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OXIDATION OF STERYL ESTERS

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ACADEMIC DISSERTATION

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ABSTRACT

Novel food products are fortified with plant sterols and stanols because of their ability to lower the LDL-cholesterol levels in plasma up to 10−15%. These compounds are added to food either in their free form or as fatty acyl esters. Like other unsaturated lipids, sterols are also prone to oxidation in the presence of oxygen and initiators such as heat, light, metal ions and enzymes. Oxidation may occur already during the manufacture of sterol preparations or during food processing and storage. The known adverse health effects of the oxidation products of cholesterol have prompted the evaluation of the biological effects of plant sterol oxides. The oxidation behaviour of free cholesterol has been extensively studied, whereas those of plant sterols have been less thoroughly examined. The oxidation behaviour of mainly free sterols has been investigated, but knowledge on those of fatty acyl esters or other conjugates is deficient.

This study investigated the effects of chemical (i.e., esterification, unsaturation degree of the acyl moiety and sterol structure) and external (i.e., temperature and medium) factors on the oxidation of sterols. Development of solid-phase extraction and HPLC-DAD-ELSD methods for the isolation and determination of intact steryl ester monohydroperoxides allowed the primary oxidation of steryl esters to be followed both in neat preparations and in saturated lipid media. The oxidation of steryl and acyl moieties could be distinguished; therefore, the oxidation of both moieties could be followed in intact molecules. Further reactions of the monohydroperoxides were followed in terms of secondary oxidation products of sterol and oligomers.

Introduction of an acyl moiety to a sterol altered the physical state and polarity of the sterol and affected its oxidation. In neat preparations at 100 °C, esterified sterols were liquefied and thus oxidised greatly, whereas the free sterol remained in a solid state and was therefore unaltered. Increased unsaturation of the acyl moiety increased the oxidation rate of both the steryl and acyl moieties. No differences in the initial reactivities of these two moieties were observed, but they oxidised concomitantly. For esters with monounsaturated acyl moieties, greater contents of steryl than of acyl moiety hydroperoxides were measured, whereas for an ester with polyunsaturated acyl moiety, greater contents of the acyl moiety hydroperoxides were measured. Increased temperature (140 °C) induced the oxidation of both steryl esters and free sterol. In a saturated lipid medium at 100 °C, the oxidation of steryl esters was decelerated, whereas the oxidation of free sterol was accelerated. Due to reduced oxidation rates, the accumulation of further reaction products was also delayed. In neat preparations and in the lipid medium, the steryl ester hydroperoxides decomposed into traditionally determined sterol secondary oxidation products and also underwent polymerisation as a rival reaction.

By altering the chemical and physical properties of sterols, their oxidisabilities may be affected. If these factors are regulated in the manufacture of the preparations and in the food processing, formation of oxidation products may be controlled and the desired functionality of plant sterols preserved.

PREFACE

This study was carried out during the years 2007–2012 in the Division of Food Chemistry at the Department of Food and Environmental Sciences (formerly known as the Department of Applied Chemistry and Microbiology). In order to complete accomplish this intricate sterol puzzle, all the small pieces had to fall into their places in a right order. Devoted and professional supervisors served as the corner stones of this work and outlined something that was to become a coherent output. The framework was comprised of financial supporters, skilled colleagues, and home front support. The rest of the pieces fell into their places with a mixture of knowledge, understanding and open-mindedness spiked with a pinch of luck. Hence, I wish to acknowledge all the parties for their share in this work.

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Helsinki, May 2012

Mari Lehtonen

”Each smallest act of kindness
–even just words of hope when they are needed,
the remembrance of a birthday, a compliment that engenders a smile–
reverberates across great distances and spans of time,
affecting lives unknown to the one whose generous spirit was the source of the good echo,
because kindness is passed on and grows each time it is passed,
until a simple courtesy becomes an act of selfless courage years later and far away.”

-This Momentous Day, H.R. White-

CONTENTS

ABBREVIATIONS

APCI	Atmospheric chemical ionization
BDE	Bond dissociation enthalpy
BHT	Butylated hydroxytoluene
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
CIS-MS	Coordination ion mass spectrometry
CL	Chemiluminescence detection
DAD	Diode-array detector
DEE	Diethyl ether
EC	Electrochemical detection
ELSD	Evaporative light-scattering detector
ESI	Electrospray ionization
EtAc	Ethyl acetate
EtOH	Ethanol
ExSTD	External standard
Fa-OOH	Acyl moiety hydropreoxide of steryl ester
FID	Flame ionization detector
GC	Gas chromatography
HDL	High-density lipoprotein
HMDS	Hexamethyldisilazane
HPLC	High-performance liquid chromatography
(HP)SEC	(High-performance) size-exclusion chromatography
IPA	2-Propanol
ISTD	Internal standard
KOH	Potassium hydroxide
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MS	Mass spectrometry
MTBE	Methyl-*tert*-butyl ether
PCA	Principal component analysis
PV	Peroxide value
RI	Refractive index
SIM	Selected ion monitoring
SPE	Solid-phase extraction
St-OOH	Steryl moiety hydroperoxide of steryl ester
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
TMCS	Trimethylchlorosilane

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred in the text by Roman numerals I−IV.

I) Lehtonen M, Kemmo S, Lampi A-M, Piironen V. 2011. Effects of esterification on the formation and decomposition of steryl hydroperoxides. European Food Research and Technology 232:255-64.

II) Lehtonen M, Lampi A-M, Ollilainen V, Struijs K, Piironen V. 2011. The role of acyl moiety in the formation and reactions of steryl ester hydroperoxides. European Food Research and Technology 233:51-61.

III) Lehtonen M, Lampi A-M, Agalga F, Struijs K, Piironen V. 2011. The effects of acyl moiety and temperature in the polymerization of sterols. European Journal of Lipid Science and Technology, *in press*.

IV) Lehtonen M, Lampi A-M, Riuttamäki M-A, Piironen V. 2012. Oxidation reactions of steryl esters in a lipid matrix. Food Chemistry, *in press*.

RESEARCH INPUT AND AUTHORSHIP OF ARTICLES (I−IV)

I−II) The planning of these studies was carried out by all of the authors. The experimental work was carried out by MSc Mari Lehtonen. She also had the main responsibility for interpreting the results and preparing the manuscript.

III−IV) The planning of these studies was carried out by all of the authors. MSc Mari Lehtonen had the main responsibility for the experimental work, result interpretation and manuscript preparation.

1 INTRODUCTION

Plant sterol and stanol enriched food products have been available for consumers since the 90s and the variety of different products is constantly increasing. Dietary plant sterols are known to reduce serum LDL-cholesterol levels up to 10−20% (Ostlund 2002; Gylling and Miettinen 2005; [EFSA] European Food Safety Authority 2009), and thus aid in the prevention of cardiovascular diseases. Plant sterols inhibit the uptake of cholesterol mainly by competing with its solubilisation into bile salt micelles in the intestine and by co-crystallizing cholesterol from the micelles (Rozner and Garti 2006). They may also interfere in the enzyme catalysed (e.g., lipase and esterase) esterification of cholesterol at the absorption sites, thereby inhibiting cholesterol uptake.

Plant sterols, also known as phytosterols, are produced only by plants and are constituents of plant cell membranes. They are structurally very similar to cholesterol, the major sterol of animal origin. Thus, plant sterols have similar functions in plants as cholesterol has in animals: Sterols regulate the physical properties of membranes and participate in lipid metabolism. People in developed countries typically consume a daily intake of 138−437 mg of plant sterols (Ostlund 2002, 2007; Piironen and Lampi 2004; Kuhlmann et al. 2005). Rich sources include vegetable oils (60−1100 mg/100 g), nuts (55−160 mg/100 g), cereals (50−180 mg/100 g) and vegetables (5−40 mg/100 g). However, daily doses of 1.5−2.4 g are needed for a reduction of serum cholesterol levels of up to 10% (EFSA 2009). Therefore various food products, such as margarines, yoghurts, dairy drinks and bread, are supplemented with plant based sterols or stanols.

Sterols are added to food products either in their free form or as fatty acyl esters. Esterification of sterols with fatty acids increases their solubility in fat and therefore promotes their incorporation into fat-based products. Sterols may be introduced to food products as such, or in microcrystalline dispersions, in liquid suspensions or in microemulsions. To ensure their availability at the site of formation of bile salt micelles, sterols are often accompanied by other agents, such as emulsifiers or other suitable vehicles.

Like all unsaturated lipids, sterols are prone to oxidation in the presence of oxygen and initiators; e.g., heat, light, metals and enzymes. This may occur, for example, during food processing and storage. The oxidation products of cholesterol are known to have adverse health effects, so the potential for plant sterol oxidation has raised concerns about the possible biological effects of plant sterol oxides. Oxidation products of cholesterol can be mutagenic, carcinogenic, cytotoxic and atherogenic (Garcia-Cruset et al. 2002; Osada 2002). Cholesterol oxides may also inhibit the biosynthesis and membrane functions of cholesterol (Rozner and Garti 2006). In vitro studies have demonstrated that, compared to their cholesterol analogues, higher doses of plant sterol oxidation products are needed to elicit biological effects (Hovenkamp et al. 2008; Ryan et al. 2009; García-Llatas and Rodriguez-Estrada 2011).

In neat preparations and in lipid media at 100 °C, the steryl moiety in esterified sterols has been found to oxidise more than in the corresponding free sterol (Korahani et al. 1982; Soupas et al. 2005). At elevated temperature (180 °C), however, free sterol oxidised to a somewhat greater extent than the steryl moiety in esterified sterols. Thus, esterification of a sterol with an unsaturated fatty acid was suggested to slow down the oxidation of steryl moiety. Although the matrix itself may have an effect on the oxidative stability of incorporated sterols, the polarity differences of free and esterified sterols may also lead to their different distributions in the medium, and this might affect their oxidative stabilities.

The oxidation reactions of free cholesterol have been extensively studied, whereas those of plant sterols have been less thoroughly examined. Depending on the physical state of sterol, the oxidation of free sterols begins by abstraction of hydrogen either from the allylic carbon C-7 in the sterol backbone or from a carbon in the side chain (Smith 1981, 1987, 1996; Sevilla et al. 1986; Maerker 1987; Kamal-Eldin and Lampi 2008). As the oxidation proceeds, oxygenated sites may be found both in the sterol backbone and in the side chain.

Fortification of foods is mainly accomplished with esterified sterols; however, very little is known about their oxidation reactions, especially when studied as intact molecules. The oxidation of steryl esters has been suggested to begin by the abstraction of hydrogen from the acyl moiety (Smith 1981, 1987, 1996). The resulting radical may react directly with oxygen, leading to an oxygenated site in the acyl moiety, or it may undergo inter- or intramolecular radical propagation, which ultimately leads to the oxidation of the steryl moiety. Products having oxygenated sites both in the steryl and in the acyl moieties may also form.

Henceforth, this discussion will concentrate primarily on the oxidation of Δ^5-sterols and their conjugates and on the analysis of their reaction products. Reactions of stanols (i.e., 5α-saturated analogues of Δ^5-sterols) will not be discussed in detail.

The purpose of this Thesis was to investigate the oxidation of steryl esters. The formation and further reactions of primary oxidation products were examined. The effects of chemical and physical properties of sterols as well as the effects of media and temperature on the oxidation were investigated. The obtained results improve the understanding of the formation of primary oxidation products in steryl esters and their reactions that lead to secondary oxidation products and oligomers with greater stability. These products may accumulate in food products, for example, during prolonged heating and storage.

2 REVIEW OF THE LITERATURE

2.1 Sterols

Sterols are triterpenes consisting of a monounsaturated tetracyclic cyclopenta[a]phenanthrene structure and a hydrocarbon side chain (Figure 1). Sterols may be divided into four main groups: 4-desmethylsterols, which contain a double bond in the ring structure (e.g., Δ^5-sterols, Δ^7-sterols and $\Delta^{5,7}$-sterols); their 5α-staturated derivatives, stanols; 4α-monomethyl sterols and 4,4-dimethyl sterols. Henceforth in this thesis, Δ^5-sterols will be referred as sterols, unless mentioned otherwise. Free sterols and stanols occur as alcohols that contain a hydroxyl group at carbon C-3. They may also occur as conjugates of fatty acids, ferulic acids and carbohydrates. The additional moieties are mainly linked to the sterol at C-3. The most common sterol, cholesterol, regulates the fluidity of membranes and functions as a precursor of bile acids, vitamin D and steroid hormones. Plant sterols, e.g., brassicasterol, campesterol, sitosterol, stigmasterol and Δ^5-avenasterol, are structurally very similar to cholesterol, with only an extra methyl or ethyl group and double bond in the hydrocarbon side chain differentiating them (Figure 1). Plant sterols are produced solely by plants, and they have similar functions in the plant membranes as cholesterol has in animal cells.

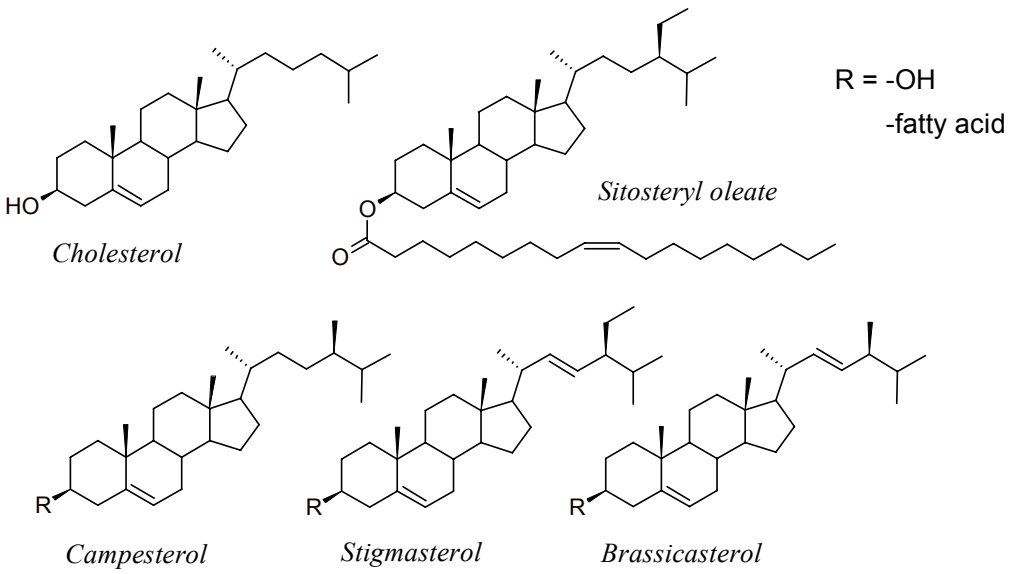

Figure 1. Structure examples of Δ^5-sterols and their acyl esters

Plant sterol and stanol enriched food products have been available to consumers since 1995, and the variety of these products is constantly increasing. Since the 1950s, plant-based sterols and stanols have been recognised for their ability to lower serum cholesterol levels and thereby they also aid in the prevention of cardiovascular diseases. The small structural differences in plant sterols and stanols, compared to cholesterol, lead to their reduced

solubilisation in bile salt micelles and decreased uptake by enterocytes. Cholesterol is absorbed up to 60%, whilst plant sterols to less than 2% (Ostlund 2007). The absorption of plant stanols is even lower; absorption is as low as 0.04%. The most accepted mechanism proposed to explain the cholesterol lowering effects of plant sterols and stanols is that they compete with and displace cholesterol during solubilisation and entry into bile salt/phospholipid micelles in the intestine (Miettinen et al. 2000; Nissinen et al. 2002, 2007; Trautwein et al. 2003; Rozner and Garti 2006; Jones and AbuMweis 2009). Plant sterols may also co-precipitate cholesterol and in this way also inhibit its micellar entry. They may also interfere with the enzymatic hydrolysis of cholesteryl esters and impede the esterification of cholesterol in the enterocytes. Hence, plant sterols/stanols would limit the availability of cholesterol at the absorption sites, thereby reducing the uptake of cholesterol. These mechanisms affect the uptake of both dietary and endogenous cholesterol. Plant sterols/stanols may also regulate the metabolism of cholesterol and enhance its excretion in the bile. The effects of lowered serum cholesterol levels have been observed in total and LDL-cholesterol levels but not in HDL-cholesterol levels.

Plant sterols and stanols may be incorporated into food products as either free or esterified forms. Esterification of a sterol/stanol with a fatty acid increases its solubility in lipid media (e.g., vegetable oils). The LDL-cholesterol lowering efficiency of free and esterified sterols and stanols has been found to be similar in magnitude (EFSA 2009). However, the matrices in which the sterols or stanols are incorporated play a significant role in the cholesterol lowering efficiency of plant sterols/stanols (Clifton et al. 2004; MacKay and Jones 2011). Transportation of the sterols to the absorption sites requires that they first enter mixed micelles consisting of fatty acids, phospholipids, bile salts and sterols (Trautwein et al. 2003). Esterified sterols are more soluble in the oil phase of emulsions than are free sterols and thus they are transported along with triacylglycerols to the site where mixed micelles are formed. While triacylglycerols are hydrolysed into fatty acids and monoacylglycerols (i.e., the constituents of mixed micelles), esterified sterols are hydrolysed into free sterols by pancreatic cholesterol hydrolase (Miettinen et al. 2000). As free fatty acids and monoacylglycerols combine with bile salts and phospholipids to form the mixed micelles, plant sterols interfere with the hydrolysis of cholesteryl ester and with the micellar entry of cholesterol (Miettinen et al. 2000, Nissinen et al. 2002, 2007). In order to enhance the transportation of the less lipid-soluble free plant sterols and stanols to the site and phase where mixed micelles are formed, they need to be accompanied by other agents, such as emulsifiers, or suitable vehicles (Woollett et al. 2006). After entering the micellar phase, sterols are transported to the absorption sites (Trautwein et al. 2003). Thus, the efficiency of plant sterols and stanols at lowering cholesterol levels depends on their solubilisation, availability and delivery (MacKay and Jones 2011). The effects of new enriched food matrices on the delivery of plant sterols and stanols should be studied individually to quantify the cholesterol lowering effects. In the scientific opinion of the EFSA (European Food Safety Authority, 2009), the scientific panel concluded that the consumption of plant sterol/stanol enriched spreads, mayonnaises, salad dressings and dairy products led to reduced

LDL-cholesterol levels, but similar effects during consumption of other types of food matrices have not been well demonstrated.

Modifying the chemical properties of sterols and stanols, for example by esterification, also can alter their physical properties and change their oxidation susceptibilities and reactions. The surrounding compounds (e.g., medium) and environmental factors (e.g., temperature) also affect the physical properties and oxidation reactions of sterols.

2.2 Sterol oxidation

Oxidation of sterols (i.e., Δ^5-sterols) may occur via enzymatic or non-enzymatic pathways. In food processing and food products, exogenous oxidation occurs mainly via non-enzymatic pathways that include autoxidation and photoxidation. Therefore, these mechanisms will be discussed in more detail in the following chapter.

Autoxidation is a radical-mediated oxidation reaction that occurs when compounds are exposed to molecular oxygen ($^3O_2$) at moderate temperatures (below 120 ºC) (Kamal-Eldin et al. 2003a; Schaich 2005; Choe and Min 2006, 2007). The oxidation of unsaturated lipids is divided into three phases: initiation, propagation and termination (Equations 1−7). In the initiation stage, carbon radicals are formed primarily by abstraction of hydrogen atoms from the allylic carbons of unsaturated lipids (Equation 1). This abstraction may be induced by heat or metal ions, or by hydroperoxides and radicals present in the reaction mixture (Bawn 1953, Uri 1956; Labuza and Dugan 1971). In the latter case, branching or re-initiation occurs (Equations 1a-f) (Kamal-Eldin et al. 2003a; Schaich 2005). As soon as the carbon radicals start to react with molecular oxygen, producing peroxyl radicals and subsequently hydroperoxides, the oxidation proceeds into the propagation stage. The formed peroxyl radicals react with other unsaturated lipids producing hydroperoxides and causing re-initiation (i.e., formation of new carbon, peroxyl and alkoxyl radicals). The formed hydroperoxides may also decompose into secondary radicals (Kamal-Eldin et al. 2003a; Schaich 2005; Choe and Min 2006, 2007; Bartosz and Kołakowska 2011). In the termination stage, the primary and secondary carbon, peroxyl and alkoxyl radicals may undergo radical recombinations or radical scissions forming oligomers (e.g., dimers and trimers) and non-radical monomers (i.e., secondary oxidation products) (Schaich 2005; Choe and Min 2006, 2007). While these rather stable oxidation products are formed, new radicals and hydroperoxides continue to be produced. The reactions are presented in the following equations 1−7 (Kamal-Eldin et al. 2003a; Schaich 2005):

Initiation:	RH	→	$R^{\bullet} + H^{\bullet}$	(1)
Branching	ROOH	→	$RO^{\bullet} + HO^{\bullet}$	(1a)
	ROOH + RH	→	$RO^{\bullet} + R^{\bullet} + H_2O$	(1b)
	2ROOH	→	$ROO^{\bullet} + RO^{\bullet} + H_2O$	(1c)
Re-initiation	$RH + RO^{\bullet}$	→	$R^{\bullet}$ + ROH	(1d)
	$RH + HO^{\bullet}$	→	$R^{\bullet} + H_2O$	(1e)
	$RH + ROO^{\bullet}$	→	$R^{\bullet}$ + ROOH	(1f)
Propagation:	$R^{\bullet} + O_2$	↔	$ROO^{\bullet}$	(2)
	$ROO^{\bullet}$ + RH	→	ROOH + $R^{\bullet}$	(3)
Termination:	$2R^{\bullet}$	→	RR	(4)
	$ROO^{\bullet} + R^{\bullet}$	→	ROOR	(5)
	$2ROO^{\bullet}$	→	O_2 + ROOR	(6)
	$2ROO^{\bullet}$	→	O_2 + ROH + R	(7)

Hydroperoxides are mainly decomposed into hydroxides and ketones. As the oxidation proceeds and most of the accessible hydrogens are abstracted, the addition of peroxyl radical to the double bonds begins to dominate (Kamal-Eldin et al. 2003a; Schaich 2005). This leads to the formation of epoxides. Oligomers are also formed, either by recombination of two allyl radicals or by addition of a radical into a double bond (Schaich 2005; Choe and Min 2006, 2007; Dobarganes and Márquez-Ruiz 2007). In both of these cases, two unsaturated moieties are connected at the position of the double bonds. Polar oligomers form in a similar manner, only these contain oxygenated sites. The rate of oxidation and the availability of oxygen affect the composition of formed oxidation products. At ambient temperatures (60−120 °C), radical peroxidation has been suggested to predominate, while at elevated temperatures (140−200 °C) non-radical reactions, such as elimination and nucleophilic substitution, become more important (Schaich 2005; Choe and Min 2006, 2007). The limited content of oxygen and restricted mobility favour polymerisation during lipid autoxidation. In the absence of oxygen, nonpolar oligomers are formed mainly via C-C linkages (Choe and Min 2006, 2007; Dobarganes and Márquez-Ruiz 2007). When an excess of oxygen is available, ether (C-O-C) and peroxide (C-O-O-C) linked oligomers are likely to form. Formation of nonpolar dimers has been found to predominate in sunflower oil heated at 190 °C (Márquez-Ruiz et al. 1995). The number of polar dimers increased along with increasing heating time. Polymerisation of lipids changes the fluidity and properties of the frying oil and is therefore considered as degradation parameter of the oil.

Oxidation of lipids may also occur via light induced oxidation: photoxidation. This occurs most often at storage conditions when food products are exposed to visible light. At the wavelengths of visible light (>400 nm), the energy of photons is not sufficient to induce a

direct radical formation in lipids; instead, the oxidation occurs via sensitization; i.e., photosensitization (Kamal-Eldin et al. 2003a; Schaich 2005; Choe and Min 2006, 2007; Bartosz and Kołakowska 2011). The energy of photons may transfer to chemical energy via excitation of a photosensitizer, which is a molecule that is in the singlet state (e.g., chlorophyll). The excited photosensitizer becomes an excited triplet state molecule, which may react with triplet state oxygen ($^3O_2$) to form singlet state oxygen ($^1O_2$). Non-radical lipids exist at singlet state and may thus react directly with singlet oxygen. The reaction occurs via a non-radical mechanism involving direct addition of oxygen. The resulting products are hydroperoxides. Different hydroperoxide isomers are formed than those produced by the reactions with triplet state oxygen (Kamal-Eldin et al. 2003a; Schaich 2005; Choe and Min 2006, 2007). Depending on the type of excited photosensitizer, initiation of radical mediated oxidation of lipids is also possible (Schaich 2005; Choe and Min 2006). When hydroperoxides are present, light and radicals are able to catalyse the formation of secondary radicals. The termination stage of photoxidation occurs via similar patterns as presented for autoxidation.

2.2.1 Autoxidation

Autoxidation of free sterols and steryl esters involves both monomolecular and bimolecular reaction mechanisms. The oxidation of free sterol or of the steryl moiety in steryl esters begins, as in other unsaturated lipids, by abstraction of hydrogen, which leads to the formation of a radical. The hydrogen is most likely abstracted from the allylic carbon C-7 in the sterol backbone (Smith 1981, 1987, 1996; Kamal-Eldin and Lampi 2008; Yin et al. 2011). This is due to the lowest theoretical bond dissociation enthalpy (BDE) of C7-αH and due to the cyclic structure (Lengyel et al. 2012; Yin et al. 2011). The formed radical reacts quickly with oxygen and forms a peroxyl radical. This radical then abstracts hydrogen either from another sterol or from the surrounding medium and forms a steryl moiety 7-hydroperoxide. Alternatively, the peroxyl radical may react directly with a double bond. In Δ^5-sterols, the addition most likely occurs at C-5 or C-6 leading to the formation of 5,6α- or 5,6β-epoxide, respectively (Kamal-Eldin and Lampi 2008). The abstraction of 7α-H has been suggested to be favoured over the 7β-H (Smith 1981, 1987, 1996; Yin et al. 2011). This most likely ensues from the orientation of the hydrogen on the opposite side of the planar sterol backbone, compared to the side chain and substituted groups at C-3, C-10 and C-13. Some authors have suggested that the steric hindrance caused by the OH-group at position C-3 would affect this as well (Lecker and Rodriguez-Estrada 2002); whereas others have proposed that the β-epimers are energetically favoured (Kamal-Eldin and Lampi 2008). However, the formed radical or peroxyl radical is readily epimerized into a thermodynamically more stable 7β-epimer. Thus, greater contents of St-7β-OOHs or St-7β-OHs than of St-7α-OOHs or St-7α-OHs have been measured for free sterols (Kemmo et al. 2005; Soupas et al. 2005). In the solid state, the crystalline structure has been suggested to protect carbon C-7 of the free sterol against oxidation (Korahani et al. 1982). Oxidation of free cholesterol was negligible in the solid state at 100 °C and side chain oxidation products were mainly measured. In

Δ^5-sterols, the addition of a peroxyl radical to less sterically hindered C-6 is favoured over the addition to C-5 (Kamal-Eldin and Lampi 2008). Therefore, greater contents of 5,6β-epoxides than of 5,6α-epoxides have been measured (Kemmo et al. 2005; Soupas et al. 2005).

When cholesterol was irradiated, radical formation was also observed in the side chain of the sterol backbone (Sevilla et al. 1986). The hydrogen is abstracted from tertiary carbons, which are C-20 and C-25 in cholesterol and C-20, C-24 and C-25 in plant sterols (Kamal-Eldin and Lampi 2008). Since the side chain is situated outwards from the planar sterol backbone, it is able to rotate freely (Sevilla et al. 1986). This ability means that the formed side chain radical is not stable and it quickly reacts further via intramolecular radical propagation to form a more stable allylic radical C-7. Thus, side chain hydroperoxides and secondary oxidation products are detected only at very low contents, but the main products have an oxygenated site in the C-7. Moreover, the BDEs of these side chain C-H bonds has been calculated to vary between 376–398 kJ/mol while the BDE of C7-H was 328 kJ/mol (Lengyel et al. 2012). In the solid state, when the side chain is not free to rotate, oxidation of the side chain has mainly been observed (Korahani et al. 1982). The calculated BDEs also vary depending on the physical state of the compound.

In steryl esters, both the steryl and acyl moieties may oxidise. Thus, three types of oxidation products may form: a steryl ester with an oxidised steryl moiety (i.e., esterified oxysterol), a steryl ester with an oxidised acyl moiety and a steryl ester with oxidised steryl and acyl moieties. The oxidation of steryl esters with polyunsaturated acyl moiety has been suggested to begin by radical abstraction from the acyl moiety rather than from the steryl moiety (Smith 1981, 1987, 1996; Lund et al. 1992). The formed acyl moiety radical would then react with oxygen to form a peroxyl radical and subsequently a hydroperoxide or an epoxide, or alternatively, intra- or intermolecular radical propagation would take place (Figure 2). Intramolecular radical propagation would result in the transfer of the acyl moiety radical to the steryl moiety radical, which then could react with oxygen to form a steryl moiety hydroperoxide. Intermolecular radical propagation, on the other hand, would result in a reaction of the acyl moiety radical with another steryl ester and lead to radical formation either in the steryl or acyl moiety of the other ester. Similar reactions of peroxyl radicals have been suggested to occur as well. These chain reactions are suggested to lead to the formation of both steryl and acyl moiety hydroperoxides, and possibly even to the formation of dihydroperoxides and epoxides. These proposed oxidation reaction mechanisms of steryl esters are based on results obtained with a mixture of free sterols and fatty acids, and not with intact steryl esters. According to the calculated BDEs, the abstraction of hydrogen from the sterol C-7 and the methyl oleate C-8 or C-11 would require same amount of energy (328 kJ/mol and 331 kJ/mol, respectively) (Pajunen et al. 2008; Lengyel et al. 2012). The abstraction of hydrogen from C-11 in methyl linoleate, on the other hand, would require only 283 kJ/mol. These different BDEs support the obtained differences between the oxidisabilities of monoenic and polyenic lipids. Yin et al. (2011) also suggested that cholesterol would be a better hydrogen donor, and thus more reactive, than monounsaturated fatty acid due to its cyclic structure and orientation of C7-αH. However, knowledge regarding the oxidisabilities

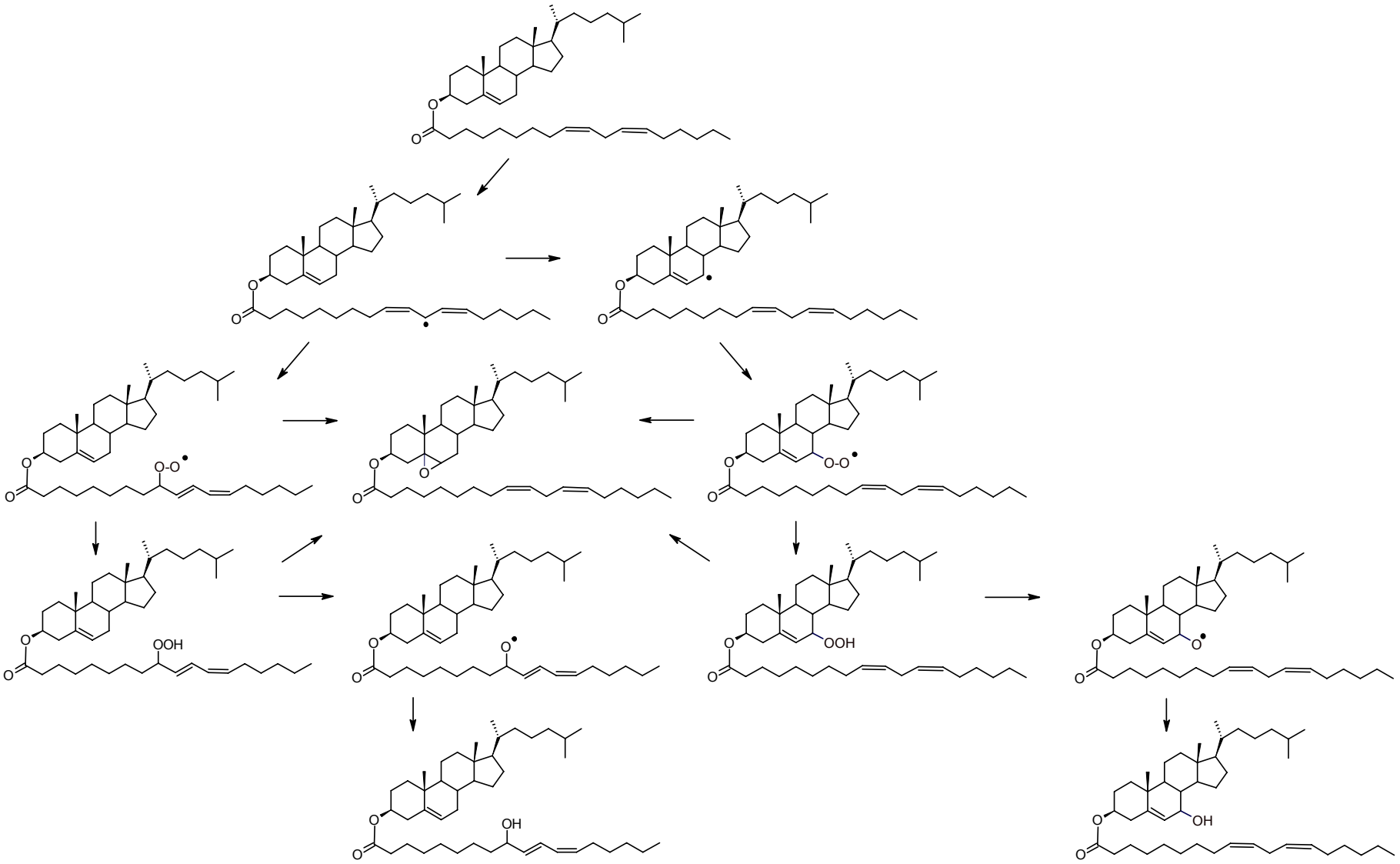

Figure 2. Formation and further reactions of steryl and acyl moiety radicals of cholesteryl linoleate.

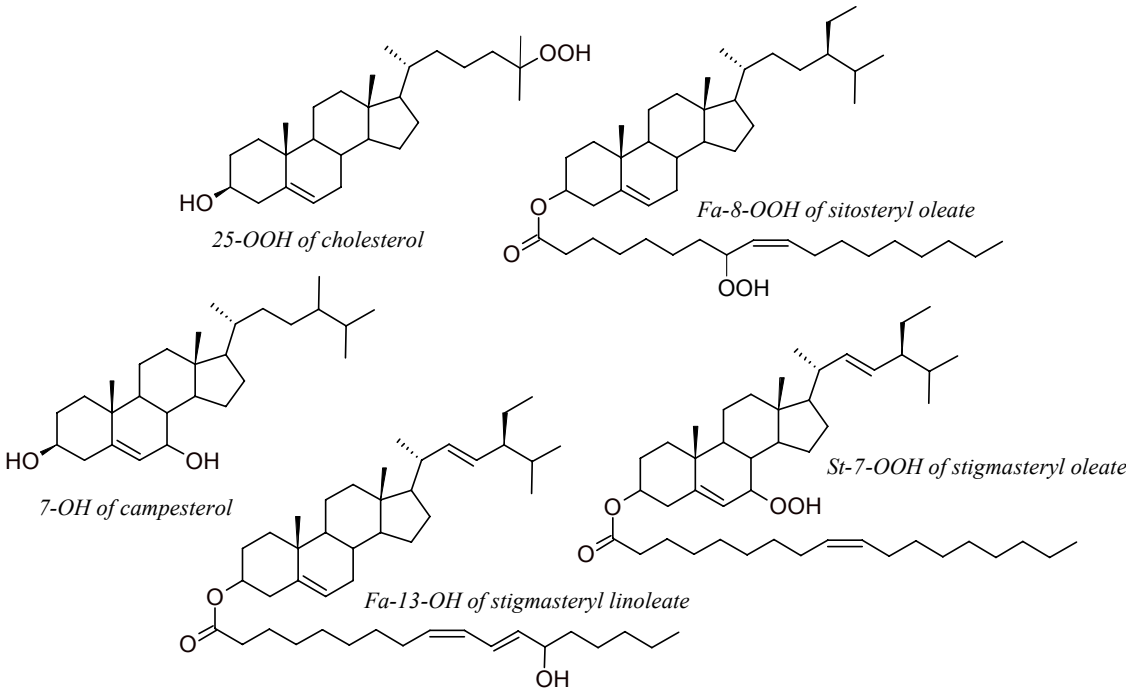

Figure 3. Structure examples of sterol and steryl ester oxidation products.

of steryl and acyl moieties in intact esters is lacking. The formation rates and ratios of steryl and acyl moiety oxidation products are also not known.

The thermal degradation of hydroperoxides has been suggested to occur via monomolecular reactions (Lercker et al. 1996; Bortolomeazzi et al. 2000). Epoxidation and polymerisation, on the other hand, are considered to be bimolecular reactions (Kamal-Eldin and Lampi 2008). As the formed steryl and acyl moiety hydroperoxides decompose, they react further into secondary oxides (i.e., hydroxides, ketones and epoxides) (Figure 3). In the steryl moiety, the main secondary oxidation products are St-6β-OH, St-5,6α/β-epoxide, St-7α/β-OH, St-7-ketone (Smith 1981, 1987, 1996; Dutta et al. 2004). Steryl esters are also known to form the side chain St-20-OH and St-25-OH. For plant steryl esters, St-24-OH may also form.

The acyl moiety oxidation products depend on the type of the acyl moiety. According to the studies conducted with fatty acids, the oxygenated sites in oleate would be C-8, C-9, C-10 and C-11 (Porter et al. 1980). Similarly, in steryl linoleate, the sites would be C-9 and C-13 (Porter et al. 1994). In physiology-related studies, cholesteryl linoleate acyl moiety Fa-9-OOHs and Fa-13-OOHs have been determined (Table 2). In addition to these polar oxidation products, nonpolar compounds may also form via elimination of the OH-group at C-3 in free sterols or after cleavage of the acyl moiety in steryl esters (Smith 1981, 1987, 1996; Menéndez-Carreño et al. 2010). Polymerisation of free sterols is also known to occur (Lampi et al. 2009; Rudzinska et al. 2010; Struijs et al. 2010). Polymerisation of steryl esters most likely occurs as well, but studies in this area are lacking.

Secondary oxidation products of sterols do not appear to account for all of the sterol losses obtained in oxidised samples. When free and esterified sitosterols were oxidised in a tripalmitin matrix at 180 °C for three hours, 28% of the free sitosterol and 13% of the esterified sitosterol could not be recovered as unchanged sterol and sterol oxides (Soupas et al. 2005). Since this gap in the recoveries was acknowledged and polymerisation was considered to be the underlying reason for it, more detailed studies on the sterol oligomers have been conducted (Lampi et al. 2009; Rudzinska et al. 2010; Struijs et al. 2010). After neat stigmasterol was oxidised at 180 °C for three hours, 21 % of the sterol was recovered as oligomers (Lampi et al. 2009). Of the formed dimers, 60% were polar (i.e., contained an oxygenated group in the molecule) and 15% were nonpolar. Moreover, 78% of the higher oligomers were polar. Recently, structures of some free sterol dimers have been indicated (Rudzinska et al. 2010; Struijs et al. 2010). Stigmasterol dimers were found to combine via C-C, C-O-C and C-O-O-C linkages (Struijs et al. 2010). The connection of two sterol molecules was proposed to occur most likely between C-7 and C-7'. Even though most of the naturally occurring sterols in vegetable oils are present in esterified form, the polymerisation reactions of steryl esters have not been studied. According to the results obtained for free sterols and for fatty acids, steryl esters would be expected to form dimers by coupling steryl or acyl moieties next to the double bonds via C-C, C-O-C and C-O-O-C linkages. Junctions between steryl and acyl moieties may also form. The formation of both nonpolar and polar dimers and oligomers would be expected in the presence of several unsaturated sites.

The importance of identifying primary oxidation products has been widely accepted in the oxidation studies of fatty acids since secondary oxidation products and, in part, the polymerisation of lipids, are continuations of the primary products, hydroperoxides. Studies on steryl ester hydroperoxides in food related systems, however, are scarce. When the primary oxidation (120 °C) of a mixture of tripalmitin and sitosteryl stearate (5%) or free sterol (5%) was investigated in terms of peroxide values, a higher rate of oxidation was found for esterified sitosterol than for free sitosterol (Yanishlieva et al. 1985).

Previous oxidation studies concerning plant sterols and their fatty acyl esters in lipid matrices have mainly focused on the formation of secondary oxidation products of steryl moiety (Yanishlieva et al. 1985; Blekas and Boskou 1989; Soupas et al. 2004b, 2005, 2007) or on the changes occurring in the lipid medium (Lampi et al. 1999; Winkler and Warner 2008). In one of these studies, the steryl moiety in steryl esters having rapeseed oil acyl moieties (7.5% saturated, 66% monounsaturated, 26% polyunsaturated) oxidised more than the corresponding free sterols, when present in a saturated lipid medium at moderate temperatures (100 °C) (Soupas et al. 2005). However, at elevated temperatures (180 °C) the opposite occurred: The oxidation of steryl moiety was greater for free sterol than for the esterified sterols.

In food products, functional lipid components, such as plant sterols, are present in a complex mixture of other compounds. The presence and reactions of these surrounding compounds also affect the reactions of the functional lipids. In the oxidation reactions, other lipids function both as hydrogen sources and as radical formers (Labuza and Dugan 1971; Schaich

2005). As other components participate in the oxidation reactions, they affect not only the rate of oxidation, but they may also modify the profile of the formed products. For example, in lipid model systems, triacylglycerols have been suggested to induce the oxidation of free cholesterol at 25−180 °C (Kim and Nawar 1991; Nawar et al. 1991; Li et al. 1994). Free fatty acids, on the other hand, not only increased the oxidation of cholesterol, but also changed the profile of the formed oxidation products (Kim and Nawar 1991; Xu et al. 2011). The unsaturation of the lipid medium has also been suggested to alter the oxidation of sterols: In a saturated medium, the oxidation of sterols would increase, but in an unsaturated medium, the high reactivity of the medium would lead to its oxidation rather than to the oxidation of sterols (Lercker and Rodriguez-Estrada 2002). The presence of water in the lipid medium may also affect sterol oxidation. For example, the oxidation of free sitosterol and sitosteryl esters was greater in margarine than in rapeseed oil used for pan-frying at 160 °C (Soupas et al. 2007). Moreover, in both margarine and butter oil, the oxidation of free sterols was greater than of the corresponding steryl esters. In emulsions, the surface area of lipids is enlarged and thus the contact with air and catalysts is increased. These changes greatly induced the oxidation of both unsaturated lipid media and free plant sterols (Cercaci et al. 2007). Since the solubility of free sterols in nonpolar lipids is limited and they have been found to act as surface active compounds, they were most likely located in the interphase of the lipid droplets and water. This likely induced the oxidation of the sterols.

2.2.2 Photoxidation

Like other unsaturated lipids, sterols also may oxidise via light induced oxidation: photoxidation. This process is mainly considered to occur via addition of singlet oxygen into a double bond in the sterol or steryl ester molecule (Kamal-Eldin and Lampi 2008). For free sterols and for the steryl moiety of steryl esters, the first detected oxidation products formed in photoxidation are St-5α-OOH and St-6α/β-OOH (El Hafidi et al. 1999; Säynäjoki et al. 2003; Kamal-Eldin and Lampi 2008). However, the St-5α-OOH is easily converted into St-7α-OOH and further isomerised into thermodynamically more stable St-7β-OOH. Especially in steryl esters, the absence of St-4-OOHs has been suggested to result from the steric hindrance caused by the surrounding groups (Lercker and Rodriguez-Estrada 2002). Even though some plant sterols contain a double bond in the side chain of the steryl moiety, photoxidation of this site has not been reported.

In steryl esters, hydroperoxyl groups may also locate in the acyl moiety. Like in fatty acids and their methyl esters, in oleyl moiety of steryl esters, Fa-9-OOH and Fa-10-OOH are formed. Similarly, in linoleyl moiety Fa-9-OOH, Fa-10-OOH, Fa-12-OOH and Fa-13-OOH, and in linolenyl moiety Fa-9-OOH, Fa-10-OOH, Fa-12-OOH, Fa-13-OOH, Fa-15-OOH and Fa-16-OOH are formed (El Hafidi et al. 1999; Hui et al. 2000).

As the hydroperoxides formed in photoxidation start to decompose (i.e., the termination stage is reached), the decomposition reactions follow similar patterns as presented for autoxidation, and thus similar reaction products are detected.

2.3 Analysis of steryl ester oxidation products

Food products generally contain very low levels of oxidation products of steryl esters and free sterols and these are present in complex mixtures of other compounds. Therefore, efficient and specific sample preparation, isolation and detection methods are needed for analysis of these oxidation products. Moreover, further reactions of these rather labile oxidation products need to be prevented during the sample treatment. The oxidation products of steryl esters and free sterols are usually studied in terms of secondary oxidation products, such as their epoxides, hydroxides and ketones, by gas chromatographic methods. However, these methods are not suitable for the analysis of primary oxidation products, the hydroperoxides, or for the determination of intact ester molecules. Therefore, HPLC (high-performance liquid chromatography) methods have been developed for the analysis of intact steryl ester oxidation products. Moreover, several detection methods have been evaluated in order to improve the detection sensitivity.

Comprehensive reviews on the analysis of secondary oxidation products of sterols by gas chromatographic methods are currently available (Dutta 2002, 2004; Guardiola et al. 2002, 2004), so this topic will not be discussed in depth. However, the analysis of intact steryl ester oxidation products by HPLC will be dealt in more detail.

2.3.1 Sample treatment

The analysis of steryl ester or free sterol oxidation products begins with lipid extraction. The extraction solvents need to be efficient at impregnating the matrix and releasing lipids. These solvents also need to be inert to avoid causing further reactions of labile oxidation products. Traditionally, the extraction is performed according to Folch et al. (1957) using chloroform/methanol (2:1, v/v) or alternatively according to Hara and Radin (1978) using hexane/IPA (3:2, v/v).

By far, food scientists have focused on studying the oxidation products of the steryl moiety. Therefore, the collected lipid extract has been traditionally exposed to cold saponification with KOH in methanol or in ethanol in order to isolate and concentrate the steryl moieties of steryl esters and free sterols (Table 1). Alternatively, transesterification with 10% sodium methylate in methanol may be performed (Johnsson and Dutta 2006). Lipid based samples, such as vegetable oils and spreads, may be directly saponified or transesterified without a prior lipid extraction step. Since the ester bonds are hydrolysed or the acyl moieties are exchanged in these procedures, the isolation of intact steryl ester oxidation products is not possible. Publications on the isolation of intact steryl ester oxidation products from lipid

media, as well as studies on the oxidation products of intact steryl esters occurring in food products, are lacking at this point in time.

After the hydrolytic treatment, the unsaponifiables (i.e., the steryl moieties and free sterols) are isolated from the sample solution by extraction with nonpolar solvents having high dielectric constant and dipole moment (e.g., diethyl ether and chloroform) or with polar aprotic solvents (e.g., dichloromethane) (Table 1). The extracts may be further purified by washing with aqueous KOH, water or water based salt solutions, such as sodium sulphate.

Chromatographic separation of sterol oxides may be enhanced and the baseline may be stabilised by further purification and fractionation of the unsaponifiables. This is usually performed by solid-phase extraction (SPE) using either silica (SiOH) or aminopropyl (NH_2) sorbents with nonpolar solvents (e.g., heptane) with increasing polarity (e.g., diethyl ether) (Table 1). These methods have resulted in the successful isolation of oxidation products from residual impurities and non-oxidised sterols, and their subsequent concentration.

In all of the sample treatment steps, the contact with air, heat, metals and light needs to be restricted in order to avoid further oxidation and artefact formation. Thus, recommended working conditions include use of peroxide and metal free solvents, and performing the sample treatments at ambient temperatures, protected from light and preferably surrounded by inert gases, such as nitrogen. Antioxidants, such as BHT, may also be added prior the sample treatment steps.

2.3.2 Gas chromatography

For the separation and detection of sterol oxidation products, mainly gas chromatographic (GC) methods have been applied so far. In the traditional analysis of secondary oxidation products of sterol, the steryl moiety of steryl esters is determined after removal of the acyl moiety by saponification or transesterification. The unsaponified or transesterified steryl moieties are extracted, purified, derivatized into more volatile compounds and determined by gas chromatographic methods.

For gas chromatographic analysis, sterol oxides are traditionally derivatized to increase their volatility and to improve their peak shapes in the chromatograms. Derivatization is usually performed by transforming the oxides into trimethylsilyl ethers (Table 1). The procedure is performed at room temperature using a mixture of either BSTFA (*N,O*-bis(trimethylsilyl)-trifluoroacetamide) or HMDS (hexamethyldisilazane) and TMCS (trimethylchlorosilane) dissolved in anhydrous pyridine. After the derivatization, the solvents are evaporated and the TMS ethers are dissolved in heptane for introduction into the GC-FID and GC-MS.

The introduction of the TMS ethers of sterol oxides to the GC has been performed by conventional split injection (Conchillo et al. 2005; Menéndez-Carreño et al. 2008; González-

Larena et al. 2011), by a falling-needle technique (Grandgirard et al. 2004) or by on-column injection (Soupas et al. 2004−2007; Julien-David et al. 2009). The separation has been achieved on 30−50 m columns having internal diameters of 0.20−0.25 mm and 5%-phenyl methylpolysiloxane stationary phases with film thicknesses of 0.1−0.25 μm (Table 1). The use of a more polar 35%-phenyl methylpolysiloxane stationary phase has also been reported (Johnsson and Dutta 2005, 2006). Helium has been the typical carrier gas, at a flow rate of 1 mL/min. Temperature programs have mainly depended on the injection technique: In on-column injections, the programs start from 50−70 °C and increase up to 275−290 °C; whereas, in the split injections, the programs start from 250−280 °C and increase up to 310−320 °C.

Separated TMS ethers of sterol oxides have mainly been detected by using FID or MS. FID is sensitive and stabile, but MS may provide improvements in both sensitivity and selectivity of the analysis (Soupas et al. 2004a). This is achieved especially by using selected ion monitoring (SIM). Correct identification of peaks is also possible based on their mass spectra.

Quantification of steryl moiety oxides has mainly been performed using 19-OH-cholesterol as an internal standard (Table 1). Either FID or MS signals may be used for the quantification purposes. Soupas et al. (2004a,b) reported higher contents for the oxides when they were quantified based on the MS signal (using SIM) than when quantified based on the FID signal. Overlapping of some peaks did not interfere with the quantification on the SIM-MS, whereas it did on the FID signal. Since individual plant sterol oxides are not commercially available, most authors have quantified the plant sterol oxides by comparing the peak areas of the oxides to the area of ISTD and using response factors (Conchillo et al. 2005; Julien-David et al. 2009; González-Larena et al. 2011). Synthesis of plant sterol and plant steryl ester oxidation products is however possible but somewhat laborious (Geoffroy et al. 2008; Julien-David et al. 2008). Calibration curves for plant sterol oxides may also be constructed by indirect methods using dilutions of oxidised neat preparations (Soupas et al. 2004b; Menéndez-Carreño et al. 2008; González-Larena et al. 2011). For example, use of indirect dilution method for stigmasterol oxides generated linear ranges of 0.3−293 μg/g for the calibration curves (Soupas et al. 2004b).

In conclusion, the analysis of intact steryl ester oxidation products or primary oxidation products cannot be achieved by gas chromatographic methods. However, determination of secondary oxidation products of steryl moiety from complex mixtures may be achieved with high sensitivity and selectivity.

Table 1 Examples of gas chromatographic methods used for the analysis of secondary oxidation products of sterol in steryl esters (continues).

Compounds	Matrix	Extraction	Separation[†]	Detection	Quantification	Reference
Cholesteryl ester oxides	Neat	Cold saponification (KOH/MeOH) → $CHCl_3$ → TLC	OV-101 (25 m x 0.25 mm, *nr* µm) He 1 mL/min, 285 °C SE-30 (25 m x 0.25 mm, *nr* µm) He 4 mL/min, 250−310 °C	FID (350 °C)	ISTD *nr*	(Korahani et al. 1982)
Plant steryl ester oxides	Enriched spread	Cold saponification (KOH/MeOH) → CH_2Cl_2 → SiOH-SPE	DB5-MS (30 m x 0.25 mm, 0.25 µm) + DB1-MS (30 m x 0.25 mm, 0.25 µm) He *nr* mL/min 50−290 °C	FID (300 °C) MS (70 eV)	STD 5α-Ch	(Grandgirard et al. 2004)
Plant steryl and -stanyl ester oxides	Tripalmitin Rapeseed oil	Cold saponification (KOH/EtOH) → DEE → SiOH-SPE	Rtx-5 w/Integra Guard (60 m x 0.32 mm, 0.1 µm) He 1.4 mL/min 70−275 °C (60 m x 0.25 mm, 0.1 µm) He 1.2 mL/min	FID (300 °C) MS (70 eV)	ISTD Ch 19-OH	(Soupas et al. 2004−2007)
Plant steryl ester oxides	Enriched spread	Cold saponification (KOH/MeOH) → DEE → SiOH-SPE	CP-Sil 8CB (50 m x 0.25 mm, 0.25 µm) He 2.5 mL/min 280−320 °C SPB-5 (30 m x 0.25 mm, 0.25 µm) He 1 mL/min 250−310 °C	FID (325 °C) MS (70 eV)	ISTD Ch 19-OH	(Conchillo et al. 2005)

5α-Ch = 5α-cholestane , Ch 19-OH = 19-hydroxycholesterol, DEE = diethyl ether, EtOH = ethanol, MeOH = methanol, NaMe = sodium methylate, *nr* = not reported, SPE = solid-phase extraction, TMS = trimethylsilyl

[†]) Separation as TMS ethers

Table 1. (continued) Examples of gas chromatographic methods used for the analysis of secondary oxidation products of sterol in steryl esters.

Compounds	Matrix	Extraction	Separation	Detection	Quantification	Reference
Plant steryl ester oxides	Vegetable oils, enriched spread	Transesterification → $CHCl_3$ → NH_2-SPE	DB35-MS (25 m x 0.2mm, 0.33 µm) + DBS MS (25 m x 0.2mm, 0.33 µm) He 0.8 mL/min 65−305 °C	FID (320 °C)	STD 5α-Ch	(Johnsson and Dutta 2005, 2006)
Plant steryl ester oxides	Enriched milk	Transesterification (NaMe/MeOH) → DEE → SiOH-SPE	VF-5ms (50 m x 0.25 mm, 0.25 µm) He 1 mL/min 75−292 °C	MS (70 ev)	ISTD Ch 19-OH	(Menéndez-Carreño et al. 2008)
Sitosteryl ester oxides	Neat	Heptane	VF-5ht (30 m x 0.25 mm, 0.1 µm), He 1 mL/min 60−380 °C	MS (70 ev)	ISTD Ch 19-OH	(Julien-David et al. 2009)
Plant steryl ester oxides	Commercial preparation	Cold saponification (KOH/MeOH) → DEE → SiOH-SPE	CP-Sil 8 low bleed/MS (50 m x 0.25 mm, 0.25 µm) H_2 0.7 mL/min 280−320 °C TR-5 ms SGC (30 m x 0.25 mm, 0.1 µm) H_2 1 mL/min 250−310 °C	FID (325 °C) MS (70 eV)	ISTD Ch 19-OH	(González-Larena et al. 2011)

5α-Ch = 5α-cholestane, Ch 19-OH = 19-hydroxycholesterol, DEE = diethyl ether, EtOH = ethanol, MeOH = methanol, NaMe = sodium methylate, *nr* = not reported, SPE = solid-phase extraction, TMS = trimethylsilyl

†) Separation as TMS ethers

2.3.3 High-performance liquid chromatography

Since HPLC is a competent technique for the analysis of thermolabile compounds, it has also been applied for the characterisation and analysis of sterol oxidation products. Fractionation of the compounds is also possible by this method, since the compounds are not destroyed during the analysis. Most HPLC methods have been developed and applied for the analysis of non-esterified cholesterol oxidation products (Rodriguez-Estrada and Caboni 2002). These methods have been adapted and further developed for the analysis of free plant sterol oxidation products (Säynäjoki et al. 2003; Kemmo et al. 2007a,b). In HPLC, the oxidation products of free cholesterol and of free plant sterols are traditionally separated on silica columns operated in a normal-phase mode. The elution is performed in an isocratic mode with hexane- or heptane-based eluents containing 2−7% isopropanol at flow rates of 0.6−1.5 mL/min. These methods accomplish the separation of individual oxidation products and their epimers. Reversed-phase methods have also been developed for the analysis of free sterol oxides (Rodriguez-Estrada and Caboni 2002). These methods can decrease the retention of polar compounds, thereby shortening the run times. However, the separation of epimers becomes challenging. The separation is mainly conducted on C18-columns using mixtures of water or methanol and acetonitrile as eluents at flow rates of 1 mL/min. Free sterol oligomers have been measured using high performance size-exclusion chromatography (HPSEC) (Lampi et al. 2009; Rudzinska et al. 2010; Struijs et al. 2010). Fractions containing dimers have also been separated further into individual dimers using reversed-phase HPLC (Struijs et al. 2010).

The methods introduced for the analysis of free sterol oxidation products are not directly applicable for the analysis of less polar intact steryl ester oxidation products. Therefore, more selective methods have been developed.

Separation of intact steryl ester hydroperoxides

In physiology related studies, chromatographic separation and identification of LDL-related intact cholesteryl ester hydroperoxides and secondary oxidation products have been conducted (Table 2). However, these studies have focused entirely on the acyl moiety oxidation products, determined either as individual compounds or as total cholesteryl ester hydroperoxides (tot-OOH). Thus far, only one of the developed HPLC methods has been applied in a food application: Hartvigsen et al. (2000) determined the total cholesteryl ester hydroperoxide content in fish oil-enriched mayonnaise. Thus, methods for the determination and quantification of individual intact steryl and acyl moiety oxidation products, as well as studies on the intact steryl ester oxidation products in food products, are lacking.

Intact steryl ester oxidation products may be isolated from plasma or tissue samples by rather simple extraction procedures (Table 2). However, isolation of intact steryl ester oxidation products from food matrices has not been reported previously. After isolation, the oxidation

products may be directly introduced into the HPLC. Improvements in the separation, especially in normal-phase systems, have often been achieved when hydroperoxides have been reduced to their corresponding alcohols (Kenar et al. 1996; Upston et al. 1997; Hui et al. 2000; Seal and Porter 2004). However, if the studied sample already contains these further reaction products of hydroperoxides, determination of the reduced hydroperoxides becomes more complicated.

Fractionation of the steryl and acyl moiety hydroperoxides of intact cholesteryl esters by column chromatography was reported by Hui et al. (2000). They used a silica sorbent (SiO_2) and hexane-ethyl acetate (15/1, v/v) to elute the steryl moiety hydroperoxides before the acyl moiety hydroperoxides. Only one of the LDL related cholesteryl ester studies mentioned HPLC separation of steryl moiety oxidation products from the acyl moiety oxidation products. Havrilla et al. (2000) indicated that St-7-OOH eluted between the acyl moiety hydroperoxides Fa-13-OOH and Fa-9-OOH when the hydroperoxides were separated on two silica columns (Ultrasphere 5 µm Si, 4.6 x 250 mm) connected in series and hexane/IPA (0.5%, v/v) was used as the eluent at a flow rate of 1.0 mL/min. However, this study focused on the investigation of the acyl moiety hydroperoxides.

HPLC has also been used to separate individual intact cholesteryl ester acyl moiety hydroperoxides in normal-phase mode using silica columns with hexane-based eluents (Table 2). Adequate separation of these compounds has been achieved by connecting several silica columns in series (Kenar et al. 1996; Havrilla et al. 2000; Seal and Porter 2004) or by reducing the hydroperoxides to their corresponding hydroxides (Kenar et al. 1996; Hui et al. 2000; Seal and Porter 2004). The polarity of hexane-based eluents has been modified by addition of 0.35−1% IPA. Using these methods, regioisomeric acyl moiety hydroperoxides of cholesteryl linoleate have been separated and eluted in an order of Fa-*c,t*-13-OOH, Fa-*t,t*-13-OOH, Fa-*t,c*-9-OOH and Fa-*t,t*-9-OOH.

Determination of total steryl ester hydroperoxides (i.e., determined as one peak, mainly including the acyl moiety hydroperoxides) has been successful using both normal- and reversed-phase HPLC (Table 2). The main purpose has been the separation of these hydroperoxides from unoxidised cholesteryl ester and from other lipids occurring in plasma or tissue samples. For example, triacylglycerols are very similar in polarity to steryl ester hydroperoxides. Similar to determination of individual hydroperoxides in the normal-phase mode, total steryl ester hydroperoxides can be separated from other lipids on silica columns using hexane or heptane based eluents modified with 0.05−0.4% IPA. In reversed-phase mode, mainly C8- or C18-phases have been used with mixtures of water or methanol in acetonitrile as eluents at flow rates of 1−2 mL/min. For example, the separation of different classes of lipid hydroperoxides (e.g., phospholipid hydroperoxides and cholesteryl ester hydroperoxides) in plasma samples was reported by Yasuda and Narita (1997), and Sugino (1999).

Separation of intact steryl ester secondary oxidation products

The separation of intact steryl ester acyl moiety secondary oxidation products and aldehydes typically involves reversed-phase methods using C18-columns and mixtures of acetonitrile and IPA as eluents at flow rates of 0.8−1.5 mL/min (Table 2). The aim of these methods has been the separation of the secondary oxidation products from other lipids occurring in the plasma or atherosclerotic tissue. In some studies, the separation of 7-keto-cholesteryl ester core aldehyde isomers was reported, either as such (Kamido et al. 1992a,b) or as derivatives of 1,3-cyclohexanedione (CHD) (Karten et al. 1998). One of the methods has been applied to the determination of sitosteryl ester oxides from neat samples (Julien-David et al. 2008, 2009).

Although polymerisation products of free sterols have been investigated by utilising size-exclusion chromatography and reversed-phase HPLC (Lampi et al. 2009; Rudzinska et al. 2010; Struijs et al. 2010), corresponding studies have not been conducted on steryl esters.

Detection of intact steryl ester oxidation products

Intact steryl ester hydroperoxides have mainly been detected using UV-detection at wavelengths of 210 nm and 234 nm (Table 2). The detection of compounds by UV-detectors is dependent on the chromophore sites in the molecule and their ability to absorb photons at wavelengths of UV light (200−400 nm). The response is dependent on the number of absorbing sites; for example, a molecule that has two double bonds gives a higher response than the one having only one double bond. Oxidised compounds also give higher responses than do non-oxidised ones. At 210 nm, all lipids containing a chromophore site may be detected, whereas 234 nm is a specific wavelength for conjugated double bonds. The acyl moiety hydroperoxides and hydroxides of cholesteryl linoleate have been quantified from plasma samples at levels 0.2−223 μM (LOD = 10 ng) using UV detection at $\lambda = 234$ nm (Kritharides et al. 1993; Kenar et al. 1996).

The detection sensitivity of steryl esters and their oxidation products has been improved using post-column derivatisation. The most commonly applied method was introduced by Akasaka et al. (1993, 1994), who developed a post-column method for specific analysis of cholesteryl ester hydroperoxides with diphenyl-1-pyrenil-phosphine (DPPP) reagent. DPPP reacts specifically with hydroperoxides forming a fluorescent phosphine oxide, which may be detected using fluorescence detector ($\lambda ex = 352$ nm, $\lambda em = 380$ nm) (Akasaka and Ohrui 2000). Even though the reaction is conducted under elevated temperature (80 °C), the method has been found applicable for the detection of hydroperoxides (Akasaka et al. 1994, 1999; Akasaka and Ohrui 2000; Hartvigsen et al. 2000; Säynäjoki et a. 2003). Cholesteryl ester hydroperoxides present in plasma samples have been measured at levels of 7−105 nM (Akasaka et al. 1994).

Reaction of hydroperoxides with ferrous(II)/xylenol orange reagent (FeXO) and detection of the reaction product at 592 nm has also been investigated for the detection of lipid

hydroperoxides (Sugino 1999). In this method, hydroperoxides oxidise Fe^{2+} into Fe^{3+} which then reacts with xylenol orange. This detection method, in connection with RPLC, has been used to measure hydroperoxides from plasma samples at a range from 40 pmol to 2 nmol (= 27−1362 ng).

Chemiluminescence (CL) detection methods have also been applied for the detection of cholesteryl ester hydroperoxides (Table 2). The eluting compounds react in post-column system with 4-aminophthalhydrazide (isoluminol) prepared in a mixture of borate buffer and methanol (Yamamoto et al. 1987). The amplifying isoluminol reacts with oxygenated compounds and forms an excited intermediate of aminophthalate, which emits light upon relaxation (chemiluminescence) (Pastorino et al. 2000). The emitted light is measured with chemiluminescence detector (CLD). A combination of RPLC and chemiluminescence detection resulted in a detection limit of 0.5 pmol (= 0.3 ng) and a quantification limit of 1 pmol (= 0.7 ng) for cholesteryl linoleate hydroperoxides or hydroxides in plasma samples (Hui et al. 2005). In another study, a working range from 4.8 nM to 77 nM was obtained for cholesteryl linoleate hydroperoxides found in plasma samples (Yasuda and Narita 1997). The detection of hydroperoxides with CLD was also claimed to be 50−100 times more sensitive than detection with UV at 234 nm (Kritharides et al. 1993).

Electrochemical (EC) detection has also been applied for the detection of cholesteryl ester hydroperoxides, either in reductive or in oxidative mode (Table 2). The response of these detectors is dependent on the concentration of the electrochemically active compounds. With coulometric detection, specific detection of cholesteryl linoleate hydroperoxides has been achieved at a linear range from 4 pmol to 635 pmol (= 3−432 ng) (Arai et al. 1996). A LOD of 2 pmol (= 1.4 ng) was obtained with coulometric detection, while the LOD was 14 pmol (= 9.5 ng) using amperometric detection. The difference is logical, since amperometric detection results in incomplete electrolysis (1−10%), whilst in coulometric detection electrolysis is closer to 100% complete. Hydroperoxides, which were reduced into their corresponding hydroxides, were not detected by this method. In addition, peaks that interfered with the detection of the hydroperoxides with UV (235 nm) did not appear in the chromatograms obtained with amperometric detectors.

Coordination ion spray mass spectrometry (CIS-MS) has been found applicable for the characterisation and structural analysis of intact steryl esters and their hydroperoxides (Havrilla et al. 2000; Seal and Porter 2004; Yin and Porter 2007; Yin et al. 2011). In this method, a central atom (e.g., Ag^{+}) is added to the compounds and the formed charged π-complex, called coordination compound, may be detected with MS (Bayer et al. 1999). The ionisation of this complex is achieved by efficient nebulisation and requires no electric field. Thus, the technique is very soft and therefore suitable for detection of thermolabile compounds. In the analysis of the steryl moiety hydroperoxides of cholesteryl esters, the location of the hydroperoxyl group has been indicated according to Hock-fragmentation (Havrilla et al. 2000). Added silver ion coordinates the fragmentation of the acyl moiety according to the position of the hydroperoxyl group and double bond. Electrospray ionisation

of ammoniated compounds was also found suitable for the detection of cholesteryl ester Fa-OOHs (Hutchins and Murphy 2011). By this method, dehydration could be minimized and collision induced dissociation (CID) produced specific spectra for the positional isomers of cholesteryl ester Fa-OOH.

Quantification of intact steryl ester oxidation products

In quantification, the selection of standards is critical and depends mainly on the choice of detection method. For example, in UV detection, oxidised compounds give greater responses than do the corresponding non-oxidised compounds. The number of chromophore sites (e.g., double bonds and aromatic rings) also affects the detector response. When using post-column derivatization, the analytes should react similarly with the derivatization reagent as the standards do. Since commercial standards of steryl ester oxidation products are lacking, most studies involving quantification have employed standards prepared by the scientists themselves. As these studies, by far, have focused mainly on the oxidation of the acyl moiety in steryl esters, acyl moiety oxidation products, such as Fa-13-OH of cholesteryl linoleate or linolenate, have been used as standards (Table 2). The preparation of these standards often involves oxidation of certain steryl esters by either enzymatic or chemically catalysed reactions, followed by isolation and purification of the standard compounds by extraction methods and TLC (thin-layer chromatography) or HPLC, and determination of their contents. The standards have been used either as internal standards or to generate external calibration curves.

Table 2. Analysis of intact steryl ester oxidation products by HPLC (continues).

Compounds	Matrix	Isolation	Separation	Mobile phase	Detection	Quantification	Reference
Intact acyl moiety hydroperoxides							
Cholesteryl ester Fa-OOH	Human plasma	hexane/MeOH (25:5, v/v)	2 x Ultrasphere 5 µm Si (4.6 x 250 mm, 5 µm)	hexane/IPA (99.5:0.5, v/v) 1.0 mL/min	UV (234 nm)	ISTD C18:2-13-OH LOD/LOQ *nr*	(Kenar et al. 1996)
Cholesteryl ester Fa-OOH	Neat	-	2 x Ultrasphere 5 µm Si (4.6 x 250 mm, 5 µm) 2 x Ultrasphere narrowbore 5 µm Si (2.0 x 250 mm, 5 µm)	hexane/IPA (99.5:0.5, v/v) 1.0 mL/min hexane/IPA (99.65:0.35, v/v) 0.15 mL/min	UV (234 nm) UV (234 nm) Ag^{+}−CIS-MS	-	(Havrilla et al. 2000; Seal and Porter 2004)
Cholesteryl ester Fa-OOH	Neat	SiO_2, hexane/EtAc (15/1, v/v)	Mightysil Si 60 (4.5 x 250 mm, 5 µm)	hexane/IPA (100/1 or 250/1, v/v) 1 mL/min	DAD (210, 233 nm)	-	(Hui et al. 2000)
Cholesteryl ester Fa-OOH	Neat	-	Acentis Si (2.1 x 150 mm, 5 µm)	iso-octane/MTBE (95.5:4.5–55:45, v/v) *nr* mL/min	ESI-MS (NH_4OAc)	-	(Hutchins and Murphy 2011)
Cholesteryl ester Fa-OOH	Human atheroma	iso-octane/EtAc (75:25, v/v)	Ultramex Silica (4.6 x 250 mm, 5 µm)	hexane/MTBE (95.5–55:4.5–45, v/v) 1 mL/min	ESI-MS (NH_4OAc)	ISTD Ch17:1 LOD/LOQ *nr*	(Hutchins et al. 2011)

AceAc = acetic acid, CHD = 1,3-cyclohexanedione, ChN = cholesterol nervonate (cholesteryl 15Z-tetracosenoate), CLD = chemiluminesense detector, DAD = diode-array detector, DEE = diethyl ether, DNPH = dinitrophenyl hydrazone, DPPP = diphenyl-1-pyrenylphosphine, ECD = electrochemical detector, EtAc = ethyl acetate, ExSTD = external standard method, FeXO = ferrous(II) xylenol orange, FLD = fluorescence detector, LiAce = lithium acetate, LOD = limit of detection, LOQ = limit of quantification, MeOH = methanol, MTBE = methyl-*tert*-butyl ether, NH_4Ac = ammonium acetate, OOH = hydroperoxide, *nr* = not reported, THF = tetrahydrofuran

Table 2.(continued) Analysis of intact steryl ester oxidation products by HPLC (continues).

Compounds	Matrix	Isolation	Separation	Mobile phase	Detection	Quantification	References
Intact total hydroperoxides							
Cholesteryl ester tot-OOH	Plasma LDL, intima	$NaBH_4$ → hexane/MeOH (5:1, v/v)	SupelcoSil (4.6 x 250 mm, 5 µm)	heptane/DEE/IPA (100:0.5:0.175, v/v/v) 2 mL/min	UV (234, 270 nm)	ExSTD Ch18:2-OH LOD/LOQ *nr*	(Suarna et al. 1995; Upston et al. 1996, 1997)
Cholesteryl ester tot-OOH	Human plasma	hexane/MeOH (7:1, v/v) → SPE (NH_2)	SupelcoSil (4.6 x 250 mm, 5 µm)	hexane/IPA (1000:5, v/v) 1.0 mL/min	UV (234 nm)	ISTD Ch18:3-OH LOD/LOQ *nr*	(Mashima et al. 2000)
Cholesteryl ester tot-OOH	Plasma HDL	hexane/MeOH (2:1, v/v)	Develosil 60-3 (4.6 x 100 mm, *nr* µm)	A: hexane B: hexane/butanol (500:25, v/v) A/B: 92:8−10:90, v/v 0.6 mL/min	UV (268 nm) FLD (DPPP) (λ_{ex} = 352 nm, λ_{em} = 380 nm)	ISTD Ch18:2-OH LOD/LOQ *nr*	(Akasaka et al. 1994)
Cholesteryl ester tot-OOH	Human plasma	hexane/MeOH (10:1, v/v) → SiO_2, hexane/EtAc (6:1, v/v)	Mightysil Si 60 (4.6 x 250 mm, 5 µm) Inertsil ODS-2 (4.6 x 250 mm, 5 µm)	hexane/IPA (250:1, v/v) 1 mL/min $EtOH/H_2O$ (50:1, v/v) 0.5 mL/min	UV (210 nm) CLD (luminol)	ISTD Fa-OOH-Ch-(24-14:0) -18:1 LOD 0.5 pmol LOQ 1 pmol	(Hui et al. 2005)
Cholesteryl ester tot-OOH	Human plasma	*n*-hexane/EtAc (1:1, v/v)	LiChrospher 100 NH_2 (4 x 250 mm, 5 µm) + 2xLiChrospher 100 RP-8 (4 x 75 mm, 5 µm)	CH_3CN/MeOH /H_2O/AceAc (65:30:5:0.1, v/v/v/v) *nr* mL/min	CLD (isoluminol)	ExSTD Ch18:2-OH LOQ 4.8 nM	(Yamamoto et al. 1987; Yamamoto and Niki 1989; Upston et al. 1997; Mashima et al 2000)

AceAc = acetic acid, CHD = 1,3-cyclohexanedione, ChN = cholesterol nervonate (cholesteryl 15Z-tetracosenoate), CLD = chemiluminesense detector, DAD = diode-array detector, DEE = diethyl ether, DNPH = dinitrophenyl hydrazone, DPPP = diphenyl-1-pyrenylphosphine, ECD = electrochemical detector, EtAc = ethyl acetate, ExSTD = external standard method, FeXO = ferrous(II) xylenol orange, FLD = fluorescence detector, LiAce = lithium acetate, LOD = limit of detection, LOQ = limit of quantification, MeOH = methanol, MTBE = methyl-*tert*-butyl ether, NH_4Ac = ammonium acetate, OOH = hydroperoxide, *nr* = not reported, THF = tetrahydrofuran

Table 2.(continued) Analysis of intact steryl ester oxidation products by HPLC (continues).

Compounds	Matrix	Isolation	Separation	Mobile phase	Detection	Quantification	References
Cholesteryl ester tot-Fa-OOH	Human plasma	EtAc/hexane (1:1, v/v)	LiChrospher 100 NH_2 (4 x 250 mm, 5 µm) + 2xLiChrospher 100 RP-8 (4 x 75 mm, 5 µm)	$CH_3CN/MeOH/H_2O/AceAc$ (65:30:5:0.01, v/v) *nr* mL/min	CLD (isoluminol)	STD *nr* LOD/LOQ *nr*	(Yasuda and Narita 1997)
Cholesteryl ester tot-Fa-OOH + oxides	Plasma LDL	hexane/MeOH (10:2.5, v/v)	Supelco C-18 (4.6 x 250 mm, 5 µm)	$CH_3CN/IPA/H_2O$ (44:54:2, v/v/v) *nr* mL/min	UV (210, 234 nm) CLD (isoluminol) MS (CI)	STD *nr* ExSTD LOD 10 ng	(Kritharides et al. 1993; Brown et al. 1996; Christison et al. 1996; Noguchi et al. 1998)
Cholesteryl ester tot-Fa-OOH	Neat	-	Ultrasphere C18 (4.6 x 150 mm, 5 µm)	I: $MeOH/CH_3CN/H_2O$ (81:10.5:8.5, v/v/v), II: $IPA/CH_3CN/MeOH/H_2O$ (62:24:9.2:4.8, v/v/v/v), III: $MeOH:CH_3CN:H_2O$ (50:22.5:27.5, v/v/v) 1.0−2.0 mL/min	ECD	STD *nr* LOD 0.1 pmol	(Korytowski et al. 1995)
Cholesteryl ester tot-Fa-OOH	Neat Human plasma	SiO_2, hexane/$CHCl_3$ (1:0, 2:1, 0:1, v/v) hexane/MeOH (1:1, v/v)	TSKgel Octyl-80Ts (4.6 x 150 mm, 5 µm)	$MeOH/H_2O/AceAc$ (97:3:0.1, v/v/v) + 50 mM LiAce 1 mL/min	UV (235 nm) ECD	ISTD ChN-OOH LOD 14 pmol	(Arai et al. 1996)
Cholesteryl ester tot-Fa-OOH	Plasma LDL	hexane/MeOH (10:2.5, v/v)	LiChrospher 100 RP-18 (*nr*)	$CH_3CN/IPA/H_2O$ (44:54:2, v/v/v) 1 mL/min	UV (210, 234 nm)	STD *nr* LOD/LOQ *nr*	(Vieira et al. 1998)

AceAc = acetic acid, CHD = 1,3-cyclohexanedione, ChN = cholesterol nervonate (cholesteryl 15Z-tetracosenoate), CLD = chemiluminesense detector, DAD = diode-array detector, DEE = diethyl ether, DNPH = dinitrophenyl hydrazone, DPPP = diphenyl-1-pyrenylphosphine, ECD = electrochemical detector, EtAc = ethyl acetate, ExSTD = external standard method, FeXO = ferrous(II) xylenol orange, FLD = fluorescence detector, LiAce = lithium acetate, LOD = limit of detection, LOQ = limit of quantification, MeOH = methanol, MTBE = methyl-*tert*-butyl ether, NH_4Ac = ammonium acetate, OOH = hydroperoxide, *nr* = not reported, THF = tetrahydrofuran

Table 2.(continued) Analysis of intact steryl ester oxidation products by HPLC (continues).

Compounds	Matrix	Isolation	Separation	Mobile phase	Detection	Quantification	References
Cholesteryl ester tot-Fa-OOH	Neat	-	Develosil ODS HG-5 (4.6 x 100 mm, 5 µm)	A: MeOH/H_2O (70:30, v/v), B: MeOH/IPA (40:60, v/v) A/B: 99:1−15:85, v/v 0.8 mL/min	FLD (DPPP) (λ_{ex} = 352 nm, λ_{em} = 380 nm)	STD *nr* LOD 1.5−3.0 pmol	(Akasaka et al. 1999)
			Develosil ODS HG-5 (2.0 x 100 mm, 5 µm)	A: MeOH/H_2O (70:30, v/v), B: MeOH/IPA (40:60, v/v) A/B: 100:0−17:83, v/v 0.2 mL/min			
Cholesteryl ester tot-Fa-OOH	Neat Plasma	- $CHCl_3$/MeOH/ acetone (1:4:5, v/v/v)	Asahipak C8P50 (4.6 x 150 mm, 5 µm)	A: H_2O/CH_3CN/MeOH (45:35:20, v/v/v), B: CH_3CN:MeOH (80:20, v/v) A/B: 90:10−10:90, v/v 0.8 mL/min	UV (FeXO) (592 nm)	STD *nr* LOD 10 pmol	(Sugino 1999)
Cholesteryl ester tot-OOH	Plasma LDL, aorta tissue	hexane/MeOH (4:1, v/v)	Vydac 201TP C18 (2.1 x 250 mm, 5 µm)	A: H_2O/THF (50:50, v/v) + NH_4Ac B: THF A/B: 100:0−55:45, v/v 0.3 mL/min	MS (CI)	-	(Harkewicz et al. 2008)
Cholesteryl ester tot-OOH	Plasma LDL	hexane	C18 (4.5 x 250 mm, *nr* µm) + C8 (4.6 x 150 mm, *nr* µm)	MeOH:NH_4Ac (94:6, v/v) 1.2 mL/min	CLD (isoluminol)	ExSTD Ch 18:2-OOH LOD 30 pmol	(Marchetti et al. 2011)

AceAc = acetic acid, CHD = 1,3-cyclohexanedione, ChN = cholesterol nervonate (cholesteryl 15Z-tetracosenoate), CLD = chemiluminesense detector, DAD = diode-array detector, DEE = diethyl ether, DNPH = dinitrophenyl hydrazone, DPPP = diphenyl-1-pyrenylphosphine, ECD = electrochemical detector, EtAc = ethyl acetate, ExSTD = external standard method, FeXO = ferrous(II) xylenol orange, FLD = fluorescence detector, LiAce = lithium acetate, LOD = limit of detection, LOQ = limit of quantification, MeOH = methanol, MTBE = methyl-*tert*-butyl ether, NH_4Ac = ammonium acetate, OOH = hydroperoxide, *nr* = not reported, THF = tetrahydrofuran

Table 2.(continued) Analysis of intact steryl ester oxidation products by HPLC.

Compounds	Matrix	Isolation	Separation	Mobile phase	Detection	Quantification	References
Cholesteryl ester tot-OOH	Fish oil enriched mayonnaise	CH_2Cl_2	PLgel 2 x 100 Å + 1 x 50 Å (7.5 x 300 mm, 5 µm)	CH_2Cl_2 0.6 mL/min	FLD (DPPP) (λ_{ex} = 352 nm, λ_{em} = 380 nm)	ExSTD TAG 18:2-OOH LOD 5.0 pmol LOQ 40 pmol	(Hartvigsen et al. 2000)
Intact secondary oxidation products							
Cholesteryl ester oxides	Human plasma, atheros-clerotic tissue	$CHCl_3$/MeOH (1:1, v/v) → TLC → DNPH	Supelcosil LC-18 (4.6 x 250 mm, 5 µm) Hewlett Packard C-18 (2.1 x 100 mm, *nr* µm)	MeOH/IPA (80−20:20−80, v/v) 0.8 mL/min A: MeOH/H_2O/NH_4OH (80:12:0.5, v/v/v) B: MeOH/hexane/NH_4OH (80:12:0.5, v/v) A/B: 100:0−0:100, v/v	ESI-MS	-	(Hoppe et al. 1997)
Sitosteryl ester oxides	Neat	-	Polaris C8-A (4.6 x 250 mm, 5 µm)	CH_3CN/H_2O (95:5, v/v) 1 mL/min	ELSD	-	(Julien-David et al. 2008, 2009)
Cholesteryl ester aldehydes	Plasma LDL	$CHCl_3$/MeOH (2:1, v/v) → TLC → DNPH	Supelcosil LC-18 (4.6 x 250 mm, 5 µm)	I: CH_3CN/IPA (4:1, v/v) II: CH_3CN/C_3H_5N (70−10:30−90, v/v) 1.0−1.5 mL/min	UV (358 nm) MS (CI)	-	(Kamido et al. 1992a,b)
Cholesteryl ester aldehydes	Plasma atheros-clerotic tissue	$CHCl_3$/MeOH (2:1, v/v) → CHD → SPE (C-18)	Ultrasphere ODS (4.6 x 75 mm, 3 µm)	CH_3CN/MeOH/IPA (68:17:15, v/v/v) 1 mL/min	FLD (CHD) λ_{ex} = 366 nm λ_{em} = 455 nm	ISTD Ch 9:9-O LOD 15 fmol	(Karten et al. 1998, 1999)

AceAc = acetic acid, CHD = 1,3-cyclohexanedione, ChN = cholesterol nervonate (cholesteryl 15Z-tetracosenoate), CLD = chemiluminesense detector, DAD = diode-array detector, DEE = diethyl ether, DNPH = dinitrophenyl hydrazone, DPPP = diphenyl-1-pyrenylphosphine, ECD = electrochemical detector, EtAc = ethyl acetate, ExSTD = external standard method, FeXO = ferrous(II) xylenol orange, FLD = fluorescence detector, LiAce = lithium acetate, LOD = limit of detection, LOQ = limit of quantification, MeOH = methanol, MTBE = methyl-*tert*-butyl ether, NH_4Ac = ammonium acetate, OOH = hydroperoxide, *nr* = not reported, THF = tetrahydrofuran

2.4 Biological effects of plant sterol oxidation products

Oxidation products of cholesterol can be cytotoxic, atherogenic, mutagenic and carcinogenic (Garcia-Cruset et al. 2002; Osada 2002; Olkkonen et al. 2012). They may also inhibit the biosynthesis and membrane function of cholesterol (Rozner and Garti 2006; Olkkonen et al. 2012). Due to their structural differences, cholesterol and plant sterols have been found to differ in their absorption by the human intestine: While about 60% of consumed cholesterol may be absorbed, less than 2% of the plant sterols have been found to be absorbed (Ostlund 2007). The reduced absorptivity may also reflect the absorptivity of the corresponding oxidation products. The incorporation of POPs into animal tissues has been found to be dose dependent (Grandgirard 2002; Hovenkamp et al. 2008; Ryan et al. 2009). In the case of single free plant sterol oxides, these can be absorbed at different affinities depending on their structure. Koschutnig et al. (2009) observed that human hepatoma cells (HepG2) absorbed more 7-ketone of sitosterol than 7α-OH. In addition, equal amounts of 7β-OH were found inside the cells and in the surrounding serum media.

In vitro studies have demonstrated that higher doses of plant sterol oxidation products are needed, compared to their cholesterol analogues, to produce diverse biological effects (Hovenkamp et al. 2008; Ryan et al. 2009; García-Llatas and Rodriguez-Estrada 2011). The sitosterol oxides 7α-OH and 7-ketone (30−120 μM) were found to be toxic in the human hepatoma cellular assays, whereas the cytotoxicity of 7β-OH was less definite (Koschutnig et al. 2009). In addition, 7-ketone showed a mutagenic tendency (Koschutnig et al. 2010). The stigma- and sitosterol 5,6α- and 5,6β-epoxides, were found to increase mortality in mealworms (Tenebrio molitor L.) (Meyer et al. 1998) and both epimers had similar effects. The degradation product of these epoxides (i.e., 3,5,6-triol) showed the highest death rates. Conflicting reports of the cytotoxicity and mutagenicity of cholesterol epoxides have appeared (Sevanian and Peterson 1986; Cheng et al. 2005). However, corresponding studies on the plant sterol epoxides have not been reported. Studies on the biological effects of plant steryl ester oxidation products are also lacking.

Due to the conflicting toxicity data, toxicity limits and safe levels of exposure have not been addressed for oxidation products of cholesterol and plant sterols. Threshold of toxicological concern (TCC) may be applied for evaluation of safe exposure levels of chemicals that are present in food products at very low contents (Kroes et al. 2004). These levels may be discovered even without the experimental data on the toxicity by utilising the knowledge on the chemical structures and on the estimated daily intakes. According to the TCC principle, the lowest limit for daily dietary consumption of unclassified compounds is 0.15 μg per day.

2.5 Occurrence of plant sterol oxidation products in food

The content of plant sterols, and thus also the content of their oxidation products, is highly related to the fat content of a food product. Vegetable oils contain 60−1100 mg/100 g of plant sterols, nuts 55−160 g/100 g, cereals 50−180 g/100 g and vegetables 5−40 g/100 g (Piironen and Lampi 2004). The contents of plant sterol oxidation products (POPs) in food products are rather low (Table 3). In vegetable oils, 80−6750 µg/100 g of POPs has been measured (Table 3). Food processing conditions may also alter the POP contents. For example, a POP range of 10−1989 µg/100 g has been found in deep-fried French fries, depending on the frying conditions and the cooking oil (Table 3). Consuming plant sterol enriched food products at a daily intake of 2−4 grams of plant sterols may lead to accumulation of 2−4 mg of POPs (Ryan et al. 2009).

Reports on the POP contents in commercial sterol preparations are scarce. Gonzalez-Larena et al. (2011) reported certain preparations containing 13−86% of sterols to include 1.7−27 µg/100 g of POPs. Dutta (1999), on the other hand, reported contents as high as 1000−6000 µg/100 g in plant sterol preparations and in tablet supplements. If the preparations themselves already contain POPs, they are transferred to the food products and may even induce further oxidation in the food processing and storage. Therefore, it is important to regulate the initial content of POPs in preparations that are to be used for supplementation. In plant sterol enriched (6−8% sterols) margarines and spreads, 1200−6800 µg/100 g of POPs have been measured whereas in non-supplemented spreads POP content can be as high as 1330−7400 µg/100 g (Table 3). However, no guidelines or recommendations on the consumption of sterol oxides have yet been proposed.

Table 3. Contents of plant sterol secondary oxidation products (POPs) in foods (continues).

Food product	Fat content [g/100 g]†	Sterol content [g/100 g]†	POP content [µg/100 g]‡	Reference
Sterol preparations	*nr*	13−86	1.7−27	(González-Larena et al. 2011)
	nr	*nr*	1000−6000	(Dutta 1999)
Enriched foods				
milk	18	0.4	210	(Menéndez-Carreño et al. 2008)
non-fat milk	*nr*	0.4−0.5	202−218	(Soupas et al. 2006)
whole milk powder	*nr*	7	1400	(Soupas et al. 2006)
spread	34−44	6−8	1200−6800	(Grandgirard et al. 2004; Conchillo et al. 2005; Johnsson and Dutta 2006)
butter oil	*nr*	8	2020−3390	(Soupas et al. 2007)
liquid margarine	80	8	2280−3960	(Soupas et al. 2007)

† expressed as g/100 g of product, ‡ expressed as µg/100 g of product, * expressed as 7-ketone derivative, ** expressed as sitosterol, ¤ expressed as sitosterol oxides, *nr* = not reported

Table 3. (continued) Conents of plant sterol secondary oxidation products (POPs) in foods.

Food product	Fat content [g/100 g][†]	Sterol content [g/100 g][†]	POP content [μg/100 g][‡]	Reference
Deep fried foods				
Potato crisps	15−38	0.04−0.25	50−1158	(Dutta and Appelqvist 1996, 1997; Tabee et al. 2008a)
French fries				
commercial	0−8	*nr*	10−210	(Lee et al. 1985;
restaurant	14−19	*nr*	51−1989	Tabee et al. 2008b)
Bakery products				
Biscuit	8.9	0.02	166[*]	(Cercaci et al. 2006)
Cake	18	0.03	173[*]	(Cercaci et al. 2006)
Roll	19	0.06	189[*]	(Cercaci et al. 2006)
Pastry	40	0.03	286[*]	(Cercaci et al. 2006)
Other food products				
Infant milk cereal	6.5−15	0.01−0.02[**]	6.6−45[*]	(Zunin et al. 1998)
Wheat flour	1.4	*nr*	49[¤]	(Nourooz-Zadeh and Appelqvist 1992)
Infant foods	2.6	0.01	64−70	(García-Llatas et al. 2008)
Infant milk formula	21−30	0.01−0.03[**]	15−116[*]	(Zunin et al. 1998)
Peanuts	*nr*	0.47	4900	(Rudzińska et al. 2005)
Margarine	*nr*	0.68	7400	(Rudzińska et al. 2005)
	63	0.33	1331	(Conchillo et al. 2005)
Vegetable oils				
Soybean	*nr*	0.17	0−80	(Nourooz-Zadeh and Appelqvist 1992; Zhang et al. 2006)
Olive	*nr*	0.1−0.26	0−770	(D'Evoli et al. 2006; Johnsson and Dutta 2006; Zhang et al. 2005, 2006)
Maize	*nr*	0.26	430	(Johnsson and Dutta 2006)
Palm	*nr*	0.11	550	(Bortolomeazzi et al. 2003)
Peanut	*nr*	0.21	260−710	(Bortolomeazzi et al. 2003; Johnsson and Dutta 2006)
Rapeseed	*nr*	0.35−0.79	190−800	(Rudzińska et al. 2005; Zhang et al. 2006; Soupas et al. 2007)
Lampante olive	*nr*	*nr*	1500−2500	(Bortolomeazzi et al. 2003)
Rapeseed/palm blend	*nr*	0.53	4100	(Dutta and Appelqvist 1996; Dutta 1997)
High-oleic sunflower	*nr*	0.44−0.55	4670	(Dutta and Appelqvist 1996; Dutta 1997)
Corn	*nr*	0.77−0.99	410−6010	(Bortolomeazzi et al. 2003; Rudzińska et al. 2005)
Sunflower	*nr*	0.23−0.71	600−6750	(Dutta and Appelqvist 1996; Dutta 1997; Bortolomeazzi et al. 2003; Zhang et al. 2005, 2006)

[†] expressed as g/100 g of product, [‡] expressed as μg/100 g of product, [*] expressed as 7-ketone derivative, [**] expressed as sitosterol, [¤] expressed as sitosterol oxides, *nr* = not reported

3 AIMS OF THE STUDY

The overall aim was to study the oxidation behaviour of steryl esters. In order to follow the oxidation, isolation and separation methods for the analysis of intact steryl ester monohydroperoxides had to be developed. The methods had to be sufficiently efficient to distinguish between the oxidation products of steryl and acyl moieties in an intact ester molecule. The effects of esterification on the formation and further reactions of steryl ester hydroperoxides were studied using the developed methods. The roles of unsaturation degree of the acyl moiety, sterol structure, temperature and lipid medium were examined with respect to the primary oxidation and further reactions. Cholesteryl esters with varying unsaturation of the acyl moiety were used as model compounds. The oxidation of cholesteryl esters was compared to that of free cholesterol. The oxidation reactions were studied by following the formation and decomposition of intact steryl ester monohydroperoxides.

Detailed objectives were:

1. To develop isolation and separation methods for the determination of intact steryl ester monohydroperoxides (I−II).

2. To investigate the effects of chemical factors (i.e., esterification, unsaturation degree of the acyl moiety and sterol structure) on the oxidation of sterol (I−IV).

3. To investigate the effects of external factors (i.e., temperature and medium) on the oxidation of sterol (III−IV).

4 MATERIALS AND METHODS

This section summarises the materials and methods used in this study. More detailed information may be gained from the original papers (I−IV).

4.1 Materials

4.1.1 Reagents

Since single compound plant steryl esters (i.e., having only one type of steryl moieties or/and one type of acyl moieties) were not commercially available, cholesteryl esters were used as model compounds in the oxidation studies. Cholesteryl stearate (cholest-5-en-3β-yl octadecanoate; ≥ 99%), cholestryl oleate (cholest-5-en-3β-yl 9-octadecenoate; ≥ 99%) and cholesteryl linoleate (cholest-5-en-3β-yl 9,12-octadecadienoate; ≥ 99%) used in the oxidation studies were purchased from Nu-Chek Prep (Elysian, MN, USA) (studies I−IV). Tripalmitin (1,3-di(hexadecanoyloxy)propan-2-yl hexadecanoate; ≥ 85%) and free cholesterol (cholest-5-en-3β-ol; ≥ 99%) were acquired from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) (IV). Stigmasteryl oleate (24S-ethylcholest-5,22-dien-3β-yl 9-octadecenoate) was synthesised enzymatically using stigmasterol (24S-ethylcholest-5,22-dien-3β-ol; ≥ 95%; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), oleic acid (≥ 99%; Nu-Chek Prep, Elysian, MN, USA) and lipase (Candida rugosa, type VII, ≥ 700 unit/mg solid; Sigma-Aldrich Chemie GmbH) (IV).

4.1.2 Synthesis of plant steryl esters

Stigmasteryl oleate was synthesised by enzyme-catalysed esterification according to Kim and Akoh (2007), after scaling up the method (IV). A mixture of 50 mg of stigmasterol, 72 mg of oleic acid and 8.8 mg of lipase (Candida rugosa, type VII, ≥ 700 unit/mg solid) in 1.5 mL of heptane was maintained at 50 °C for 17 hours with gentle shaking. The formed steryl esters were extracted with diethyl ether and purified by SPE. The final yield of stigmasteryl oleate was 38% and purity was 96%.

4.1.3 Purification of steryl esters and tripalmitin

Because oxidation reactions tend to be easily affected by catalysts such as metal residues present in preparations, a solid-phase extraction method was developed for the purification of the preparations (I−II). 100 mg of steryl ester was purified on pre-conditioned (2 x 20 mL heptane) 5 g SiOH-SPE cartridge (Strata SI-1 Silica, Phenomenex, Inc.; Torrance, CA, USA) using 2 x 20 mL of heptane. The ester was collected with 2 x 20 mL of heptane/MTBE (98:2, v/v), after which the solvent was evaporated and the preparation was re-dissolved in heptane

to be stored at −20 °C for further use. Each of the steryl ester preparations were purified by the SPE method before oxidation experiments (I−IV).

Tripalmitin was purified by adsorption chromatography according to a previously described method (Lampi et al. 1999), after modification of the solvent (IV). 100 g of tripalmitin was passed through a glass column packed with 250 g of activated aluminium oxide (Al_2O_3) using 200 mL of heptane/dichloromethane (1:1, v/v).

The purities of steryl ester preparations and tripalmitin were assured by GC-FID and GC-MS (Lampi et al. 2002; Soupas et al. 2005) (I−IV). The lipid profiles were examined by TLC and the presence of tocopherols were monitored by HPLC-FLD (Ryynänen et al. 2004). No other lipids, oxidation products or tocopherols were detected in the steryl ester preparations or tripalmitin by the used methods.

4.2 Oxidation models

In order to obtain and understand how the oxidation of steryl esters occurs, the studies were started by following the oxidation in neat preparations. The sample size was remained small in order to simulate interfacial oxidation. Tripalmitin was chosen to represent a simple lipid medium, which is not oxidising itself, but which allows the oxidation behaviour of steryl esters to be followed independently. Moreover, steryl esters are considered to localize within the lipid medium in food products.

4.3 Oxidation experiments

Oxidation of cholesteryl stearate, oleate and linoleate, stigmasteryl oleate and free cholesterol were carried out in neat preparations (I−III) and in a lipid model (IV). For the autoxidation of neat preparations, 10 mg of steryl ester or free sterol was placed in a glass vial (I−III). For the oxidation of steryl esters and free sterol in a lipid medium, 92 mg of tripalmitin and 12 mg of steryl ester (corresponding to a sterol equivalent of 8%) or 8 mg of free cholesterol were mixed and placed in a glass vial (22 mm x 46 mm) (IV). The solvent was evaporated gently under N_2 stream (+30 °C) in order to create a uniform sample film. The samples were maintained in open vials in the dark at 100 ºC (I−IV) or at 140 ºC (III) for 0−5 days. After the oxidation, the samples were cooled in a desiccator and dissolved either in 2 mL of heptane/MTBE (80:20, v/v) (I−II), in 5 mL of THF (III) or in 5 mL of heptane/dichloromethane (1:1, v/v) (IV) for further analysis. For each time point, three replicate samples were oxidised and the experiment was repeated two times (n = 6).

4.4 Analysis of steryl ester oxidation products

4.4.1 Primary oxidation products

Isolation of intact steryl ester oxidation products by solid-phase extraction

Two different solid-phase extraction (SPE) methods were developed for the isolation of steryl ester oxidation products. In order to achieve the separation of steryl ester hydroperoxides in HPLC, the intact oxidation products had to be segregated from the unoxidised steryl ester (I−II). 5−10 mg of oxidised steryl ester preparation was introduced to a pre-conditioned (2 x 2.5 mL of heptane) 500 mg SiOH-SPE cartridge (Strata SI-1 Silica, Phenomenex, Inc.; Torrance, CA, USA) in 1 mL of heptane/IPA (98:2, v/v). Unoxidised material was eluted with 2 x 2.5 mL of heptane/MTBE (98:2, v/v), after which the oxidation products were collected with 2 x 2.5 mL of heptane/MTBE (80:20, v/v). The remaining solvent was evaporated and depending on the further HPLC analysis, the residue was dissolved either in 2 mL of heptane/MTBE (90:10, v/v) (I) or in 2 mL of heptane/IPA (98:2, v/v) (II).

Another SPE method was developed for the isolation of intact steryl ester oxidation products from a lipid matrix (IV). Silica (SiOH) and aluminium oxide (Al_2O_3) sorbents were evaluated for the isolation purposes. Since the hydroperoxides were reduced to corresponding hydroxides in the Al_2O_3, a SiOH-sorbent was chosen for further development. Varying

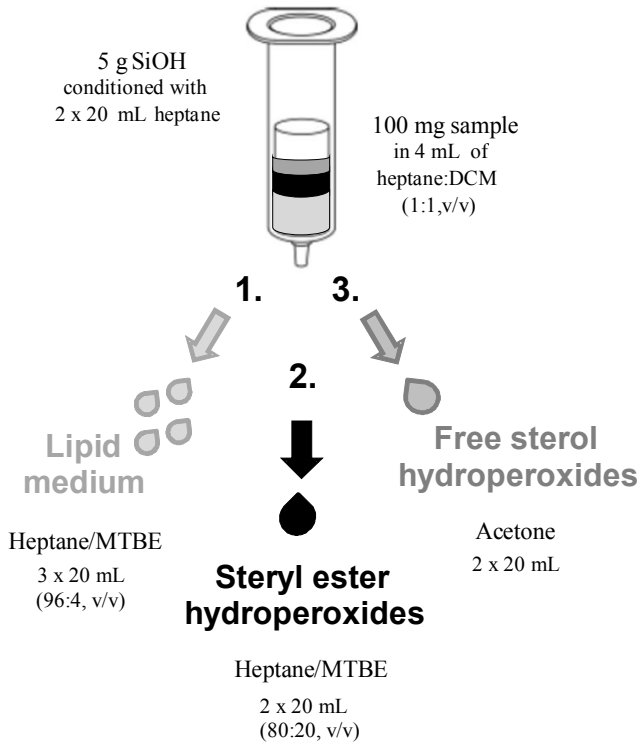

Figure 4. Isolation of steryl ester and free sterol oxidation products from lipid medium by SiOH-SPE (IV).

volumes (1−4 x 20 mL) of heptane/MTBE (0−20% of MTBE) and heptane/EtAc (0−20% of EtAc) were evaluated for the fractionation of different compounds present in the oxidised sample. In the final method (
Figure 4), 80−100 mg of the oxidised mixture of steryl ester and tripalmitin was loaded on a pre-conditioned (2 x 20 mL heptane) 5 g SiOH-SPE cartridge (Strata SI-1 Silica) in 4−5 mL of heptane/dichloromethane (1:1, v/v). Tripalmitin was eluted with 3 x 20 mL of heptane/MTBE (96:4, v/v), after which intact steryl ester hydroperoxides or free sterol hydroperoxides were collected with 2 x 20 mL of heptane/MTBE (80:20, v/v) or acetone, respectively. The remaining solvent was evaporated and the residue was dissolved in 2 mL of heptane/IPA (98:2, v/v) for immediate analysis with HPLC-DAD-ELSD.

The efficiencies of the isolation methods were evaluated by TLC and HPLC-DAD-ELSD (I−II, IV). The presence of different compounds in the collected fractions was evaluated qualitatively. Recovery efficiency was determined for the method where steryl ester hydroperoxides were isolated from a lipid matrix (IV).

Determination of intact steryl ester and free sterol monohydroperoxides by HPLC-DAD-ELSD

In order to distinguish between the oxidation of steryl and acyl moieties in intact steryl ester molecules, an efficient HPLC method for the separation of steryl and acyl moiety oxidation products had to be developed.

Separation of steryl moiety hydroperoxides of cholesteryl stearate was achieved by silica column (Supelcosil, 250 x 2.1 mm i.d., particle size 5 µm; Supelco, Bellefonte, PA, USA) using gradient elution with MTBE in heptane (0.5−10%) (I). However, this method was unable to resolve steryl and acyl moiety hydroperoxides of cholesteryl oleate and linoleate. Therefore, the method was further developed (II). Increasing the polarity of the stationary phase resulted in separation of St-OOHs and Fa-OOHs. Using a neutral alumina column (125 x 4.0 mm i.d., particle size 5 µm; Merck, Darmstadt, Germany) with a gradient elution of IPA in heptane (0−2% + 0.5% THF) at a flow rate of 1.0 mL/min, St-OOHs eluted before the Fa-OOHs, and isomers and epimers of St-OOHs and of Fa-OOHs could be separated as well.

Detection of all of the compounds present in the sample was achieved with diode-array detector (DAD) at 206 nm and with evaporative light-scattering detector (ELSD) (I−IV). Conjugated hydroperoxides of cholesteryl linoleate (i.e., Fa-9-OOH and Fa-13-OOH) could be indicated with DAD at 234 nm. Quantification of the steryl ester hydroperoxides was performed by external standard method. Unoxidised steryl esters were used as standards. Quantification was performed assuming equal responses of oxidised and unoxidised compounds at ELSD.

Free sterol monohydroperoxides were determined by a previously described HPLC-DAD method (Säynäjoki et al. 2003; Kemmo et al. 2005), after some modification (I, IV). The

separation was performed on a silica column using a linear gradient of IPA in heptane (2−5%) at a flow rate of 0.6 mL/min. The eluting compounds were detected at a wavelength of 206 nm. Quantification of the free sterol hydroperoxides was performed by external standard method. Unoxidised cholesterol was used as a standard at a range of 300−10500 ng/injection (R^2 = 0.991−0.998).

Characterisation of steryl ester hydroperoxides by Ag^+−CIS-MS

The elution order of cholesteryl ester hydroperoxides in the alumina column was confirmed by coordination ion spray mass spectrometry (CIS-MS) (II). The MS parameters were optimised for both steryl and acyl moiety hydroperoxides using steryl ester hydroperoxides that were isolated and fractionated from oxidised cholesteryl ester preparations by SPE and HPLC. To confirm the elution order, the flow from the HPLC was split (1:10, v/v) and mixed with a flow of 203 nM $AgBF_4$ solution (500 L/min). Each sample was run with MS-parameters optimised for the steryl moiety hydroperoxides and with parameters optimised for the acyl moiety hydroperoxides. The MS was performed in the positive ion mode. Both scan and MS/MS runs with a mass range of 100−1000 m/z were performed.

Peroxide values

The total content of hydroperoxides was measured by the peroxide value (PV) (I−II, IV). Measurements were performed according to a ferric thiocyanate method (Mortensen et al. 2002), after some modifications. Oxidised samples were diluted in methanol/decanol/heptane (3:2:1, v/v/v) and the absorbance of ferric thiocyanate complex formed during a 5-min reaction period was measured at 500 nm.

4.4.2 Secondary oxidation products of sterol

The secondary oxidation products of sterol (e.g., hydroxides, epoxides and ketones) were measured to monitor the decomposition of hydroperoxides into further products (I−II, IV) and to evaluate the stage of oxidation (III). The analysis was performed according to Lampi et al. (2002) and Soupas et al. (2004), after some modifications.

Sample treatment

1−100 µg of internal standard (19-OH-cholesterol) was added to 2−10 mg of oxidised steryl ester or free sterol, or to 37 mg of oxidised tripalmitin enriched with steryl esters or free sterol. The samples were cold saponified at room temperature with saturated KOH in ethanol. The unsaponifiables were extracted with diethyl ether and the extracts were washed (aqueous KOH and Na_2SO_4). The unsaponifiables were further purified and fractionated by SiOH-SPE. The collected oxides were derivatised into TMS ethers for the analysis with GC-FID and GC-MS.

Determination of sterol oxides by GC-FID and GC-MS

The secondary oxidation products of sterol were quantified as TMS ethers with GC-FID. The compounds were separated on a Rtx-5MS w/Integra-Guard fused-silica capillary column (60 m x 0.32 mm i.d., 0.1 µm film; Restek Corporation, Bellefonte, PA, USA) using a temperature program (70−275 °C) and helium as a carrier gas (1.4 mL/min). Quantification was based on the FID response using an internal standard method and assuming equal responses for the internal standard (19-OH-cholesterol) and the quantified oxides.

The elution order of sterol oxides was confirmed by GC-MS. The same conditions and column with a 0.25 µm diameter were used. The MS was run on a full-scan mode at an m/z-range of 100−600 amu. The mass spectra of analysed sterol oxides were compared to those of the cholesterol oxide standard compounds. The epimers could be differentiated by their retention times and identification was confirmed by the main fragments for the TMS ethers.

4.4.3 Further reaction products

The oxidation of steryl esters in neat preparations and in tripalmitin at 100 °C and 140 °C were also followed in terms of polymerisation.

Determination of polymerised material by HPSEC-RI

The contents of oligomers were determined by high-performance size-exclusion chromatography and refractive index detection (HPSEC-RI), as described previously (Lampi and Kamal-Eldin 1998) (III−IV). The compounds were separated based on their size using one 100-Å and two 50-Å PLGel columns (5 µm, 300 mm x 7.5 mm i.d.; Polymer Laboratories Inc., MA, USA) connected in series and a 0.6 mL/min flow of THF (incl. 0.025% BHT). The eluting peaks were monitored with a refractive index detector. Quantification was performed using an external standard method. Linear standard curves at a range of 0.16−100 µg/injection (R^2 = 0.999−1.000) were created with monomeric cholesteryl stearate or free cholesterol. Equal responses were assumed for the oligomers and monomers.

Characterisation of steryl ester oligomers by Ag^+−CIS-MS

Ag^+–CIS-MS (III) was used to indicate the presence of oligomers and their molecular sizes in oxidised steryl ester preparations. The MS parameters were tuned by a mixture of oxidised sample (4 mg/mL) and $AgBF_4$ (60 µM) solution (1:1, v/v). The samples were investigated as mixtures using direct liquid injection.

4.5 Data analysis

The contents were expressed as mg of oxidation product per one gram of original steryl ester or free sterol (mg/g). The average and standard deviation of each content was calculated across all of the replicates ($n = 6$). One-way ANOVA and post-hoc tests (Tukey) were performed to examine the effects of esterification, unsaturation of the acyl moiety, sterol structure and temperature on the formation of hydroperoxides, secondary oxides of sterol and oligomers along the prolonged heating ($p < 0.05$, SPSS 15.0 for Windows). The formation and reduction rates were also compared. Correlations (r) of data obtained by two different methods were calculated. Principal component analysis (PCA) was performed to obtain an overview of the oxidation and to see how the used methods describe the reactions.

5 RESULTS

5.1 Analysis of intact steryl ester monohydroperoxides and further reaction products (I–IV)

In order to distinguish between the oxidation of steryl and acyl moieties in steryl esters, both isolation and separation methods had to be developed for labile hydroperoxides.

Solid-phase extraction (SPE) in normal-phase mode using silica sorbent proved to be an efficient method for the isolation of steryl ester oxidation products from unreacted steryl ester preparation and from a lipid medium (I–II, IV). Even at low hydroperoxide contents, relatively high recoveries were achieved: The recoveries of individual cholesteryl linoleate hydroperoxides from oxidised sample varied between 94 ± 6% and 118 ± 21% ($n = 6$) when the contents ranged from 23 to 233 μg/g lipid, or 70 ± 6% and 95 ± 4% ($n = 6$) when the contents were halved (IV).

The separation of intact steryl moiety hydroperoxides of steryl esters was achieved by using a silica column and heptane based eluents (I). However, a baseline separation of the intact steryl moiety hydroperoxides from the acyl moiety hydroperoxides was not possible. Separation was instead achieved with a neutral alumina column and gradient elution with IPA in heptane (0–2% + 0.5% THF) (II). By this method, the steryl moiety hydroperoxides eluted before the acyl moiety hydroperoxides (Figure 5). Separation of epimers and positional isomers was also achieved. Intact steryl ester monohydroperoxides could be quantified by comparing ELSD responses of the hydroperoxides to those of unoxidised steryl esters. The limit of detection (LOD, 3:1 signal-to-noise ratio) for unoxidised steryl esters was 20 ng/10 μl injection (2 μg/mL). Linear standard curves were attained in the range of 200–2000 ng/injection ($R^2 = 0.981–0.992$) (I) and 20–1000 ng/injection ($R^2 = 0.967–0.995$) (II, IV).

The results obtained by HPLC-DAD-ELSD method were confirmed by peroxide values (PV). In the propagation stage, the two methods gave highly correlating results. Depending on the ester, correlations of $r = 0.765–0.987$ and $r = 0.841–0.985$ were obtained when steryl esters were oxidised in neat (II) and in tripalmitin (IV), respectively. After the monohydroperoxide maxima, however, the PVs did not decrease as drastically as was implied by the content of monohydroperoxides determined by HPLC-DAD-ELSD. According to the principal component analysis (PCA), peroxide values also reflected compounds other than only the monohydroperoxides determined by HPLC (Figure 6). They described the oxidation reactions further towards the secondary oxidation products. The main secondary oxidation products of sterol (i.e., 7-OHs, 7-ketone and 5,6-epoxides) reflect the next stage of oxidation while 6β-OH, 25-OH and triols were formed at the stage when polymerisation became important. As the monomers and oligomers fall on the opposite quarters of the plot, oligomers were clearly formed from monomeric steryl esters and free sterol.

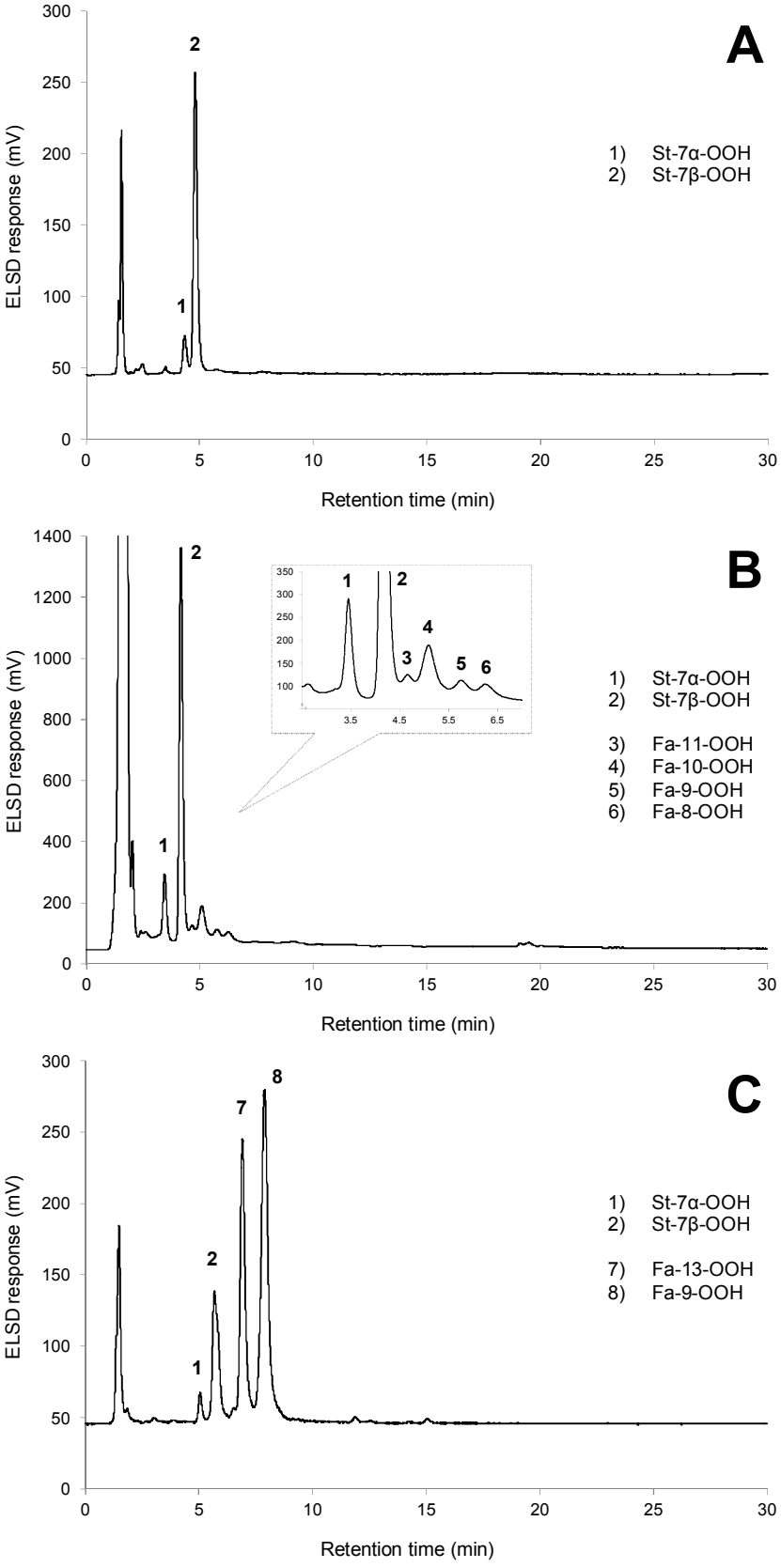

Figure 5. Separation of cholesteryl linoleate steryl (St-OOH) and acyl moiety (Fa-OOH) hydroperoxides by HPLC-DAD-ELSD using Al_2O_3-column and gradient elution with IPA in heptane (0−2% + 0.5% THF). A) Cholesteryl stearate, B) cholesteryl oleate and C) cholesteryl linoleate.

A previously described HPSEC-RI method (Lampi and Kamal-Eldin 1998) was also found suitable for the determination of steryl ester oligomers (III−IV). Oligomers could be separated from the monomeric steryl esters and their monomeric oxidation products and they could be quantified by external standard method using a monomeric steryl ester as a standard.

Coordination ion mass spectrometry (CIS-MS) proved to be a suitable technique for the detection of intact steryl ester hydroperoxides (II) and oligomers (III). According to fragmentation patterns, the steryl and acyl moiety hydroperoxides could be distinguished. With the aid of the isomeric specific Hock-fragmentation patterns, the location of the hydroperoxyl group in the acyl moiety could be indicated, and the elution order of the compounds confirmed. The dominant ions for cholesteryl stearate, oleate and linoleate hydroperoxides were $[M+^{107/109}Ag]^+$ corresponding to m/z = 792/794, 790/792 and 788/790, respectively. The location of hydroperoxyl group in the oleyl moiety could be indicated by the Hock-fragmentation of m/z = 790/792 $[M+^{107/109}Ag]^+$: m/z = 676/678 [Fa-11-OOH+$^{107/109}$Ag-Hock]$^+$, m/z = 662/664 [Fa-10-OOH+$^{107/109}$Ag-Hock]$^+$, m/z = 648/650 [Fa-9-OOH+$^{107/109}$Ag-Hock]$^+$ and m/z = 634/636 [Fa-8-OOH+$^{107/109}$Ag-Hock]$^+$. In the MS/MS of cholesteryl linoleate hydroperoxide m/z = 788/790 $[M+^{107/109}Ag]^+$, fragments of m/z = 770/772 $[M+^{107/109}Ag\text{-}H_2O]^+$, m/z = 687/689 [Fa-13-OOH+$^{107/109}$Ag-Hock]$^+$ and m/z = 648/650 [Fa-9-OOH+$^{107/109}$Ag-Hock]$^+$ revealed the presence and position of hydroperoxyl group at positions C-13 and C-9, respectively, in the acyl moiety. The presence of St-OOHs was indicated by the MS/MS of $[M+^{107/109}Ag]^+$. The fragments m/z = 507/509 and m/z = 489/491 signified the presence of St-OOH in all of the studied cholesteryl esters and fragment m/z = 475/477 indicated the presence of unoxidised steryl moiety. Indication of the presence of oligomers was also possible (III). According to the peak patterns and m/z-values obtained

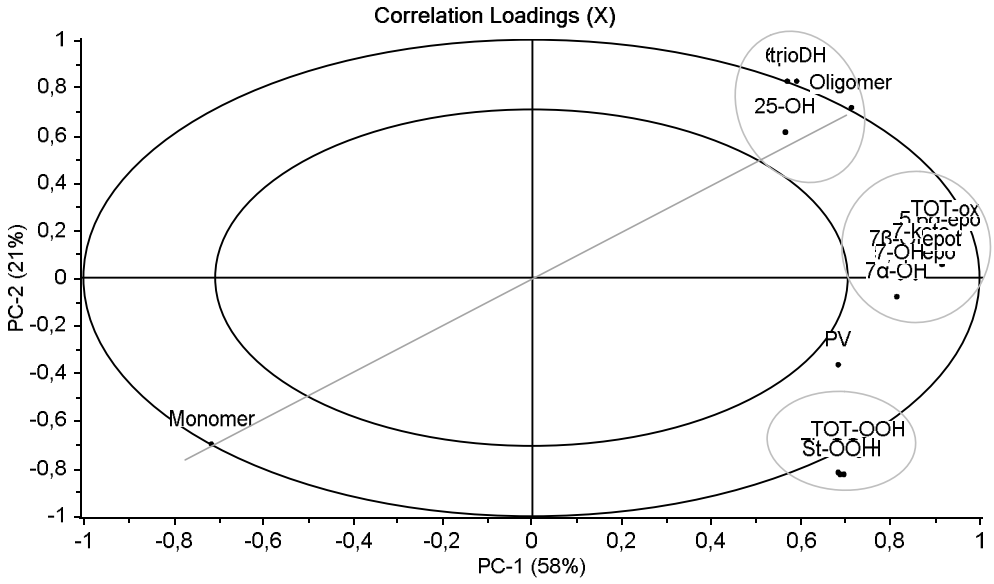

Figure 6. Correlation loadings in the principal component analysis (PCA) for the data obtained by oxidising steryl esters and free sterol in neat and in tripalmitin at 100−140 °C. The ellipse represents the 95% confidence level.

in the mass spectra, the molecular sizes of several oligomers could be indicated for the oxidised steryl esters. For example, for neat cholesteryl stearate oxidised at 140 °C for four days, m/z ranges of 360−450, 465−550, 760−840, 1040−1260, and 1435−1535 were detected. Compounds with molecular weights corresponding to mainly 2−5 steryl ester units were indicated. However, the exact compound distribution could not be determined by this method because of the large number of compounds and their broad molecular size range.

5.2 Formation of primary oxidation products

5.2.1 Effects of saturated acyl moiety (I, IV)

When steryl esters and free sterol were oxidised as neat preparations at 100 °C, esterification increased the oxidation of sterol (I−II). Free cholesterol remained solid, but the esterified sterols were liquefied. For cholesteryl stearate, which has a saturated acyl moiety, the lag phase lasted one day, after which a 2-day propagation stage began. A hydroperoxide maximum of 64 mg/g was reached after three days of heating (Figure 7). A drastic drop in the hydroperoxide content was observed after four days of heating, indicating that the termination stage was reached. In the presence of possible impurities, the initiation time was shortened to 3 hours and the oxidation rate was significantly increased (I). A hydroperoxide maximum of 102−111 mg/g was reached already after 12 hours of heating and after two days monohydroperoxides were no longer detected. For free cholesterol, barely any hydroperoxides were measured during the four day heating period. Both for cholesteryl stearate and free cholesterol, greater contents of St-7β-OOHs than of St-7α-OOH were measured.

In a saturated lipid medium, the initiation was delayed and the oxidation rate of esters was decreased (Figure 8A) (IV). For free cholesterol, the opposite occurred and the oxidation of cholesterol was highly induced. For cholesteryl stearate, oxidation was noted after two days of heating. At that point, the propagation stage was also reached. A hydroperoxide maximum of 82−88 mg/g was reached after four days of heating. For free cholesterol, on the other hand, the propagation stage was clearly evident after 16 hours of heating. The hydroperoxide maximum (206 mg/g sterol corresponding to 124 mg/g steryl ester) was reached after two days of heating, and a drastic drop in the hydroperoxide content was observed after three days of heating. As was seen for the neat preparations, the content of St-7β-OOHs was higher than of St-7α-OOHs.

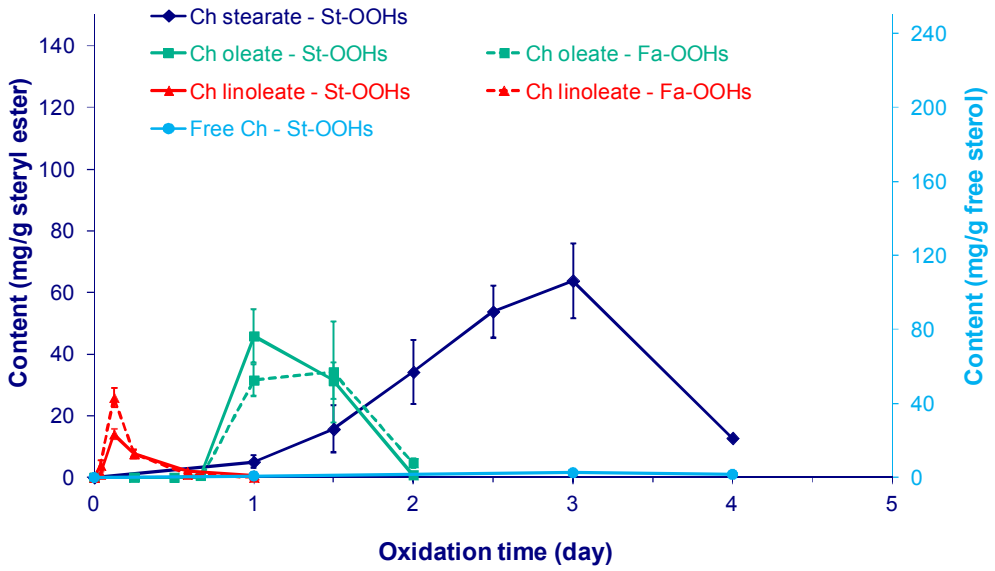

Figure 7. Formation of the steryl (St-OOH) and acyl (Fa-OOH) moiety hydroperoxides in neat steryl ester (left-hand y-axis) and free sterol (right-hand y-axis) preparations maintained at 100 °C for 0−4 days: Cholesteryl stearate (Ch stearate), cholesteryl oleate (Ch oleate), cholesteryl linoleate (Ch linoleate) and free cholesterol (Free Ch). The scale of free sterol y-axis is adjusted commensurate to the steryl ester y-axis in order to enable direct comparison.

5.2.2 Effects of unsaturation degree of the acyl moiety (II, IV)

When neat steryl esters were oxidised at 100 °C, an increased degree of unsaturation of the acyl moiety shortened the induction period and increased the oxidation of both steryl and acyl moieties (Figure 7) (II). For cholesteryl oleate, which has a monounsaturated acyl moiety, oxidation was noted after 16 hours of heating and the propagation stage was reached after one day of heating (Figure 7). At the hydroperoxide maximum (78 mg/g) at day one, higher contents of steryl than of acyl moiety hydroperoxides (46 mg and 32 mg/g, respectively) were measured. After one and a half days of heating, their contents became, however, equal (31 mg and 34 mg/g, respectively). After two days of heating, a drastic drop in the hydroperoxide content was observed. For cholesteryl linoleate, which has a polyunsaturated acyl moiety, the initiation time was further shortened and the oxidation rate was additionally increased (Figure 7) (II). Oxidation was noted within an hour of heating. At that point, the propagation stage was already reached, and a hydroperoxide maximum of 40 mg/g was reached after three hours of heating (Figure 7). At the hydroperoxide maximum, more acyl than of steryl moiety hydroperoxides were measured (26 mg/g and 14 mg/g, respectively). After three hours of heating, the hydroperoxide content started to decrease, and after 16 hours almost no hydroperoxides were detected.

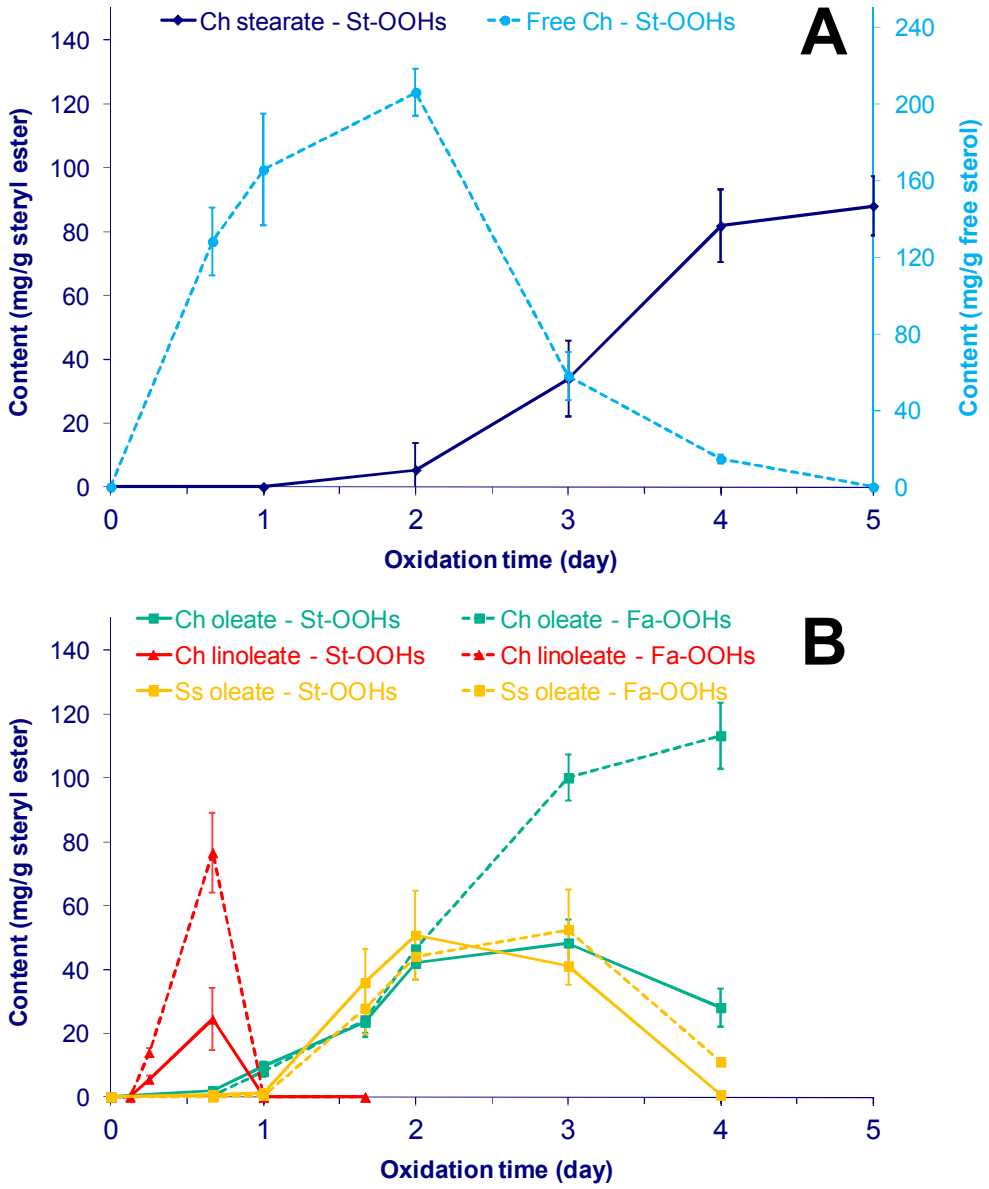

Figure 8. Formation of steryl (St-OOH) and acyl (Fa-OOH) moiety hydroperoxides in steryl esters (left-hand y-axis) and free sterol (right-hand y-axis) maintained in tripalmitin medium at 100 °C for 0−5 days: A) Cholesteryl stearate (Ch stearate) and free cholesterol (Free Ch), B) cholesteryl oleate (Ch oleate), stigmasteryl oleate (Ss oleate) and cholesteryl linoleate (Ch linoleate). The scale of free sterol y-axis is adjusted commensurate to the steryl ester y-axis in order to enable direct comparison.

In a saturated lipid medium, the lag phase of oxidation was lengthened and the oxidation rate was decreased compared to the neat preparations (Figure 8B) (IV). However, the initiation times of cholesteryl oleate and linoleate were shorter and their oxidation rates were greater than for the ester with a saturated acyl moiety. For cholesteryl oleate, oxidation was noted after 16 hours of heating, and the propagation stage was reached after one day of heating (Figure 8B). A hydroperoxide maximum of 148 mg/g was reached after three days of heating. In the initial stage, slightly greater contents of steryl than of acyl moiety hydroperoxides were

detected. At the maximum, on the other hand, greater contents of acyl than of steryl moiety hydroperoxides were measured (100−112 mg/g and 28−48 mg/g, respectively). After four days of heating, the content of steryl moiety hydroperoxides decreased, but the content of acyl moiety hydroperoxides kept on increasing.

For cholesteryl linoleate, which was oxidised in a lipid medium, the lag phase lasted for three hours, and the propagation stage was reached after six hours of heating (Figure 8B) (IV). After 16 hours of heating, a hydroperoxide maximum of 101 mg/g was reached. At the maximum, greater contents of acyl than of steryl moiety hydroperoxides were measured (77 mg/g and 24 mg/g, respectively).

As was seen for cholesteryl stearate and free cholesterol, greater contents of St-7β-OOHs than of St-7α-OOH were measured for cholesteryl oleate and linoleate at each time point both in neat preparations and in tripalmitin (I, IV). The difference was most distinctive at the hydroperoxide maxima. At the hydroperoxide maximum of neat cholesteryl oleate, that is, after one day of heating, Fa-OOHs consisted of 42% of Fa-11-OOH + Fa-10-OOH, 28−30% of Fa-9-OOH and of Fa-8-OOH each (II). In tripalmitin medium after three days of heating, 43% of the Fa-OOHs consisted of Fa-10-OOH, 23% of Fa-9-OOH, 22% of Fa-11-OOH and 12% of Fa-8-OOH (IV). For neat cholesteryl linoleate, equal contents of Fa-9-OOH and Fa-13-OOH were measured at the hydroperoxide maximum after three hours of heating (II). In tripalmitin medium, a difference in the contents was noted: At the hydroperoxide maximum, Fa-13-OOH accounted for 72% and Fa-9-OOH accounted for 28% of the acyl moiety hydroperoxides (IV).

5.2.3 Effects of unsaturation degree of the steryl moiety (IV)

In a saturated lipid medium, the addition of a double bond and methyl group into the side chain of the sterol backbone induced the oxidation of sterol (IV). The formation rate of hydroperoxides was higher for stigmasteryl oleate than for its cholesterol analogue (Figure 8B). For stigmasteryl oleate, hydroperoxides were detected after one day of heating. At the very beginning of the formation, slightly greater contents of St-OOHs than of Fa-OOHs were measured. A hydroperoxide maximum of 95 mg/g was reached after two days of heating. At the maximum, 51 mg/g of St-OOHs and 44 mg/g of Fa-OOHs were measured. In contrast to the findings with cholesteryl oleate hydroperoxides, the content of both steryl and acyl moiety hydroperoxides decreased significantly by the fourth day of heating. At day four, greater contents of acyl (11 mg/g) than of steryl moiety (0.7 mg/g) hydroperoxides were measured. In agreement with the findings for cholesteryl oleate, greater contents of St-7β-OOHs than of St-7α-OOH were measured at the hydroperoxide maximum of stigmasteryl oleate (i.e., after three days of heating). Moreover, 48% of the Fa-OOHs consisted of Fa-10-OOH, 33% of Fa-9-OOH, 11% of Fa-8-OOH and 8% of Fa-11-OOH.

5.3 Further oxidation products of steryl esters

At the termination stage of oxidation, hydroperoxides decompose into secondary radicals that may undergo reactions to form secondary oxidation products and oligomers. Therefore, the further reactions of hydroperoxides formed in steryl esters and in free sterol in neat preparations and in tripalmitin at 100 °C and 140 °C were followed in terms of the secondary oxidation products of sterol and higher molecular size products.

5.3.1 Formation of secondary oxidation products of sterol (I–IV)

As observed for the hydroperoxide contents, secondary oxidation products of sterols also showed that esterification increased the oxidation of sterol (I–II). At the hydroperoxide maximum of neat cholesteryl stearate; that is, after three days of heating at 100 °C, 294 mg/g of secondary oxides (expressed as mg of oxysteryl ester per gram of original steryl ester) were measured (Figure 9A). The content then levelled for the following two days. When possible impurities were present, higher contents of secondary oxides were measured at earlier time points. For free cholesterol, on the other hand, only 0.6 mg/g of secondary oxides were detected after five days of heating (Figure 9D).

The introduction of unsaturation to the acyl moiety increased the secondary oxidation as well (II–III). At the hydroperoxide maximum of cholesteryl oleate; that is, after one day of heating at 100 °C, 244 mg/g of secondary oxides were measured (Figure 9B). After two days of heating, the content had increased to 334 mg/g, after which a decrease was observed, and after four days of heating, a content of 237 mg/g was measured. At the hydroperoxide maximum of cholesteryl linoleate; that is, after three hours of heating, only 44 mg/g of secondary oxides were measured (Figure 9C). After a decrease in the hydroperoxide content was observed, the content of secondary oxides increased to 248 mg/g. After one day of heating, the content levelled at 335–372 mg/g for the following two days.

When the temperature was raised to 140 °C, the oxidation rate was increased, as expected, and lower contents of secondary oxides were measured for steryl esters (III). The increase in the oxidation rate was most intensive for free cholesterol. After one day of heating, 215 mg/g of secondary oxides of cholesteryl stearate (expressed as oxysteryl ester) were measured. For free cholesterol, the content was 214 mg/g. For both compounds, the content of secondary oxides decreased with prolonged heating time. For cholesteryl oleate, 202 mg/g of secondary oxides were measured after 16 hours of heating and the content decreased along the two days heating period. For cholesteryl linoleate, 262 mg/g of secondary oxides were measured after three hours of heating and the content remained at same level throughout the one-day heating period.

In a saturated lipid medium, the oxidation of steryl esters occurred more slowly than in neat preparations (IV). Moreover, the oxidation of saturated steryl ester occurred more slowly than the oxidation of free sterol. In tripalmitin at 100 °C, only 15 mg/g of secondary oxides of

sterol (expressed as oxysteryl ester) were measured for cholesteryl stearate after four days of heating (Figure 10A). When the hydroperoxide content levelled on the fifth day, the content of secondary oxides started to increase and a content of 36 mg/g was measured. While neat free cholesterol remained unaltered at 100 °C, the oxidation was highly induced in a lipid medium. Already after 16 hours of heating, 84 mg/g of secondary oxides could be measured (Figure 10D). The content increased until two days of heating, after which the content levelled at 262−287 mg/g.

The elevated oxidation rate in response to the increased degree of unsaturation was also observed in the formation of secondary oxidation products in the lipid medium (IV). For cholesteryl oleate, secondary oxides of sterol were already present at significant contents when the hydroperoxide content was still increasing; that is, 131 mg/g (expressed as oxysteryl ester) were measured after two days of heating (Figure 10B). The content increased during the four day heating period, reaching a content of 322 mg/g. For stigmasteryl oleate, the formation rate of secondary oxides of sterol was even greater. After two days of heating, 154 mg/g of secondary oxides of sterol were measured. A maximum of 306 mg/g was reached on the third day of heating and by the fourth day the content decreased to 266 mg/g. As the unsaturation of the acyl moiety was further increased, the rate of secondary oxide formation also increased significantly. For cholesteryl linoleate, 69 mg/g of secondary oxides of sterol were measured after 6 hours of heating (Figure 10C). The content continued to increase throughout the first day of heating, and 387 mg/g was reached.

The profiles of the generated secondary oxides were dependent on the rate and stage of oxidation (I−IV). At the beginning of oxidation, 7-OHs and 7-ketone were the main oxides. Even though epoxides of sterols are considered to form simultaneously with hydroperoxides, considerable amounts of epoxides were measured only after oxidation proceeded further. Increased rates of oxidation increased the formation of 7-ketone. Increased temperature did not significantly alter the profiles of formed secondary oxides. Introduction of the lipid medium, on the other hand, increased the epoxide formation. Both in neat preparations and in tripalmitin, β-epimers of 7-OHs and 5,6-epoxides predominated over the corresponding α-epimers.

5.3.2 Formation of oligomers (III−IV)

As was seen for the secondary oxidation products of sterols, hydroperoxides also decomposed into oligomers. Increased oxidation rates due to esterification and increased unsaturation also induced the polymerisation of sterols. For neat cholesteryl stearate, 79 mg/g of oligomers were already measured at the hydroperoxide maximum; that is, after three days of heating at 100 °C (Figure 9A) (III). As the hydroperoxide content decreased by the fourth day of heating, a significant increase in the oligomer content was observed. After five days of heating, oligomers and secondary oxides were measured at similar contents: 279 mg/g each. The increased oxidation of unsaturated steryl esters was also observed as greater contents of

oligomers. For cholesteryl oleate, a greater content of oligomers (456 mg/g) than of secondary oxides was measured after two days of heating (Figure 9B). The amount increased up to 788 mg/g after four days of heating. For cholesteryl linoleate, the polymerisation was most intensive: a total of 714 mg/g of oligomers were measured after only one day of heating (Figure 9C). Over the following two days, the amount rose to 809 mg/g. For free sterol, oligomers were not detected during the five days at 100 °C (Figure 9D).

When the temperature was elevated to 140 °C, the polymerisation rate of cholesteryl esters increased and the polymerisation of free cholesterol became significant (III). For cholesteryl stearate, 428 mg/g of oligomers were measured after one day of heating, and the content increased to 560 mg/g in two days. For cholesteryl oleate, 488 mg/g of oligomers were measured after 16 hours and 607 mg/g after two days of heating. For cholesteryl linoleate, a total of 513 mg/g of oligomers were measured after only three hours of heating. By day one, the amount had risen to 679 mg/g. As free cholesterol was liquefied at 140 °C, the oxidation, and especially polymerisation, became significant. After just one day of heating at 140 °C, 420 mg/g of oligomers were measured. The content increased throughout the four-day heating period, until a level of 596 mg/g was reached. These effects of temperature on the polymerisation were also seen in the principal component analysis (Figure 11). Samples oxidised at 140 °C were grouped closest to the loading of oligomers (Figure 6).

The oxidation of steryl esters was slowed down in the lipid medium, so the rates of polymerisation were also decreased (IV). Moreover, the polymerisation of free sterol became significant. For cholesteryl stearate, insignificant content of oligomers were noted even after five days of heating (Figure 10A). For cholesteryl oleate, oligomers were measured (36 mg/g) after two days of heating while hydroperoxides were still accumulating (Figure 10B). After the content of hydroperoxides levelled at day three, a significant increase in the oligomer content was observed. On the fourth day, 185 mg/g of oligomers were measured. At the early stage, the polymerisation rate of stigmasteryl oleate was similar to that of its cholesterol analogue, but at later stage it became greater. After four days of heating, 304 mg/g of oligomers were measured. For cholesteryl linoleate, 76 mg/g of oligomers were measured within six hours of heating, and the content continued to increase up to 413 mg/g during the first day of heating (Figure 10C). For free cholesterol, two types of oligomers were measured. The first type was oligomers of free sterol (i.e., eluting right after the monomeric triacylglycerol but before the monomeric sterol) and the second type was higher oligomers since they eluted before the monomeric triacylglycerol. Oligomers of free sterol (i.e., the first type) were measured at a content of 6 mg/g after 16 hours of heating (Figure 10D). The content increased up to 271 mg/g in three days. The second type of oligomers was measured after two days of heating and their content rose to 126 mg/g in three days. The influence of the lipid medium on the oxidation rate, and especially on the formation of oligomers, is shown in Figure 12: Samples that were oxidised as neat preparations are grouped close to the loading of oligomers (Figure 6), whereas the tripalmitin samples are evenly distributed around the centre of the model.

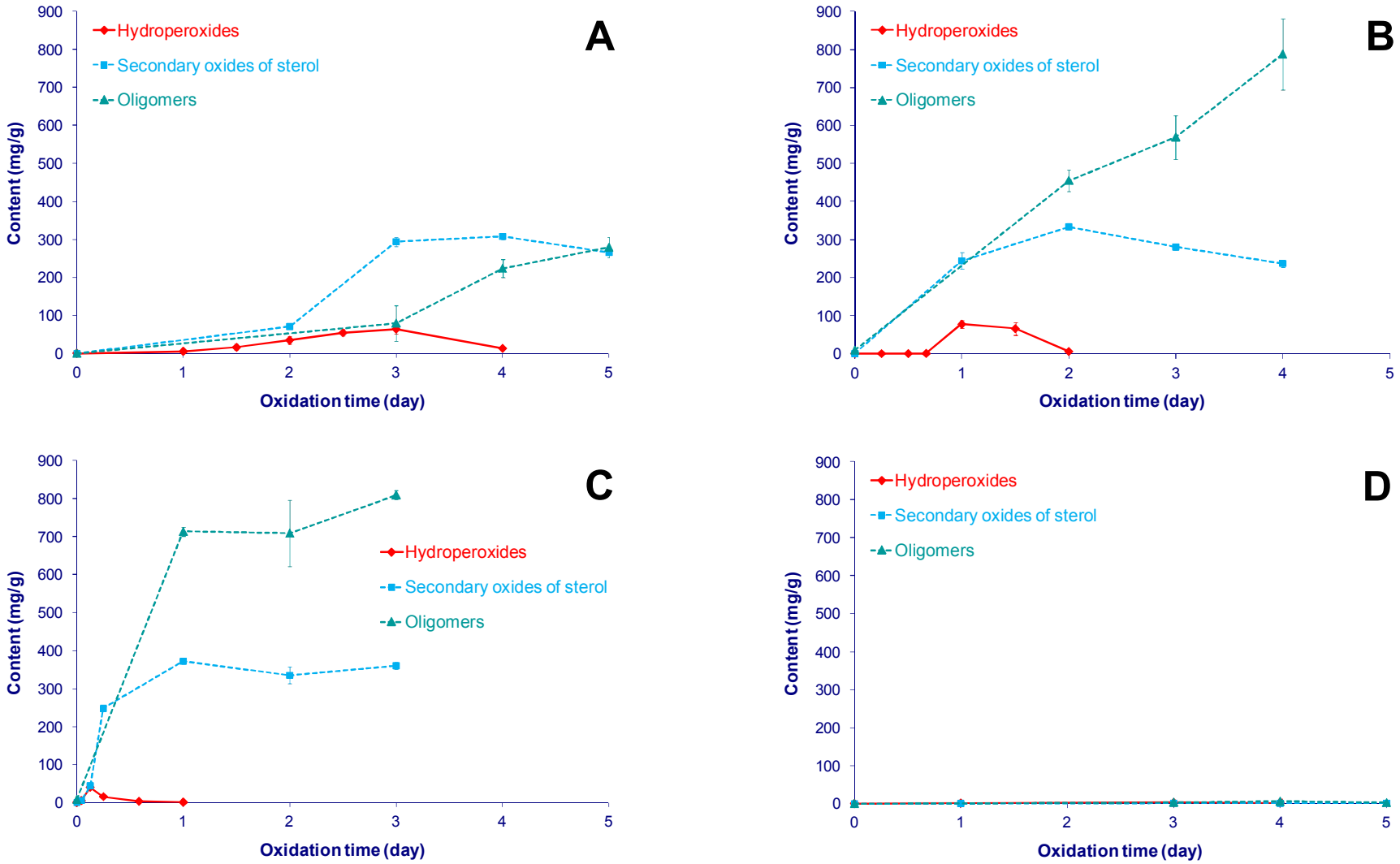

Figure 9. Formation of primary oxidation products and their decomposition into further products in neat cholesteryl esters and free cholesterol maintained at 100 °C. A) Cholesteryl stearate, B) cholesteryl oleate, C) cholesteryl linoleate and D) free cholesterol. Content represents the amount of oxidised or polymerised steryl ester or sterol (mg) per original steryl ester or sterol (g).

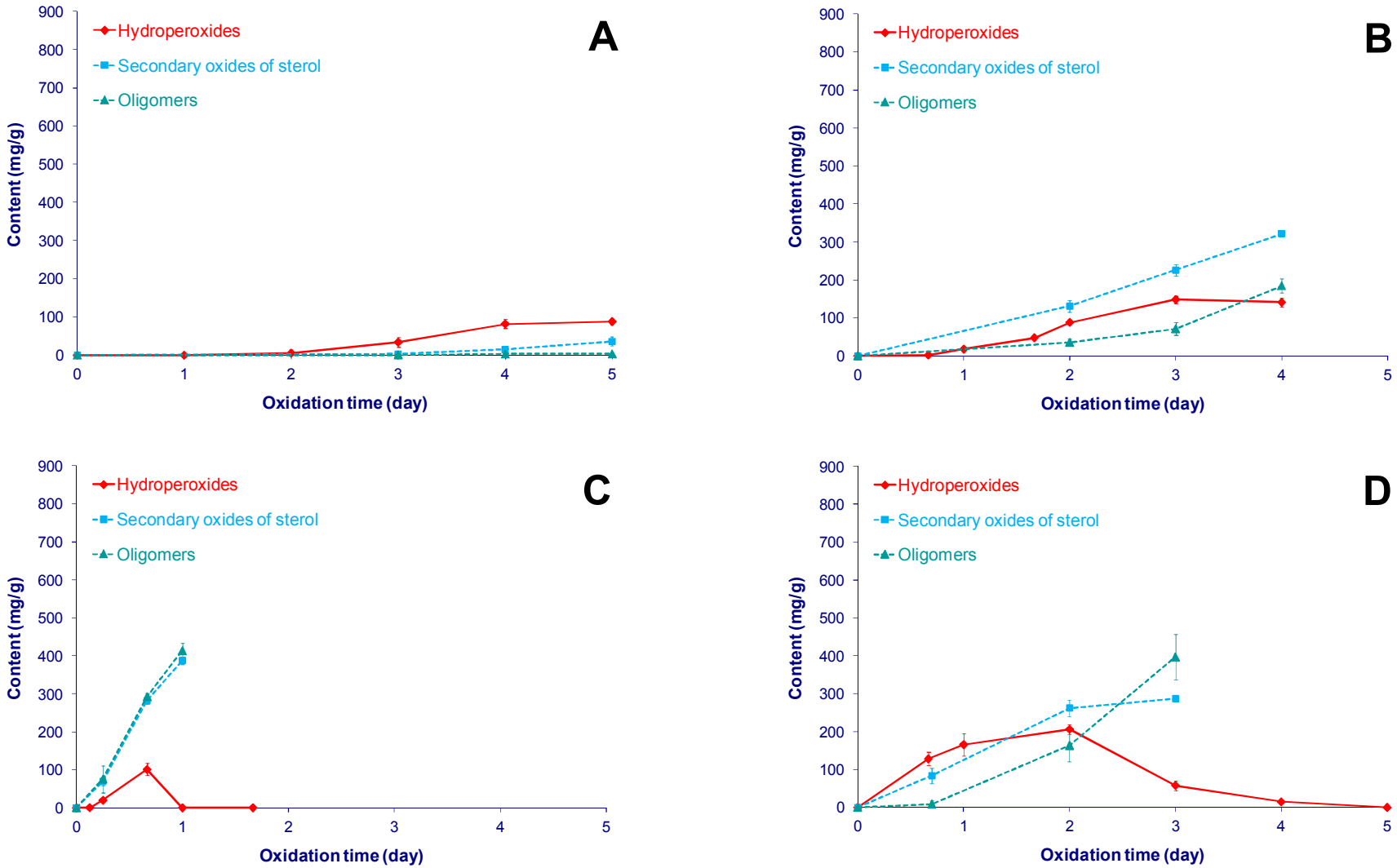

Figure 10. Formation of primary oxidation products and their decomposition into further products in a saturated lipid medium maintained at 100 °C. A) Cholesteryl stearate, B) cholesteryl oleate, C) cholesteryl linoleate and D) free cholesterol. Content represents the amount of oxidised or polymerised steryl ester or sterol (mg) per original steryl ester or sterol (g).

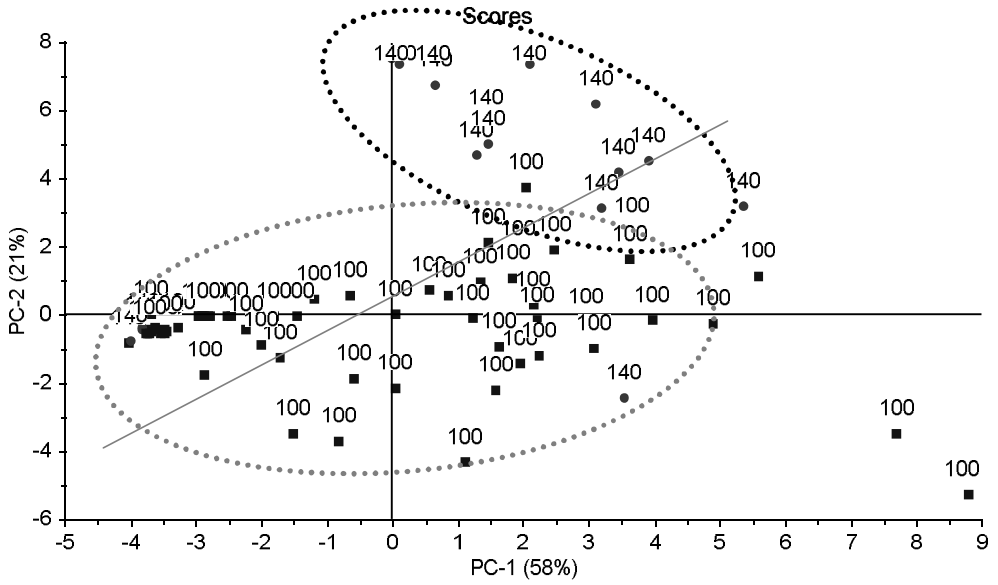

Figure 11. Influence of temperature on the oxidation of sterols. Principal component analysis (PCA) was performed using data obtained by oxidising steryl esters and free sterol in neat and in tripalmitin at 100−140 °C.

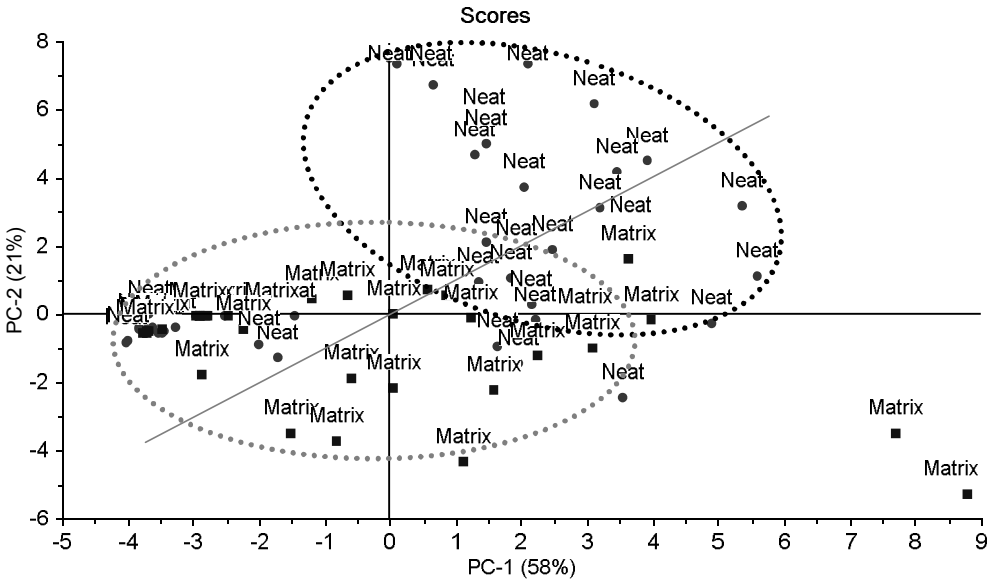

Figure 12. Influence of lipid medium on the oxidation of sterols. Principal component analysis (PCA) was performed using data obtained by oxidising steryl esters and free sterol in neat and in tripalmitin at 100−140 °C.

6 DISCUSSION

6.1 Determination of intact steryl ester monohydroperoxides and their further reaction products

Characterisation and quantification of intact St-OOHs and Fa-OOHs was accomplished

During the oxidation of lipids, the first measurable products are monohydroperoxides. As the aim of this thesis was to study the oxidation behaviour of steryl esters, the investigation began with primary oxidation and, more precisely, how the oxidation begins in steryl esters. In order to do this, methods had to be developed to determine the first oxidation products as intact molecules. These methods also had to be capable of providing information about the oxygenated site in the ester molecule. Previously, the oxidation of steryl esters has been studied in terms of steryl moiety oxidation (Korahani et al. 1982, Soupas et al. 2005, 2006, 2007). The methods used involved saponification or transesterification (Table 1) and thus the information about the oxidation of the intact molecules was not gained. During the hydrolysis, the lipid medium is also hydrolysed and unsaponifiables may be isolated by liquid extraction. In the analysis of intact steryl esters, this type of isolation is not possible and therefore other approaches had to be considered. A combination of normal-phase SPE and HPLC-DAD-ELSD using alumina sorbent proved to be efficient for the isolation and determination of intact steryl ester monohydroperoxides. By using this method combination, the oxidation of steryl and acyl moieties of intact steryl esters could be distinguished.

Isolation of intact hydroperoxides by solid-phase extraction

Oxidation products of steryl esters could be isolated by silica SPE using nonpolar solvents with increasing polarity. Since the hydroperoxides seemed to reduce to their corresponding hydroxides on alumina sorbent, cartridges packed with silica were chosen for further development. By using silica in a normal-phase mode, components were separated according to their polarity; less polar compounds eluted immediately with nonpolar solvents while the polar compounds (e.g., oxidation products) were retained on the sorbent material and could be collected with more polar solvents. Previously, silica sorbent based normal-phase SPE has been successfully used for the isolation of free cholesterol oxidation products from lipid extracts of various food products (Penazzi et al. 1995; Ulberth and Rössler 1998). Based on our experiments, the polarity of steryl ester hydroxides and free cholesterol are of similar order, since they eluted in the same fraction both in silica SPE and in HPLC. Since the steryl ester hydroperoxides are less polar than the hydroxides, isolation methods with greater selectivity had to be developed. The isolation of rather reactive hydroperoxides requires inert sorbents and solvents. By using silica sorbent and heptane-based eluents high recoveries of hydroperoxides were achieved.

Determination of intact hydroperoxides by HPLC

The HPLC separation of intact steryl and acyl moiety hydroperoxides was achieved by using neutral aluminium oxide, alumina, as a stationary phase and heptane-based eluent with increasing polarity. Alumina not only contains hydroxyl groups, but also other types of adsorption sites, such as Al^{3+} cations and oxygen, which enables efficient separation of compounds having small polarity or structural differences. The separation is mainly influenced by the polarities of compounds, but also by the increased degree of unsaturation, which increases the retention. Alumina HPLC columns have previously been used successfully for the separation of nonpolar lipids; e.g., steryl esters and triacylgycerols, in a normal-phase mode (Norbäck and Lundberg 1999; Moreau et al. 2002). A mixture of silica and alumina (84:16, w/w) has also been used for the separation of free cholesterol oxides (Lakritz and Jones 1997). In the present study, the separation of intact steryl and acyl moiety monohydroperoxides was not achieved on a silica column with heptane-based eluents. In order to separate nonpolar cholesteryl ester Fa-OOHs, methods involving combination of 2−3 silica columns in series and hexane-based eluents have been reported (Table 2). Moreover, the St-7-OOH was suggested to elute between Fa-13-OOH and Fa-9-OOH, since it was not detected by UV at 234 nm (Havrilla et al. 2000). The separation of steryl ester hydroperoxides was improved by reducing the hydroperoxides to their corresponding hydroxides. However, if the sample already contained hydroxides prior to the reduction of hydroperoxides, the content of hydroperoxides could not be determined reliably. HPLC methods for the separation and quantification of both St-OOHs and Fa-OOHs have not been reported previously. Fractionation of cholesteryl linoleate St-OOHs and Fa-OOHs has been achieved using column chromatography with SiO_2 sorbent and hexane/diethyl acetate (15/1, v/v) as an eluent (Hui et al. 2000). The less polar fraction contained St-OOHs and the more polar fraction contained Fa-OOHs.

Detection of intact hydroperoxides

Detection of unoxidised and oxidised steryl esters was possible with a diode-array detector at wavelengths 206 nm (all of the compounds) and 234 nm (compounds including conjugated double bonds). However, quantification was not feasible using this detection method since suitable hydroperoxide standards were not available. Double bonds, conjugation and oxygenated sites increase the UV response; thus, unoxidised steryl esters could not be used as standards. Moreover, the baseline drift caused by gradient elutions was cumbersome. These problems were overcome by using an evaporative light-scattering detector (ELSD). The response of this type of detector is dependent on the size of the molecule and variations in the unsaturation or oxygenated sites will not affect the response (Christie 1992). ELSD has previously been applied, for example, for the detection of nonpolar lipids (Norbäck and Lundberg 1999; Moreau et al. 2002) and oxidation products of free cholesterol (Caboni et al. 1997; Lakritz and Jones 1997). Since ELSD gives a response for all compounds that have a higher boiling point than the eluent, the isolation and separation of steryl ester oxidation products had to be optimal. For example, if too much tripalmitin was left in the isolated oxidation product fraction, baseline separation was not achieved and quantification was

affected. Other authors have reported that the linear range of the ELSD response is rather narrow (Christie 1992). In the present study, however, a linear working range of 20−1000 ng/injection was obtained after plotting square roots of the peak areas against the injected amount.

Characterisation of intact hydroperoxides and oligomers

In mass spectrometry, the ionisation of nonpolar molecules, such as steryl esters and sterols, is difficult with conventional ionisation methods; i.e., ESI and APCI. In steryl esters, the ester bond is easily broken by in-source fragmentation and thus a molecular ion is not detected. Moreover, during the ionisation of hydroperoxides, loss of water (m/z = 18 $[M\text{-}H_2O]^+$) seems to be inevitable (Kemmo et al. 2007a). In the present study, coordination ion mass spectrometry (CIS-MS) approved to be a useful technique for the characterisation of formed steryl ester hydroperoxides and their further reaction products. Using this technique, the location of the oxygenated site could be distinguished between the steryl and acyl moieties. In the MS/MS of molecular ions containing silver $[M+Ag]^+$, the fragmentations of the steryl and acyl moiety hydroperoxides were different. Moreover, the location of the oxygenated site in the acyl moiety could be determined by Hock-fragmentation (Bayer et al. 1999; Havrilla et al. 2000). CIS-MS also enabled the indication of oligomers in the oxidised steryl esters. In CIS-MS, a charged silver ion forms a complex with a nonpolar molecule, which stabilises the molecule and enables soft ionisation. This method is applicable for molecules containing π-electrons or free electron pairs, e.g., double bonds, ester moieties or oxygenated sites (Bayer et al. 1999). Ag^+−CIS-MS has been previously used for the detection of carotenoids and peptides (Bayer et al. 1999), cholesteryl ester hydroperoxides (Havrilla et al. 2000) and nonpolar unsaturated lipids (Seal et al. 2003). It has also been successfully used for the characterisation of intact steryl ester Fa-OOHs (Havrilla et al. 2000; Seal and Porter 2004) and more recently for the characterisation of free sterol dimers (Struijs et al. 2010).

As reported by Havrilla et al. (2000), the dominant ions for cholesteryl linoleate Fa-OOHs were m/z = 788/790 $[M+^{107/109}Ag]^+$. In the MS/MS of m/z = 788/790, the presence and position of hydroperoxyl group at the positions C-13 and C-9 in the acyl moiety were indicated. In addition, α-fragments have been found to differentiate hydroperoxide isomers (Seal and Porter 2004). However, these fragments were not observed in the present study. *Trans,trans*- and *cis,trans*-isomers of cholesteryl linoleate Fa-OOHs have also been differentiated by HPLC−CIS-MS (Seal et al. 2004). However, CIS-MS of cholesteryl stearate and oleate have not been reported previously. The presence of St-OOHs was indicated by the MS/MS of $[M+^{107/109}Ag]^+$. The fragments m/z = 507/509 and m/z = 489/491 indicated the presence of St-OOHs in all of the studied cholesteryl esters and fragment m/z = 475/477 indicated the presence of unoxidised steryl moiety. Previously, the presence of St-OOHs was suggested by the fragment m/z = 387 $[C_{18}H_{32}O_2 + Ag]^+$ (Seal et al. 2004). However, a fragmented unoxidised linoleyl moiety silver complex gives the same m/z-value. Therefore, this m/z-value was not considered as a clear indicator in this study. Like in the present study,

the presence of an unoxidised steryl moiety has previously been indicated by m/z = 475/477 (Havrilla et al. 2000).

Steryl ester oligomers could also be indicated by Ag^+−CIS-MS. If a lipid molecule contains more than one double bond or has an ester moiety, a silver adduct with several silver ions may form. As a result, a peak pattern specific for the number of silver ions will be observed. The charge (z) of the adduct also increases with the number of silver ions. Thus, the peak pattern indicates the number of silver ions and charge, and they can be used to calculate the molecular weight of the molecule. In the present study, adducts with mainly 3−5 silver ions were detected, which indicated thus the presence of compounds with molecular weights corresponding to dimers and trimers. The presence of oligomers up to hexamers was eventually indicated. Previously, Ag^+−CIS-MS has been found applicable for the detection of intact peroxy and ether linked dimers of free sterols (Struijs et al. 2010). In the more energetic APCI-MS, these thermolabile compounds were degraded and the dimers could not be reliably indicated. When stigmasterol was heated at 180 °C, the formed dimers were suggested to connect mainly via C-C linkages at carbon C-7 (Struijs et al. 2010). Dimers forming ring structures (i.e., two monomers connected via two C-C junctions) were also indicated. At lower temperatures in the presence of hydroperoxides, also C-O-O-C and C-O-C linkages are likely to form. Similar junctions that have been detected for free sterols and fatty acids are expected to form for steryl esters. These molecules may combine either via steryl or acyl moieties. Unsaturated acyl moieties in triacylglycerols have been suggested to combine next to the double bonds (Dobarganes and Márquez-Ruiz 2007; Omwamba et al. 2010). Similar behaviour is expected to occur in steryl esters having unsaturated acyl moieties. In the early stage of polymerization, non-polar oligomers are expected to form. In sunflower oil, the number of polar dimers was found to increase along heating time (Márquez-Ruiz et al. 1995). In order to characterise and identify exact molecular weights and structures of steryl ester oligomers, efficient fractionation and separation methods would still be needed.

6.2 Effects of esterification on the oxidation of sterol

Introduction of an acyl moiety altered the physical state and polarity of sterol, affecting its oxidation

The results of this study clearly demonstrated that esterification alters the physical state of sterol and thereby affects its oxidation. In neat preparations at 100 °C, free sterol (melting point of 148−150 °C) remained in solid state whereas the esterified sterols (melting point of 42−83 °C) were liquefied. The oxidation of the steryl moiety was considerably greater for the esterified sterols than for the free sterol. Esterification also affected the profile of the formed oxidation products: In the solid state, the side chain of the steryl moiety primarily was oxidised, whereas in the liquefied state, the ring structure was oxidised leaving the side chain unreacted. In the solid state, the side chain may be prone to oxidation due to its location in the external parts of the molecule bilayer. Moreover, the crystal structure may protect C-7 in the

steryl moiety against oxidation (Korahani et al. 1982). However, after melting, this shield is lost. This in turn may explain the difference in the amounts of 7-hydroperoxides measured in the esterified sterols and in the free sterol. Similarly, in previous studies, a higher oxidation rate has been reported for esterified sterol than for free sterol at 100 °C (Yanishlieva and Marinova 1980; Yanishlieva-Maslarova et al. 1982; Korahani et al. 1982). At 140 °C, when both the esterified and free sterols were liquefied, the oxidation of cholesteryl stearate and free cholesterol were similar.

In a lipid medium, the oxidation of steryl esters was slowed down but the oxidation of free sterol was markedly increased. The effect could be seen in the formation of primary oxidation products as well as in the formation of secondary oxides of sterol and oligomers. Free sterol was mostly solubilised in the saturated lipid medium, and therefore the oxidation occurred even at 100 °C. The high oxidation rate of free cholesterol, compared to cholesteryl stearate, may ensue from their different polarities and thus different localizations in the nonpolar medium. Free sterols, which are rather polar due to their hydroxyl group at C-3, would likely head towards the polar phase (e.g., water or air). This might increase contact with air or catalysts and thus induce oxidation. In more complex lipid models, competition with other surface active compounds (e.g., other sterols, fatty acids and phospholipids) could also alter the location of sterols and thus affect their oxidation (Cercaci et al. 2007). The concentration of sterols may also affect their oxidation susceptibilities. Since only 1−3% of free cholesterol has been found to solubilise in nonpolar lipid medium at room temperature (Jandacek et al. 1977; Qianchun et al. 2011), addition of greater amounts may lead to their congregation on the surface of the lipid phase. As a consequence, this leads to greater oxidation of the sterols. Fatty acyl esterified sterols, on the other hand, are solubilised in nonpolar lipid media to greater extent (up to 23−35% at room temperature) (Jandacek et al. 1977; Qianchun et al. 2011) and thus their reactions with oxygen are limited.

Oxidation of the steryl moiety of steryl esters mainly involved oxidation of C-7 in the sterol B-ring. The primary oxidation products were St-7α-OOH and St-7β-OOH. Higher contents of St-7β-OOH than of St-7α-OOH were measured because an equatorial OOH-group is thermodynamically more stable than an axial OOH-group. In addition, St-7α-OOH is readily converted to St-7β-OOH (Smith 1981; Kamal-Eldin and Lampi 2008). Similar ratios were obtained for St-7α-OH and St-7β-OH, as was seen for St-7α-OOH and St-7β-OOH.

All of the major secondary oxidation products of sterol (i.e., 7-OHs, 5,6-epoxides and 7-ketone) were measured both in the esterified sterols and in the free sterol. The content profiles of 7-OHs and 7-ketone were similar when steryl esters and free sterol were oxidised as neat preparations and in tripalmitin. However, a significant increase in the epoxide contents was observed when the esters were oxidised in lipid medium. In nonpolar media, the addition of peroxyl radicals is favoured because of the limited access to the abstractable hydrogens; therefore, an increase in epoxide formation is often observed (Schaich 2005). Even though sterol epoxides have been suggested to form simultaneously with the hydroperoxides, in the present study they were formed mostly as decomposition products of hydroperoxides. The

formation was most considerable for the steryl ester which contained a polyunsaturated acyl moiety. This indicates that an acyl moiety hydroperoxide reacted with a double bond in the steryl moiety.

The results of this study also show that, even at moderate temperatures, polymerisation is a significant phenomenon in the autoxidation of steryl esters. Polymerisation even competed with the formation of secondary oxidation products of sterol: After the hydroperoxides decomposed, the content of oligomers increased significantly and even exceeded the level of secondary oxides. Polymerisation was more intensive in neat preparations than in tripalmitin. This may be due to the higher oxidation rate but also to the smaller sample size. Short travelling distances may have favoured recombination of steryl esters into oligomers. Some overestimation of the contents of secondary oxides and oligomers could have occurred, especially in the advanced stage of oxidation. In the saponification process, polar monomers may be released from polar oligomers which are combined via acyl moieties, and this could lead to an overestimation of sterol oxides. Lampi et al. (2009) also observed that heated stigmasterol gave greater response in HPSEC-RI than non-heated sample: The ratio of non-heated and heated sample was 0.93. Thus, by using a standard curve created with monomeric compounds (i.e., non-heated) may have led to a slight overestimation of the oligomers.

6.3 Effects of unsaturation of the acyl moiety on the oxidation behaviour of steryl esters

Unsaturation of the acyl moiety shortened the initiation time and increased the oxidation rate

Introduction of unsaturation to the acyl moiety lowered the activation energy of radical formation, and thus led to shorter initiation time and increased oxidation rate. The increased reactivity of the acyl moiety also induced the oxidation of the steryl moiety. This was observed as shortened initiation times and as increased formation rates of hydroperoxides, secondary oxides and oligomers. Previously, when plant sterols esterified with rapeseed oil fatty acids (7.5% saturated, 66% monounsaturated and 26% polyunsaturated) were incorporated into tripalmitin and maintained at 100 °C, 9% of the steryl moieties were oxidised after 2 days (Soupas et al. 2005). In the present study, the corresponding proportion was 13% for cholesteryl oleate and 15% for stigmasteryl oleate. At elevated temperature (180 °C), on the other hand, the unsaturated acyl moieties were suggested to oxidise more than the steryl moieties, thereby enabling protection of steryl moieties against oxidation (Soupas et al. 2005).

For the esters having monounsaturated acyl moiety, greater contents of St-OOHs than of Fa-OOHs were measured at the initial stage of the oxidation. The content of St-OOHs peaked and started to decrease before the content of Fa-OOHs. This result indicates a higher reactivity for the steryl OOHs than for the acyl OOHs for conversion into further products. The difference was clearer in the saturated lipid medium than in the neat preparations, since the oxidation occurred at a slower rate. According to theoretical bond dissociation enthalpies

(BDE), the abstraction of hydrogen from the sterol C-7 and from methyl oleate C-8 or C-11 would require same amount of energy: 328 kJ/mol and 331 kJ/mol, respectively (Pajunen et al. 2008; Lengyel et al. 2012). Thus, the reactivities of the steryl and acyl moieties should be of similar order. However, Yin et al. (2011) suggested that the oxidisabilities of Δ^5-sterols would be greater than those of monounsaturated fatty acids. The cyclic structure of a sterol and the orientation of allylic C7-αH would favour the radical formation in sterol. Moreover, the ring structure and trisubstitution of C-7 would stabilise the formed allylic radical (Sevilla et al. 1986; Yin et al. 2011), whereas for the oleyl moiety only the resonance of the allyl would stabilise the carbon or peroxyl radical. The addition of oxygen to a delocalised radical also depends on the spin distribution of the radical (Pratt et al. 2003). Greater autoxidation propagation rate constant has been determined for cholesterol (11 $M^{-1}s^{-1}$) than for oleic acid (0.8 $M^{-1}s^{-1}$) in a nonpolar medium (Porter et al. 1994; Xu et al. 2009). Thus, the structural differences of the steryl and acyl moieties may be the reason for the observed differences in the hydroperoxide contents.

The reactivity of the polyunsaturated ester was very high. The oxidation of the steryl moiety was markedly increased when the oxidation of the acyl moiety increased. The high reactivity of cholesteryl linoleate ensued from the low bond dissociation energy of the bisallylic hydrogen in the polyunsaturated acyl moiety. The pentadienyl resonance system stabilised the acyl radicals and therefore could have led to the high contents of acyl moiety hydroperoxides. The oxidation of unsaturated steryl esters has been suggested to begin by radical formation in the acyl moiety, with the formed radical then being converted into a steryl moiety C-7 radical via intra- and intermolecular radical propagation (Smith 1981, 1987, 1996; Lund et al. 1992). According to the calculated bond dissociation enthalpies, the abstraction of a hydrogen from the C-11 in the linoleyl moiety requires less energy than the abstraction of C7-αH from the steryl moiety; 283 kJ/mol and 328 kJ/mol, respectively (Pajunen et al. 2008; Lengyel et al. 2012). In addition, greater autoxidation propagation rate constant has been determined for linoleic acid (62 $M^{-1}s^{-1}$) than for cholesterol (11 $M^{-1}s^{-1}$) in a nonpolar medium (Xu et al. 2009). These findings also support the observed differences in the oxidation rates of steryl and acyl moieties: Fa-OOHs were formed at a higher rate than the St-OOHs and greater contents of Fa-OOHs were measured. However, both St-OOHs and Fa-OOHs were detected at the initial stage and their formation and decomposition occurred concomitantly.

In neat preparations, the formation rates of individual acyl moiety hydroperoxides were of similar order, but when the oxidation was slowed in the lipid medium, differences in the rates were detected. In monoenic lipids, hydrogen is abstracted from the external end of the double bond system; i.e., at the C-8 or C-11 of oleyl moiety, after which the radical site may isomerise between C-8, C-9, C-10 and C-11 (Porter et al. 1994; Schaich 2005). The hydroperoxyl groups also have been shown to localise preferably to the external positions of the double bond system (Porter et al. 1994; Pratt et al. 2003). Our results suggest that this also occurs in the acyl moieties of neat steryl esters. However, in a saturated lipid medium, the C-10 was preferred over the external positions C-11 and C-8. We are not yet able to explain this observed behaviour. In dienic lipids, hydrogen is most likely abstracted from the

bisallylic carbon C-11, after which the radical site may isomerise between the external ends of the system; i.e., C-9 and C-13 (Porter et al. 1980; Schaich 2005). In the present study, equal ratios of Fa-9-OOH and Fa-13-OOH were measured when neat steryl esters were oxidised. However, in a saturated lipid medium, the unequal ratio of Fa-9-OOHs and Fa-13-OOHs may ensue either from the steric hindrance caused by the steryl moiety or from the energetically favoured localisation at C-13 in the double bond system. The orientation of the molecules in the medium may also affect. Previously, identical ratios of oleic and linoleic acid hydroperoxide isomers have been determined when 2% of the neat fatty acids were oxidised (Porter et al. 1980; Porter et al. 1994). In our study, however, over 10% of the material was oxidised. In another study, when a greater oxidation stage was reached after oxidising neat linoleic acid at 60 °C for 1 day, the ratios of Fa-13-OOH and Fa-9-OOH were 64% and 36%, respectively (Nogala-Kalucka et al. 2007). Thus, when the oxidation proceeds further at a lower rate (e.g., under mild conditions), differences in the contents of internal and external OOH isomers may appear. The medium and environment may as well affect the formed ratios. For linoleic acid and for methyl linoleate, greater contents of 13-OOH than of 9-OOH were measured in micelles whereas the opposite was obtained in bilayer environment (Barclay et al. 1997; Xu et al. 2011).

6.4 Effects of sterol structure on the oxidation behaviour of steryl esters

Plant steryl ester oxidised in a similar manner as the corresponding cholesteryl ester

A comparison of the oxidation of stigmasteryl oleate and its cholesterol analogue revealed only small differences. The oxidation rate was slightly greater for stigmasteryl oleate compared to cholesteryl oleate. This was observed both in the primary oxidation as well as in the further reactions. The observed differences were most likely caused by the increased unsaturation of the steryl moiety (i.e., presence of a double bond in the side chain of the sterol backbone). This was expected since addition of another radical formation site is likely to increase the oxidation rate. Theoretical BDEs of 376–398 kJ/mol have been reported for the cholesterol side chain C-H bonds whereas for stigmasterol they were 320–369 kJ/mol (Lengyel et al. 2012). Thus, stigmasterol has three energetically somewhat identical radical formation sites: C-20, C-24 and C-7. Even though oxidation of the side chain was not observed, radicals were most likely formed in the side chain. Due to the mobility of the side chain, the side chain radicals have been suggested to isomerise by intramolecular radical propagation into more stable C-7 radicals (Sevilla et al. 1986). This could also explain the higher oxidation rate of the stigmasteryl moiety compared to the cholesteryl moiety in the steryl esters.

In both esters, more of St-OOHs than of Fa-OOHs were detected at the early stage of oxidation. However, the difference was dissipated as the formation of hydroperoxides escalated. At the hydroperoxide maximum, equal contents of St-OOHs and Fa-OOHs were measured for each of the esters. However, the total content of hydroperoxides was lower for

stigmasteryl oleate than for cholesteryl oleate. For both esters, a decrease in the content of St-OOHs was observed before a decrease in the content of Fa-OOHs. This indicates that St-OOHs were already undergoing further reactions. Since the oxidation rate of cholesteryl oleate was lower, the difference in the oxidisabilities of the steryl and acyl moieties could be observed more clearly.

6.5 Effects of temperature on the oxidation behaviour of steryl esters

Increased temperature shortened the initiation time and increased the oxidation rate

Increased temperature induced the oxidation of steryl esters. This was observed in increased rates of secondary oxidation and polymerisation. In general, the polymerisation of lipids is expected to occur at frying conditions (i.e., at 150−190 °C) in the presence of oxygen and water (Schaich 2005; Choe and Min 2006, 2007). For a steryl ester with a saturated or monounsaturated acyl moiety, the increased rate of polymerisation seemed to depend more on increasing temperature than on the introduction of an unsaturated acyl moiety. However, for the ester with polyunsaturated acyl moiety, the reaction rate was already very high at 100 °C, and therefore an increase in the rate was not observed when the temperature was elevated to 140 °C. Previously, the polymerisation of triacylglycerols was found to depend more on the temperature (60−220 °C) than on the degree of unsaturation (Kamal-Eldin et al. 2003b). The increased unsaturation of the acyl moiety in steryl esters, on the other hand, has been suggested to protect the steryl moiety against oxidation at 180 °C (Soupas et al. 2005). In the present study, the effect of temperature on oxidation and polymerisation was most significant for free cholesterol: At 100 °C, the free sterol remained solid and therefore unaltered, but at 140 °C, the preparation was liquefied, and thus significant contents of secondary oxides and oligomers were measured. Similar effects of temperature on the oxidation of cholesterol have been reported previously (Korahani et al. 1982; Kim and Nawar 1993).

6.6 Effects of lipid medium on the oxidation behaviour of steryl esters

Oxidation reactions of steryl esters were slowed down in saturated lipid medium

As expected, the oxidation of steryl esters occurred more slowly in tripalmitin than in neat preparations. When compounds were solubilised in the lipid medium, the contact with air was most likely reduced and thus oxidation was slowed down. The primary oxidation products were more stable in the lipid medium and therefore higher contents could be measured. The hydroperoxide profiles were qualitatively similar to those obtained in neat steryl esters at 100 °C. However, the small differences in the individual OOH contents became clearer when the reactions were slowed down. The decomposition of the hydroperoxides into further products was also delayed. The decreased oxidation rate also altered the composition of oxidation products: secondary oxidation products of sterol and oligomers were measured only after the hydroperoxide contents had levelled. The profiles of individual secondary oxidation

products of sterol remained similar. For cholesteryl linoleate, however, the secondary oxides of sterol were formed at similar rates both in tripalmitin medium and in the neat preparation. A significant increase in the epoxide contents was observed when this ester was oxidised in saturated tripalmitin medium. In nonpolar media, the addition of peroxyl radicals is favoured because of the limited access to abstractable hydrogens. Therefore, an increase in the epoxide formation was expected. Polymerisation, on the other hand, was more intensive in the neat preparation, possibly due to the small sample size and short travelling distances.

Oxidation of free cholesterol was markedly increased by the introduction of a saturated lipid medium. Neat cholesterol remained unaltered at 100 °C, but as the free sterol was partly solubilised in the lipid medium, it became prone to oxidation even at 100 °C. In a saturated lipid medium, the free sterols have been found to oxidise even at 80 °C (Soupas et al. 2004b). Thus, the oxidation susceptibilities of sterols may be altered by the physical state but also by other compounds present in the sample. Since the solubilisation of free sterols in nonpolar lipid media is limited and free sterols are known to act as surface active compounds, they are most likely found in the interphase of lipid and water or air. Thus, their susceptibility to oxidation may be highly affected by their concentration. In the present study, 8% sterol equivalents were added to tripalmitin and higher oxidation rates were obtained for free sterol than for steryl esters with either saturated or monounsaturated acyl moieties. However, the oxidation rate of a sterol with a polyunsaturated acyl moiety was greater. In a previous study, when 1% sterol equivalents of plant steryl esters or free plant sterols were added to tripalmitin, 9% of the plant steryl esters were oxidised after 2 days of heating at 100 °C whereas only 0.2−0.9% of the corresponding free sterols were oxidised (Soupas et al. 2005). The acyl moieties of the plant steryl esters originated to rapeseed oil fatty acids and thus were mainly monounsaturated (66%) and partly polyunsaturated (26%). On the other hand, in other studies conducted at 130−180 °C, triacylglycerols have been found to induce the oxidation of free cholesterol (Kim and Nawar 1991; Nawar et al. 1991). In more complex lipid models, other surface active compounds, such as other sterols, fatty acids and phospholipids, may compete with sterols in the displacement and thus affect the oxidation susceptibilities of sterols (Cercaci et al. 2007).

Tripalmitin remained unchanged during the oxidation at 100 °C, and the formed oligomers all originated from the steryl esters and free sterol. In previous studies, tripalmitin or tristeartin matrices have been confirmed to remain unchanged during oxidation at 100 °C (Soupas et al. 2005). Oxidation of tripalmitin has been observed at elevated temperatures (120−180 °C) in the presence of plant sterols (1−5%) or their rapeseed oil acyl esters (1%) (Yanishlieva et al. 1985; Porter et al. 1995; Soupas et al. 2005).

6.7 Effects of other external factors on the oxidation of steryl esters

The presence of impurities increased the oxidation rate and altered the composition of oxidation products

The presence of pro-oxidants and other impurities in the sterol preparations may initiate and promote the oxidation of lipid and sterols, thereby leading to higher reaction rates and contents of oxidation products. Since the oxidation of the steryl ester was rather slow in the present study, the presence and effects of pro-oxidants were clear: The initiation time was shortened and the oxidation rate was highly increased. In neat cholesteryl stearate, the initiation time was shortened from one day to three hours and the hydroperoxide maximum was reached already after 12 hours instead of after three days of heating. Changes in the oxidation rate also influenced the composition of formed oxidation products. For example, the formation of St-7-ketone was highly induced. In the chemical synthesis of the steryl esters, acid or base catalysts are used to promote the esterification reaction. If residues of these catalysts are left in the preparation after the purification process, they may influence the stability of sterols. In the present study, the initiation was controlled by removing possible contaminants from the steryl ester preparations and tripalmitin by adsorption chromatography.

The surfaces of containers and reaction vessels may also affect the oxidation of lipids. They may affect the orientation of lipids or they may act as catalysts or contain trace levels of metals which act as initiators (Bawn 1953; Uri 1956; Togashi et al. 1961; Kamal-Eldin et al. 2003a; Schaich 2005). The most effective metals undergo one-electron transfer (e.g., Mg^{2+}, Fe^{2+}, Co^{2+} and Cu^{2+}) (Bawn 1953; Uri 1956). Even when they are present at trace levels, they may transfer electrons to double bonds in lipids or generate oxygen radicals which react with lipids. They may also catalyse the decomposition of hydroperoxides. Especially in nonpolar media, the reactions are rapid. The presence of these residual initiators is, however, difficult to control. In the present study, the large contact area of neat preparations with the glass vial may have had an effect on the oxidation. However, the experiments were repeated and all the samples were treated in a similar manner, and therefore the results were considered comparable.

In oxidation models, sample size may have a significant role in the oxidation. In large samples, the surface area is relatively small compared to the sample volume and therefore contact with air is limited. When the sample size is reduced, the proportion of the surface area to the sample volume increases and thus enables greater contact with oxygen but also with the vessel. Due to the increased accessibility to oxygen and possible initiators, the oxidation rate may be affected (Labuza and Dugan 1971; Cercaci et al. 2007). In the present study, the sample size of neat preparations was remained small and therefore the oxidation was considered to occur as interfacial oxidation. Thus, the surface-to-volume ratio had only a minor effect. In tripalmitin samples, the effect of surface area became more important. This

was evident especially when the oxidation of esterified and free sterol was compared; while cholesteryl stearate was dissolved in the lipid medium and its oxidation occurred slowly, free sterol remained on the surface of the medium and was thus oxidised. Interfacial oxidation of lipids is known to occur in food products which contain emulsions (Labuza and Dugan 1971; Cercaci et al. 2007; Bartosz and Kołakowska 2011).

Pro-oxidants as well as antioxidants appear in different ratios in different food products. The location of different sterols also varies depending on the matrix and other compounds present. Therefore, in order to predict the stability of sterols in a particular food product, the oxidation of sterols needs to be studied in that product. If the sterols incorporated to food products are already oxidised, other compounds in the food product may promote further oxidation and thus increase the contents of sterol oxides during manufacture and storage.

7 CONCLUSIONS

Control of the formation and accumulation of sterol oxidation products in plant sterol enriched food products requires a thorough understanding of the oxidation behaviours of sterols and their conjugates. The present study focused on the development of analysis methods for the determination of intact steryl ester oxidation products and on the investigation of the oxidation behaviour of steryl esters. The effects of the acyl moiety and its unsaturation degree, as well as the effects of sterol structure, on the oxidation of sterols were examined while the oxidation was performed at different temperatures and in different media.

A combination of normal-phase solid-phase extraction and HPLC-DAD-ELSD proved to be the most efficient method for the determination of intact steryl ester monohydroperoxides. The distinction between the oxidation of the steryl and acyl moieties was achieved and the determination of individual monohydroperoxide isomers and epimers was accomplished at levels of 10−233 μg/g lipid. This method allowed the primary oxidation of steryl esters to be followed both in neat preparations and in a saturated lipid medium. Further reactions of the monohydroperoxides were followed in terms of secondary oxidation products of sterol and in terms of oligomers.

The results of the oxidation studies clearly demonstrated that, in neat preparations at 100 °C, esterification altered the physical state of the preparations and thus led to different oxidation behaviours of esterified and free sterols. The oxidation of the steryl moiety was considerably greater for esterified sterols than for free sterol. Increased unsaturation of the steryl and acyl moieties shortened the induction period of the oxidation and increased the formation rate of primary hydroperoxides of both steryl and acyl moieties. The hydroperoxide contents also started to decrease earlier and consequently the formation of secondary oxides of sterol and oligomers advanced. The hydroperoxides not only decomposed into traditionally determined secondary oxidation products of sterol, but also polymerised in a rival reaction. Differences in the initial reactivities of the steryl and acyl moieties were not observed, but steryl and acyl moiety hydroperoxides were formed concomitantly. Increased unsaturation of the acyl moiety did not postpone the oxidation of steryl moiety, as expected, but instead increased it. Thus, protection of the steryl moiety against oxidation was not achieved by addition of a more reactive counterpart. At the termination stage, oligomers were initially formed alongside with the secondary oxidation products, but after prolonged heating, polymerisation became dominant.

Increased temperature shortened the induction period and increased the oxidation rates of both esterified and free sterols. At 140 °C, a steryl ester with a saturated acyl moiety and free sterol oxidised in a similar manner. Polymerisation was pronounced at higher temperatures. Introduction of a saturated lipid medium at 100 °C decelerated the oxidation of steryl esters, as expected, but induced the oxidation of free sterol. Due to the dissimilar polarities of

esterified and free sterols, their displacement in the lipid medium was likely to differ and lead to their different oxidisabilities. As the oxidation of steryl esters occurred more slowly in a lipid medium than in neat preparations, small differences in the initial reactivities of the steryl and acyl moieties were observed: Slightly greater contents of steryl than of acyl moiety hydroperoxides were detected for the esters with monounsaturated acyl moieties. For an ester with a polyunsaturated acyl moiety, the reaction rate was still great in the lipid medium, and differences in the reactivities of the steryl and acyl moieties were not observed.

By modifying the chemical and physical properties of sterols as well as by controlling the external factors, the oxidation of sterols may be altered significantly. As the present study demonstrated, it is important to monitor the oxidation of both steryl and acyl moieties of steryl esters, since they have an impact on each other. The results obtained for the oxidation behaviours of steryl esters may be utilised when new processing methods are developed for incorporation or manufacture of plant sterol fortified food products.

In the future, further development of isolation and determination methods used for the analysis of intact steryl ester oxidation products from more complex food models and food products is still required. Extraction methods, in which the separation is based on the sterol structure, are particularly needed for selective isolation of intact steryl ester oxidation products from other lipid oxidation products with similar polarities. Along with the evolved analysis methods, knowledge of the oxidation reactions of steryl esters in unsaturated lipid media, emulsions and food products may be gained. As the location and presence of other compounds in the food products may affect the oxidation of sterols and their conjugates in different manners, it is important to study their reactions in the food products in question.

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