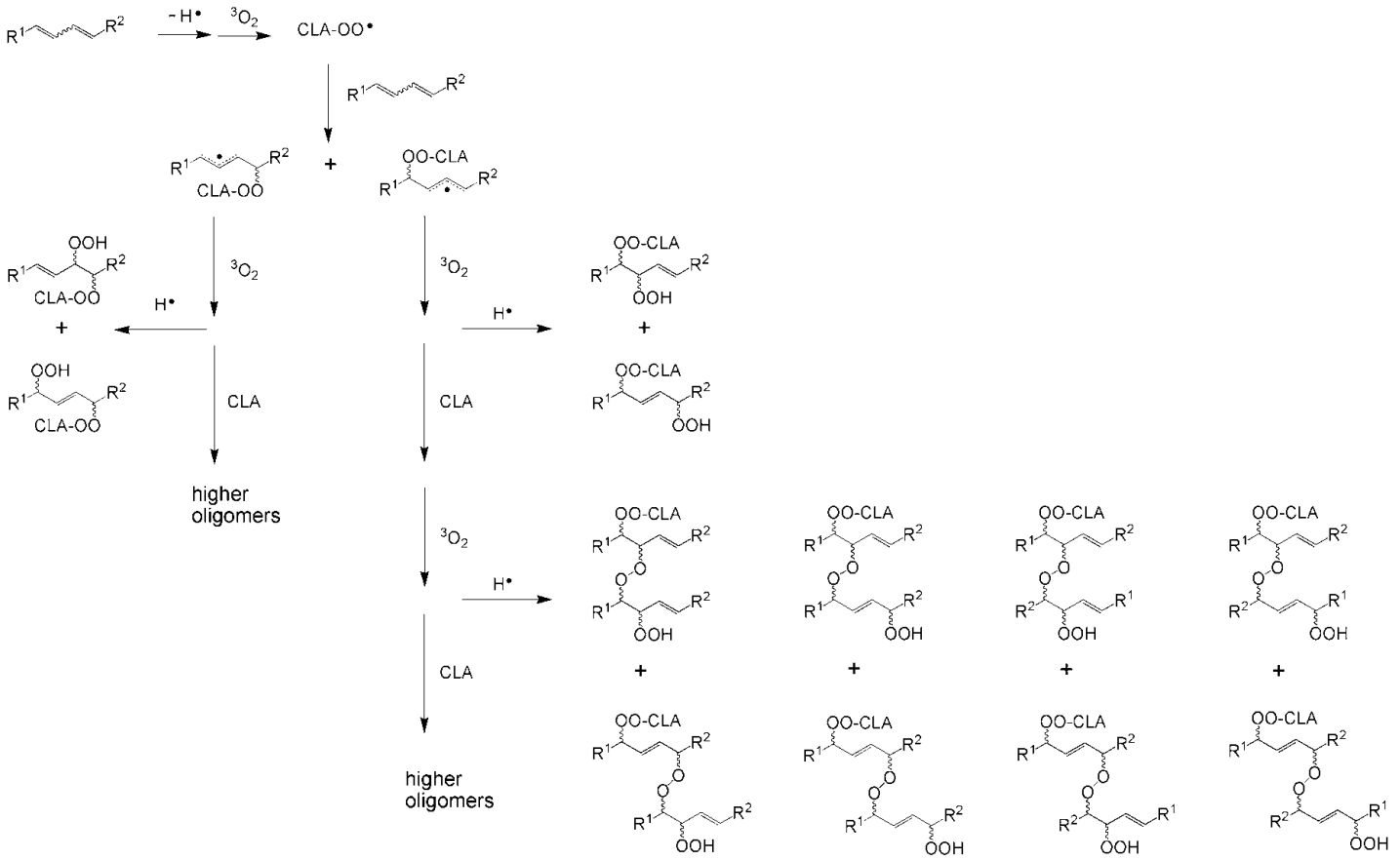


Figure 19 Postulated autoxidative polymerization of CLA methyl ester resulting from addition of peroxyl radicals (CLA-OO•) to the double bonds of unoxidized CLA methyl ester.

with oxygen-carbon rather than carbon-carbon links, and it would result in disappearance of conjugated double bonds and appearance of isolated unsaturation. Importantly, these dimers would have 1,2- or 1,4-addition of oxygen with respect to one of the monomers, agreeing with the data of Kern et al. (1955).

Empirical kinetic reassessment of the early data by Brimberg & Kamal-Eldin (2003) suggests that oligomers having an average of three monomers would be kinetically favoured. The addition mechanism would produce trimers with peroxide crosslinks (see examples in **Fig. 19**). However, this does not explain the observed heterogeneity of the oligomeric fraction (Muizebelt & Nielen 1996). In effect, the autoxidative polymerization can be envisioned to involve several basic chemical reactions in the propagation and termination phases of the autoxidation, as discussed in Pajunen & Kamal-Eldin 2008, pp. 95-99. Importantly, as observed by Mayo (1968) in alkene oxidation studies, whenever competition between addition and abstraction favours addition, the competition between peroxidation, which leads to dimeric or higher peroxides, and unzipping of the polyperoxides, which leads to epoxides and alkoxyl radicals, becomes important. The unzipping refers to a de-polymerization reaction where the oligomeric allyl radical reacts back with the peroxide bridge instead of adding a molecule of oxygen. This homolytic displacement reaction, as demonstrated by a few examples in **Fig. 20**, may lead to very complex mixtures of oligomeric products and thus explain the observed heterogeneity. It is of interest to note that the unzipping would not only supply radicals to the autocatalytic pool of the autoxidation mixture but would also produce aldehydes and ketones. It is plausible that this is, in effect, the main route for the formation of the volatile CLA autoxidation products that have been identified in CLA autoxidation mixtures (Yurawecz et al. 1995, 1997; Sehat et al. 1998; Eulitz et al. 1999).

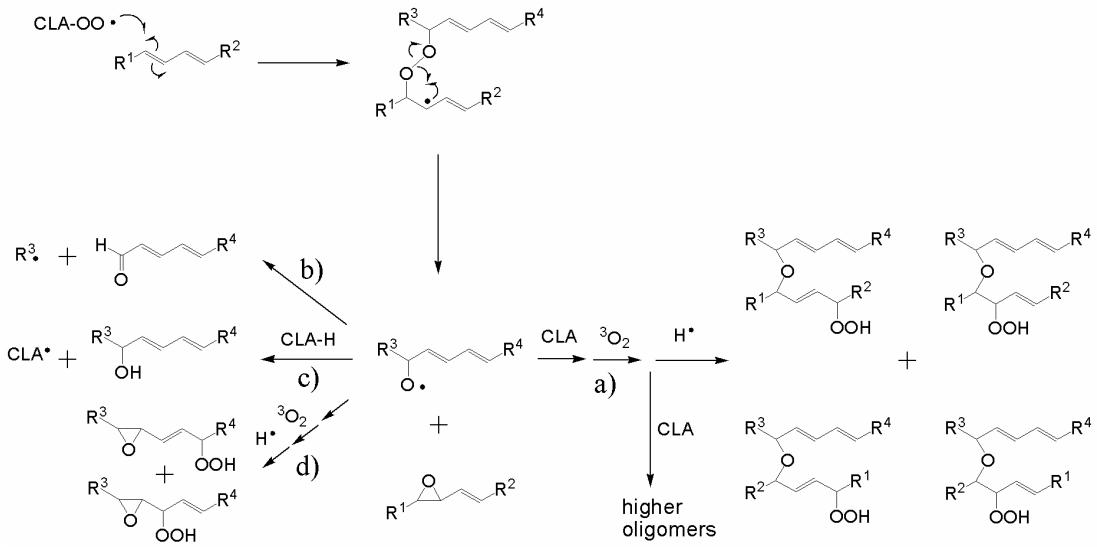


Figure 20 Unzipping of the postulated dimeric radical leading to an epoxide and alkoxyl radical, and subsequent propagation reactions of the alkoxyl radical a) addition reaction, b) β-scission, c) H-atom abstraction, and d) intramolecular cyclization (Pajunen & Kamal-Eldin 2008).

Formation of furans, and monomeric products similar to those produced in the singlet oxygen oxidation of conjugated linoleic acid (**Fig. 11**, Pathways B and C) Bascetta et al. (1984) report the formation of a 1,2-dioxine as the major product in the photo-oxidation of Me 9*t*,11*t*-CLA CLA. As depicted in **Fig. 21**, the treatment of 1,2-dioxine with ferrous ion results in FFA formation. Therefore, it seems possible that 1,2-dioxanes are precursors for FFAs formed during autoxidation of CLA (**Fig. 18**, Pathways 2 and 3).

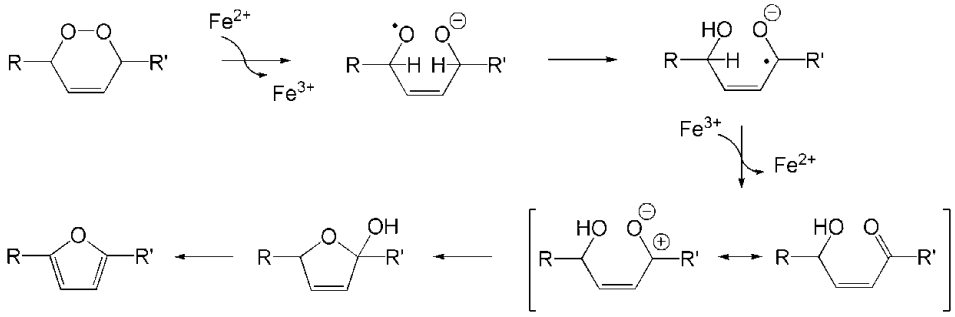


Figure 21 Formation of a FFA by treatment of a 1,2-dioxine with ferrous ion in aqueous tetrahydrofuran (Modified from Bascetta et al. 1984).

The autoxidation of CLA has been suggested to yield two nonconjugated diene monohydroperoxides, one 1,2-dioxine, and two 1,2-dioxetanes as primary products (Eulitz et al. 1999; **Fig. 11**, Pathway C). Formation of the nonconjugated diene hydroperoxides could be envisioned to occur, albeit not by a concerted ene-reaction as in the photo-oxidation but rather through a free radical mechanism (**I**, **III**, **Fig. 17**). Formation of the cyclic peroxides as primary products in the autoxidation of CLA is, however, difficult to understand. According to Wigner's spin-conservation rule the direct addition of triplet oxygen to the diene π system gives a product in its triplet state. Because the formation of such products has a high energy barrier, the 1,2- and 1,4-addition reactions of oxygen are not plausible pathways for the formation of cyclic peroxides. Formation of these peroxides through splitting of oligomers as suggested by Brimberg & Kamal-Eldin (2003) seems more probable but remains to be confirmed. It could, for example, be envisioned that decomposition of a dimeric alkoxyl radical, formed by the unzipping of a trimeric addition product, might yield a bisepoxide and the subsequent rearrangement of this epoxide a 1,2-dioxine (**Fig. 22**A). Alternatively, the 1,2-dioxines might be formed by intramolecular cyclization of the unusual *cis,trans* peroxyl radical followed by a disproportionation reaction (**Fig. 22**B; **Fig. 18**, Pathway 3). In accordance with this suggestion, the autoxidation of the principal CLA isomers (9*c*,11*t*/9*t*,11*c*-CLA, 41%; 10*t*,12*c*-CLA 44%) of the CLA mixture used in the autoxidation study by Yurawecz et al. (1995) would produce, according to the mechanism in **Fig. 15**, those *cis,trans* peroxyl radical isomers that would ultimately yield $F_{8,11}$, $F_{10,13}$, $F_{11,14}$, which were, indeed, identified from the autoxidation mixture. However, formation of a localized carbon radical and loss of diene conjugation and of resonance stabilization of the allylperoxyl group makes the cyclization step less favourable. Moreover, if oxygen is present in sufficient concentration, this route would also give rise to α-hydroperoxy-1,2-dioxines.

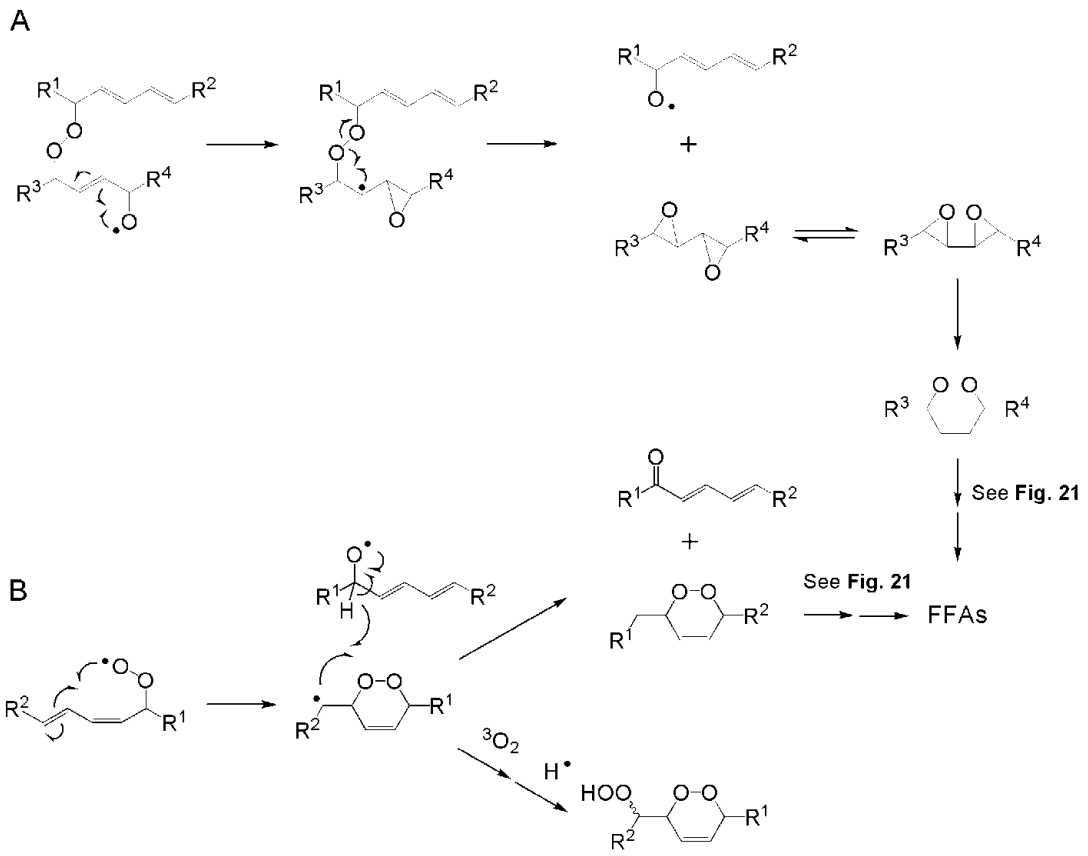


Figure 22 Postulated formation of 1,2-dioxines from A) dimeric alkoxyl radicals and B) *cis,trans* peroxyl radicals where the double bond adjacent to the methine carbon has *cis* geometry (Pajunen & Kamal-Eldin 2008).

5 Conclusions and suggestions for further work

The present study established that conjugated diene hydroperoxides are, contrary to earlier assumptions, one type of primary product in the autoxidation of the two major CLA methyl esters in the presence and absence of α-tocopherol and that the autoxidation of the CLA methyl esters follows, at least in part, Farmer's hydroperoxide theory. Autoxidative polymerization has been reported to be the major pathway in the autoxidation of CLA (Kern et al. 1955, 1956; Privett 1959; Luna et al. 2007). This study, however, demonstrated that the autoxidation may be directed towards hydroperoxide formation by performing the autoxidation in the presence of a good hydrogen atom donor, *i.e.* α-tocopherol.

The present study described the production of conjugated diene allylic monohydroperoxides from the two major CLA methyl esters by autoxidation in the presence of a high amount of α-tocopherol. These hydroperoxides were analysed and separated first as methyl hydroxyoctadecadienoates and then as intact hydroperoxides by HPLC, and the separated products were characterized by UV, GC-MS, 1D and 2D NMR techniques. Autoxidation of the two major CLA isomers results in a mixture of six kinetically-controlled primary products with diene conjugation that are positional and geometric isomers of methyl hydroperoxyoctadecadienoates. Not only is the kinetic product mixture more complex than those from the autoxidation of methyl oleate and methyl linoleate, but the hydroperoxides are produced in unequal proportions. Differing from methyl linoleate autoxidation, the autoxidation of the two major CLA methyl esters is diastereoselective in favour of one geometric isomer as a pair of enantiomers. Moreover, the autoxidation reactions of the two major CLA methyl esters produce new positional isomers of conjugated diene monohydroperoxides, *i.e.* the 8-, 10-, 12- and 14-hydroperoxyoctadecadienoates. Interestingly two of these new positional isomers have an unusual structure for a *cis,trans* lipid hydroperoxide where the allylic methine carbon is adjacent to the *cis* instead of the usual *trans* double bond.

The present study proposes a mechanism for the hydroperoxide pathway of CLA autoxidation in the presence of a high amount of α-tocopherol based on characterized primary products, their relative distribution and theoretical calculations. This is an important step forward in CLA research, where exact mechanisms for the autoxidation of CLA have not been presented before. This mechanism is in line with kinetic evidence (Kern et al. 1955; Minemoto et al. 2003) and it serves not only as a means of predicting the hydroperoxides and their isomeric distribution from any other CLA isomer but it also facilitates further investigations directed to complete elucidation of the autoxidation mechanism. More research is required, for example, to establish the formation of the nonconjugated hydroperoxides as well as the details of the suggested isomerizations of the bisallylic peroxyl radicals. In addition, the importance of the reactions that contribute to the pro-oxidant properties of α-tocopherol and the role of the oligomeric products in the autocatalytic radical supply are of interest.

Knowledge of the hydroperoxide formation steps is of crucial importance for understanding the subsequent steps and the different pathways of the autoxidation of CLA methyl ester. Moreover, knowledge of the structures of the CLA methyl ester

hydroperoxides allows the prediction of possible structures for the oligomeric products that can be envisioned to arise by the addition mechanism and thereafter the possible reaction pathways that would explain the observed heterogeneity of the oligomeric products formed on CLA autoxidation. The confirmation of these predictions needs extensive and thorough investigation.

A deeper understanding of the mechanism(s) of the autoxidation of CLA may aid the development of an efficient method(s) to monitor CLA autoxidation and the interpretation of the results obtained by conventional methods. In addition, the autoxidation procedure and the SPE and the semi-preparative NP-HPLC separation methods developed in this study, offer for the first time a means of producing and separating intact CLA methyl ester hydroperoxides from the autoxidation of the two biologically active CLA isomers in amounts sufficient for investigation of their secondary autoxidation, rearrangement, and oligomerization reactions, and for evaluating their biological activity.

The present study provides basic knowledge of lipid hydroperoxides and their hydroxy derivatives. The full assignments of the ^{1}H and ^{13}C NMR spectra of nine isomeric methyl hydroxyoctadecadienoates and of ten methyl hydroperoxyoctadecadienoates, including the unique *cis,trans* hydroperoxides *i.e.* Me 8-OOH-9c,11*t* and Me 14-OOH-10*t*,12*c*, were accomplished with the aid of 2D NMR spectroscopy. This demonstrated that NMR spectroscopy is an excellent tool for determining the structures of CLA methyl ester hydroperoxides and of their hydroxy derivatives. The assigned ^{13}C NMR resonance data allowed determination of the effects of the hydroxyl and hydroperoxyl groups on the carbon chemical shifts of CLA isomers, identification of diagnostic signals, and determination of chemical shift differences of the olefinic resonances that may help assignment of structure to as yet unknown lipid hydroperoxides either as hydroxy derivatives or as intact hydroperoxides produced, for example, from other geometric and positional isomers of CLA methyl ester. Information regarding solvent effects on the chemical shifts of the hydroperoxides may be utilized for choosing the appropriate solvent for an investigation of mixtures of lipid hydroperoxides by NMR spectroscopy.

In biological systems CLA is present only in small amounts and the likelihood of autoxidative polymerization can be expected to be low. Therefore, clarification of the mechanism of hydroperoxide formation and of the differences in hydroperoxide formation between CLA and monoene and nonconjugated diene fatty acids may be considered to be of high importance when studying the biological significance of CLA isomers. The results of this study will thus be of interest in currently ongoing research on biological mechanisms of CLA action. Moreover, a deeper understanding of the autoxidation mechanisms is required for ensuring the safety of CLA-rich foods. The results of this study may assist in the design of CLA enriched food and food supplements.

6 Experimental

This section summarizes the experimental methods for the preparation of the methyl hydroxystearate standards that have not been included in the original articles **I**-**IV**.

6.1 General

Chemicals were obtained unless otherwise stated from commercial sources and used without further purification. Crystalline trimethylamine *N*-oxide was prepared (Söderquist & Andersson 1986) from trimethylamine *N*-oxide dihydrate (Eastman Organic Chemicals, Kingsport, USA). Methyl 8-iodooctanoate was prepared (Quinton & Le Gall 1991) from 8-bromooctanoic acid (Fluka, Buchs, Switzerland). Zinc powder (Merck, Darmstadt, Germany) was activated using aqueous HCl (5%, w/w) (Newman & Evans 1955). NaI was dried at 100 °C under high vacuum (oil pump) for 4 h. Oleic acid methyl ester, 1,2-dibromoethane, 1-bromononane and 1-bromodecane (Fluka, Buchs, Switzerland) were distilled under high vacuum before use. 1-Bromopentane (Fluka, Buchs, Switzerland) was pre-dried over anhydrous $CaCl_2$, filtered and distilled before use. All solvents were of analytical grade. Acetone was dried over K_2CO_3 and dichloromethane (DCM) over anhydrous $CaCl_2$. Diethyl ether was washed, pre-dried, and distilled before use over sodium in the presence of benzophenone (0.1 g/100 mL). Dimethylformamide and methanol were dried by distillation over CaH_2. Dimethylsulfoxide was distilled over CaH_2 under high vacuum (oil pump).

The reactions were monitored by thin-layer chromatography (TLC) and the R_f values were determined by TLC on Merck precoated silica gel 60 F_{254} with the indicated eluents. Compounds were visualized by dipping the plate in H_2SO_4/methanol (1/9, v/v) and heating for a few minutes. After the washing procedures, organic layers were dried over anhydrous $MgSO_4$ or Na_2SO_4, filtered, and concentrated *in vacuo*. Column chromatography was performed on Silica gel 60 (230-400 Mesh) with the indicated eluents.

Melting points were measured on an electrothermal, digital melting point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded in deuterochloroform on a NMR Gemini 2000 spectrometer (Palo Alto, CA, USA) at room temperature. Chemical shifts are given in parts per million relative to tetramethylsilane (δ 0.00). The coupling constants were measured in hertz (Hz). The hydroxystearate standards were derivatized to trimethylsilyl ethers and analysed by GC-MS with the same procedure and equipment as reported in **II**.

6.2 Preparation of the hydroxystearates

Methyl 8-, 9-, and 13-hydroxyoctadecanoates were prepared by the Grignard reaction (**Fig. 23**) using alkylbromides and oxoesters (Noller & Adams 1926). It is noteworthy that oxoesters are secondary autoxidation products of CLA autoxidation (Yurawecz et al. 1997).

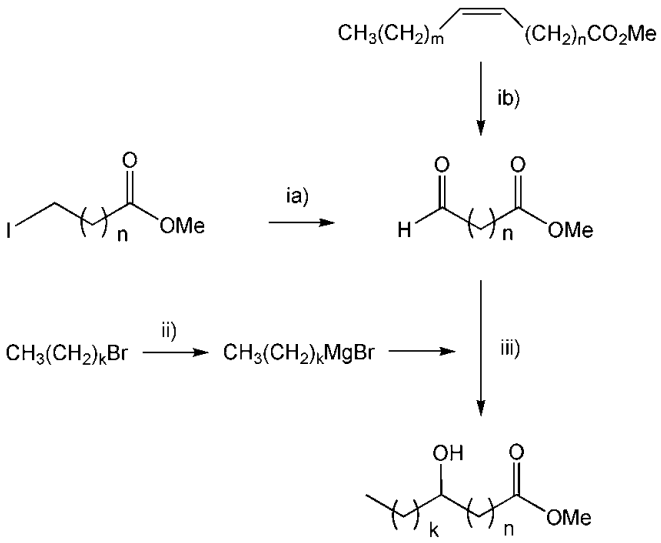


Figure 23 Synthesis of methyl 8-, 9-, and 13-hydroxyoctadecanoates ia) anhydrous trimethylamine *N*-oxide (n=6); ib) 1. ozone, 2. glacial acetic acid, activated Zn (m=7,7 and n=7,11); ii) Mg turnings, 1,2-dibromoethane; iii) Grignard reaction (n=6, 7, 11 and k=9, 8, 4).

Methyl 8-oxooctanoate was prepared from methyl 8-iodooctanoate (4.90 g, 17.25 mmol) applying the procedure developed for the direct oxidation of primary iodides to aldehydes using trimethylamine *N*-oxide in dimethylsulfoxide (Godfrey & Ganem 1990). Purification by silica gel column chromatography (DCM) afforded methyl 8-oxooctanoate (1.24 g, 7.20 mmol, 42%) as a colourless oil, R_f(EtOAc/DCM=1/10, v/v)=0.59. The ^{1}H and ^{13}C NMR data agreed with published data (Lee et al. 1994).

Methyl 9-oxononanoate and **methyl 13-oxotridecanoate** were prepared from oleic acid methyl ester (7.90 g, 26.65 mmol) and erucic acid methyl ester (4.00 g, 11.34 mmol) by cleavage of the double bond by ozone according to the procedure of Pryde et al. (1960) with the exception that erucic acid methyl ester was dissolved in MeOH/DCM (1/4, v/v, 50 ml). Purification by silica gel column chromatography (EtOAc/hexane=1/1, v/v) and drying under high vacuum for 3 h afforded methyl 9-oxononanoate (3.44 g, 18.47 mmol, 69%) as a colourless oil, R_f(EtOAc/DCM=1/10, v/v)=0.71. Methyl 13-oxotridecanoate (2.17 g, 9.59 mmol, 85%) was obtained as a colourless oil, R_f(EtOAc/DCM=1/10, v/v)=0.70. The ^{1}H and ^{13}C NMR data of these oxoesters agreed with published data (Waugh & Berlin 1984; Kann et al. 1990).

Methyl 8-hydroxyoctadecanoate was prepared from methyl oxooctanoate (0.50 g, 2.90 mmol) and 1-bromodecane (0.71 g, 3.19 mmol). Methyl oxooctanoate was dissolved

in dry diethyl ether (5 mL) under argon and the solution was cooled to -5 °C. Freshly prepared Grignard reagent, decanylmagnesium bromide, was added dropwise with a syringe pump (0.2 mL/min). The reaction mixture was stirred at 0 °C for 2 h and quenched by pouring the reaction mixture into a beaker containing ice water (40 mL). The reaction vessel was rinsed with diethyl ether (2x10 mL) and water (2x10 mL). The solution was made slightly acidic using aqueous HCl (5%, w/w). The layers were separated and the water phase was extracted with diethyl ether (3x30 mL). The combined organic layers were washed with water (3x30 mL). Purification by column chromatography (EtOAc/hexane=1/19, v/v) afforded methyl 8-hydroxyoctadecanoate (0.14 g, 0.45 mmol, 15%) as a white solid (m.p. 46.9-50.4 °C); R_f(EtOAc/DCM=1/10)=0.42; ^{1}H NMR (200 MHz) δ 3.67 (*s*, 3 H, OCH_3), 3.64-3.52 (*m*, 1 H, H-8), 2.31 (*t*, 2 H, H-2, $J_{2,3}$=7.5 Hz), 1.72-1.52 (*m*, 2 H, H-3), 1.51-1.16 (*m*, 26 H), 0.94-0.83 (*m*, 3 H, H-18); *m/z* 243, 245.

Methyl 9-hydroxyoctadecanoate and **methyl 13-hydroxyoctadecanoate** were prepared in the similar manner to methyl 8-hydroxyoctadecanoate. Methyl 9-hydroxyoctadecanoate was obtained as a white solid (m.p. 43.0-45.2 °C) in a yield of 8%; R_f(EtOAc/DCM=1/10)=0.50; ^{1}H NMR (200 MHz) δ 3.67 (*s*, 3 H, OCH_3), 3.66-3.50 (*m*, 1 H, H-9), 2.30 (*t*, 2 H, H-2, $J_{2,3}$=7.5 Hz), 1.72-1.52 (*m*, 2 H, H-3), 1.50-1.16 (*m*, 26 H, CH_2), 0.88 (*t*, 3 H, H-18, $J_{18,17}$=6.4 Hz); *m/z* 229, 259. Methyl 13-hydroxyoctadecanoate was obtained as a white solid (m.p. 55.4-56.1 °C) in a yield of 19%; R_f(EtOAc/DCM=1/10)=0.58; ^{1}H NMR (200 MHz) δ 3.67 (*s*, 3 H, OCH_3), 3.64-3.52 (*m*, 1 H, H-13), 2.30 (*t*, 2 H, H-2, $J_{2,3}$=7.5 Hz), 1.68-1.52 (*m*, 2 H, H-3), 1.50-1.16 (*m*, 26 H, CH_2), 0.89 (*t*, 3 H, H-18, $J_{18,17}$=6.4 Hz); *m/z* 173, 315.

The ^{13}C NMR data of the synthesized methyl hydroxyoctadecanoates agreed with published data (Tulloch 1978).

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In the printed version of this thesis, articles **I-IV** follow. Legalities prevent their appearance in the electronic version.

I Hämäläinen T.I., Sundberg S., Mäkinen M., Kaltia S., Hase T., and Hopia A., Hydroperoxide formation during autoxidation of conjugated linoleic acid methyl ester, *Eur. J. Lipid Sci. Technol.*, 2001, **103**, 588-593. Copyright Wiley-VCH Verlag GmbH & Co.KGaA.

II Hämäläinen T.I., Sundberg S., Hase T., and Hopia A., Stereochemistry of the hydroperoxides formed during autoxidation of CLA methyl ester in the presence of α-tocopherol, *Lipids*, 2002, **37**, 533-540. Copyright Springer-Verlag Heidelberg.

III Pajunen T.I., Johansson M.P., Hase T., and Hopia A., Autoxidation of conjugated linoleic acid methyl ester in the presence of α-tocopherol: the hydroperoxide pathway, *Lipids*, 2008, **43**, 599-610. Copyright Springer-Verlag Heidelberg.

IV Pajunen T.I., Koskela H., Hase T., and Hopia A., NMR properties of conjugated linoleic acid (CLA) methyl ester hydroperoxides, *Chem. Phys. Lipids*, 2008, **154**, 105-114. Copyright Elsevier.

Erratum to article **I**, Page 591. Unfortunately Tab. 1 contains a few errors and the corrections are the following: 9-OOH isomer: 131.00 (C-10), 130.11 (C-11), 127.30 (C-12) and 134.16 (C-13); 13-OOH isomer: 133.82 (C-9), 127.47 (C-10), 129.96 (C-11) and 131.21 (C-12). We apologize for this oversight.