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Phytosterol and Acyl Chain Oxidation Products Formed upon Thermal Treatment of Phytosteryl/-stanyl Fatty Acid Esters

Stefan Paul Wocheslander

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Vorsitzender: Univ.-Prof. Dr. Wilfried Schwab

Prüfer der Dissertation: 1. Univ.-Prof. Dr. Karl-Heinz Engel

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ABBREVIATIONS

ABC	ATP-binding cassette transporter
ACAT 2	acyl CoA cholesteryl acyltransferase 2
ACEH	lysosomal acid cholesteryl ester hydrolase
ACOP(s)	acyl chain oxidation product(s)
ApoB48	apoprotein B48
BD	bile duct
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
CAD	coronary artery disease
Caco	colonic adenocarcinoma
Chol	cholesterol
CoA	coenzyme A
COSY	correlation spectroscopy
Cyp7α1	cholesteryl-7-α-hydroxylase
DMAPP	dimethylallyl diphosphate
DNPH	dinitrophenylhydrazone
EFSA	European Food Safety Authority
ELSD	evaporative light scattering detector
FID	flame ionization detector
FPP	farnesyl diphosphate
GC	gas chromatography
HDL	high-density lipoprotein
HMBC	heteronuclear multiple-bond correlation spectroscopy
HPLC	high performance liquid chromatography
HSQC	heteronuclear single-quantum correlation spectroscopy
i.d.	internal diameter
IL	interleukin
IPP	isopentenyl diphosphate
IS	internal standard
IUPAC	International Union of Pure and Applied Chemistry
LC	liquid chromatography

LDL	low-density lipoprotein
LDLR	LDL receptor
LM	liquid margarine
LRP	LDL receptor-related protein
LXR	liver X receptor
MCP	monocyte chemotactic protein
MMI	multimode inlet
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTBE	methyl *tert*-butyl ether
MVA	cytosolic mevalonate
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
NPC1L1	Niemann-Pick C1-like protein 1
9-ONC	cholesteryl 9-oxononanoate
ORP	oxidation rate
PAE	phytostanyl fatty acid ester
PCM	pneumatics control module
Phyto	plant sterols
POP(s)	phytosterol oxidation product(s)
PS	free phytosterols
PSE	phytosteryl fatty acid ester
PTV	programmable temperature vaporizer
ROS	reactive oxygen species
RRT	relative retention time
SC	side chain
SPE	solid-phase extraction
SR-B1	scavenger receptor class B
SREBPs	sterol regulatory element-binding proteins
SSD	sterol-sensing domain
TCMS	trichloromethylsilane

TG	triacylglycerides
TGFβ1	transforming growth factor β1
TLC	thin layer chromatography
TMS	trimethylsilyl
TNF-α	tumor necrosis factor-α
U937	human myelomonocytic leukemia
VLDL	very low-density lipoprotein

1. INTRODUCTION AND OBJECTIVES

A high plasma level of low-density lipoprotein (LDL)-cholesterol has been identified as a major risk factor for the development of cardiovascular diseases (Roeters van Lennep *et al.*, 2002), the prevalent cause of death in developed countries (WHO, 2015). A daily dietary intake of 2-3 g phytosterols/-stanols was shown to reduce total and LDL-cholesterol levels in hypercholesterolemic patients by approximately 9-12 % (Katan *et al.*, 2003; AbuMweis *et al.*, 2008; Demonty *et al.*, 2009; Talati *et al.*, 2010; Ras *et al.*, 2014) and thus, represents a possible therapeutic approach to counteract a high level of low-density lipoprotein (LDL)-cholesterol. On the basis of their cholesterol-lowering properties, phytosterols/-stanols have been used for the enrichment of a broad range of foods (EFSA, 2008). In order to improve the intestinal solubility and the solubility in the mostly fat-based food matrices, phytosterols/-stanols are not added in their free forms but rather as fatty acid esters (Moreau *et al.*, 2002; Trautwein *et al.*, 2003; MacKay and Jones, 2011). Phytosterols/-stanols and their fatty acid esters are classified as "*novel food ingredients*" since their dietary intake via enriched foods is eight- to twelvefold higher than that expected from a traditional diet. Therefore, in the European Union, foods enriched with phytosteryl/-stanyl fatty acid esters fall in the scope of the Regulation (EC) No. 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients (EU, 1997) that was amended and replaced by Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 (EU, 2015).

In agreement with the reactivity of the structurally related cholesterol, phytosterols are susceptible to oxidations on the basis of their unsaturation in the sterol ring system. In foods, the oxidation of phytosterols proceeds via autooxidation, a free radical mechanism that is catalysed in the presence of heat, light, air, water and transition metals (Porter *et al.*, 1995; Garcia-Llatas and Rodriguez-Estrada, 2011). Similarly to cholesterol oxidation products, the formation and dietary intake of the resulting phytosterol oxidation products is discussed to cause harmful effects in humans, such as e.g. cytotoxic and pro-inflammatory properties (Garcia-Llatas and Rodriguez-Estrada, 2011; Vanmierlo *et al.*, 2013). In addition, the oxidation of phytosterols may decrease the intial cholesterol-lowering efficiency of phytosterols (Liang *et al.*, 2011).

Cooking and baking applications may provide conditions that initiate the formation of phytosterol oxidation products in foods. The European Food Safety Authority (EFSA) was asked to provide its scientific opinion on an application requesting the use of phytosteryl fatty

acid ester-enriched liquid margarines for cooking and backing purposes (Unilever, 2013). To provide data on the formation of phytosterol oxidation products in an equal type of food, in the course of the present study the formation of phytosterol oxidation products in a non-enriched and in a phytosteryl/-stanyl fatty acid ester-enriched liquid margarine should be compared upon various heat treatments that may represent typical applications in the home. Moreover, key parameters that may influence the oxidation of esterified phytosterols in the course of cooking or baking applications should be identified. For this purpose, a method based on on-line LC-GC that was previously developed for the analysis of phytosterol oxidation products in a phytosteryl/-stanyl fatty acid ester-enriched margarine after cleavage of the ester bonds should be applied (Scholz *et al.*, 2015b).

The identities of phytosterol oxidation products formed upon thermo-oxidations of phytosteryl fatty acid esters are well-known. However, in a recent study the formation of POPs in an enriched margarine upon various heat treatments was shown to account for only approx. 20 % of degraded phytosteryl/-stanyl fatty acid esters; in a phyostanyl fatty acid ester-enriched margarine, POPs accounted for even < 1 % (Scholz *et al.*, 2016). This indicated that oxidative modifications in the fatty acid moieties may account for part of the degraded phytosteryl/-stanyl fatty acid esters, particularly for those of unsaturated fatty acids. However, the current knowledge on acyl chain oxidation products of phytosteryl/-stanyl fatty acid esters is almost exclusively limited to the formation of unstable, primary hydroperoxides in the fatty acid moieties (Lehtonen *et al.*, 2012b). Therefore, acyl chain oxidation products formed upon thermal treatment of phytosteryl/-stanyl fatty acid esters should be identified in the second part of this thesis, taking the existing knowledge on oxidized cholesteryl fatty acid esters and oxidized fatty acids into account. In addition, a method for the quantitative determination of acyl chain oxidation products should be developed to investigate the extent of oxidations in the fatty acid moieties in different model mixtures of phytosteryl/-stanyl fatty acid esters.

Analyses of oxidized acyl moieties of triacylglycerides are commonly done after conversion of the oxidized triacylglycerides into FAME. This enables the collective analysis of fatty acid methyl esters that exhibit the same oxidized acyl moiety, however, the structural information on the original triacylglycerides is lost (Dobarganes and Márquez-Ruiz, 2007). As a spectrum of individual phytosteryl/-stanyl fatty acid esters oxidized in the fatty acid moieties should be analyzed, the conversion of esters into FAME was not applicable. Gas chromatography was previously shown to be a suitable platform for the analysis of intact phytosteryl/-stanyl fatty acid esters (Barnsteiner *et al.*, 2012). Therefore, the analysis of individual acyl chain oxidation

products of phytosteryl/-stanyl fatty acid esters via gas chromatography was also considered suitable, particularly for those oxidation products that result from the cleavage of the fatty acid moieties and thus showing lower molecular weights than that of the original phytosteryl/-stanyl fatty acid esters.

2. BACKGROUND

2.1. Phytosterols/-stanols and Their Conjugates

2.1.1. Structural Properties, Biosynthesis and Biological Function in Plants

Phytosterols are the plant-derived structural analogues of cholesterol, the predominant sterol found in mammalian cells. Both phytosterols and cholesterol are amphipatic steroid alcohols biosynthetically derived from squalene and form a group of triterpenes. They are characterized by a tetracyclic cyclo-penta[*α*]phenanthrene ring, two methyl groups at C_{10} and C_{13}, a varying side chain at C_{17} consisting of 9-10 C-atoms, and a polar hydroxyl group at C_3 contributing to hydrogen-bond interactions (Piironen *et al.*, 2000; Nes, 2011). The two existing systems recognized for the numbering of carbon atoms of the sterol nucleus and side chain are shown in Figure 1.

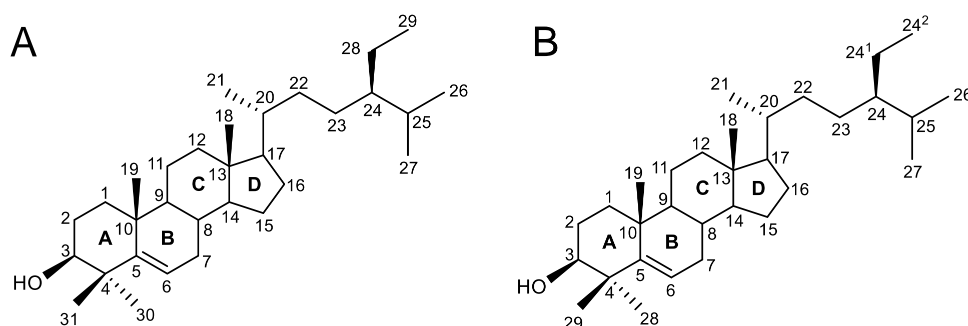

Figure 1. Numbering of basic sterol structures according to (A) Fieser (Fieser and Fieser, 1959) and (B) IUPAC recommendations (Moss, 1989).

Chemical surveys on the sterol compositions of prokaryotes, eukaryotes, and sedimentary organic matter show that there are at least 250 sterols and related steranes (Nes, 2011). Based on their structural properties, phytosterols can be grouped in three classes: 4-desmethyl sterols, 4*α*-monomethyl sterols, and 4,4-dimethyl sterols (Akihisa *et al.*, 1991). The quantitatively dominating 4-desmethyl sterols can be further categorized into Δ^5-sterols, Δ^7-sterols and $\Delta^{5,7}$-sterols, pursuant to the position and number of double bonds in the B-ring (Nes, 1987). Essential differences between phytosterols and the related cholesterol are in the side chain (C_{20}-C_{26}/C_{27}), which exhibits various degrees of substitution and unsaturation depending on the individual phytosterols (Nes, 2011). In particular, the alkylation of C_{24} occurs only in plant sterols. The most abundant phytosterols belong to the Δ^5-sterols, exhibit an additional methyl or ethyl group at C_{24}, and may have a *trans*-Δ^{22}-double bond in the side chain (Figure 2) (Piironen *et al.*, 2000).

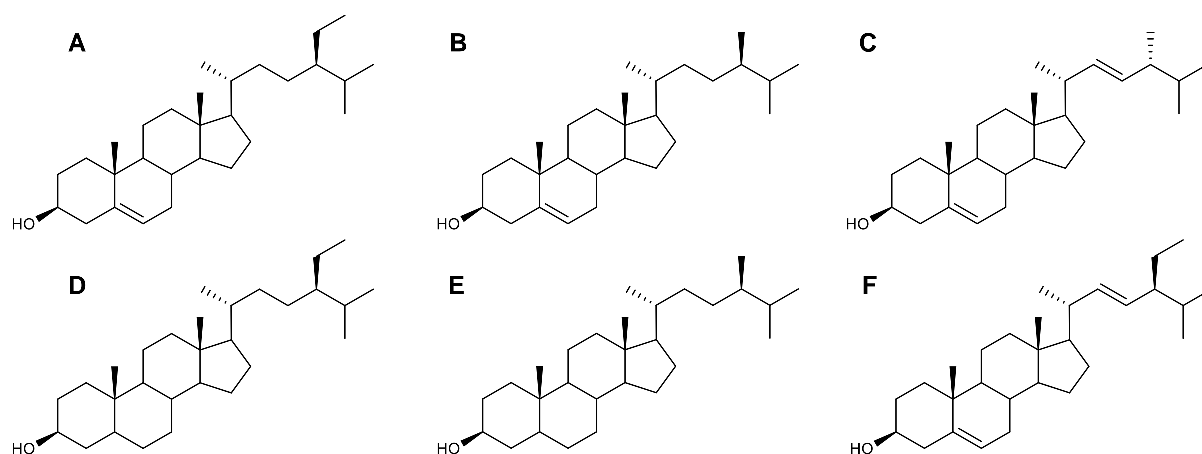

Figure 2. Structures of most abundant plant sterols and stanols: (**A**) sitosterol, (**B**), campesterol, (**C**) brassicasterol, (**D**) sitostanol (stigmastanol), (**E**) campestanol, and (**F**) stigmasterol.

Phytosterols are products of the isoprenoid biosynthetic pathway which is characterized by a sequence of more than 30 enzyme-catalyzed reactions, all associated with membranes (Hartmann, 1998; Piironen *et al.*, 2000; Benveniste, 2004). A pair of isomeric C_5 diphosphates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), represents the two initial precursors of the isoprenoid biosynthetic pathway which are mainly formed via the cytosolic mevalonate (MVA) pathway. The subsequent condensation of three C_5 units leads to the formation of farnesyl diphosphate (FPP, C_{15}). The transformation of two molecules FPP into squalene (C_{30}), the central precursor for all sterols, is controlled by the activity of squalene synthase (Piironen *et al.*, 2000; Miras-Moreno *et al.*, 2016). The following enzymatic steps taken by plants with a phytosynthetic apparatus in the course of the post-squalene pathway differ significantly from those of fungi and mammals (Figure 3). In plants, oxidized squalene is converted into cycloartenol followed by a series of methylation, desmethylation and desaturation reactions, eventually resulting in a mixture of phytosterols consisting of mainly campesterol, sitosterol, and stigmasterol. The post-squalene pathway is characterized by critical rate-limiting steps (e.g. the methylation of cycloartenol). By contrast, in non-photosynthetic fungi and mammals oxidized squalene is transformed into lanosterol, finally leading to the formation of ergosterol and cholesterol, respectively (Hartmann, 1998; Piironen *et al.*, 2000).

In their capacity as essential membrane compounds, phytosterols are capable of changing the biophysical properties of membranes, i.e. the fluidity and permeability, and may play a role in the adaption to temperature (Piironen *et al.*, 2000; Neelakandan *et al.*, 2010). They ensure a proper transport of vesicles and interact with integral membrane proteins, which regulate the

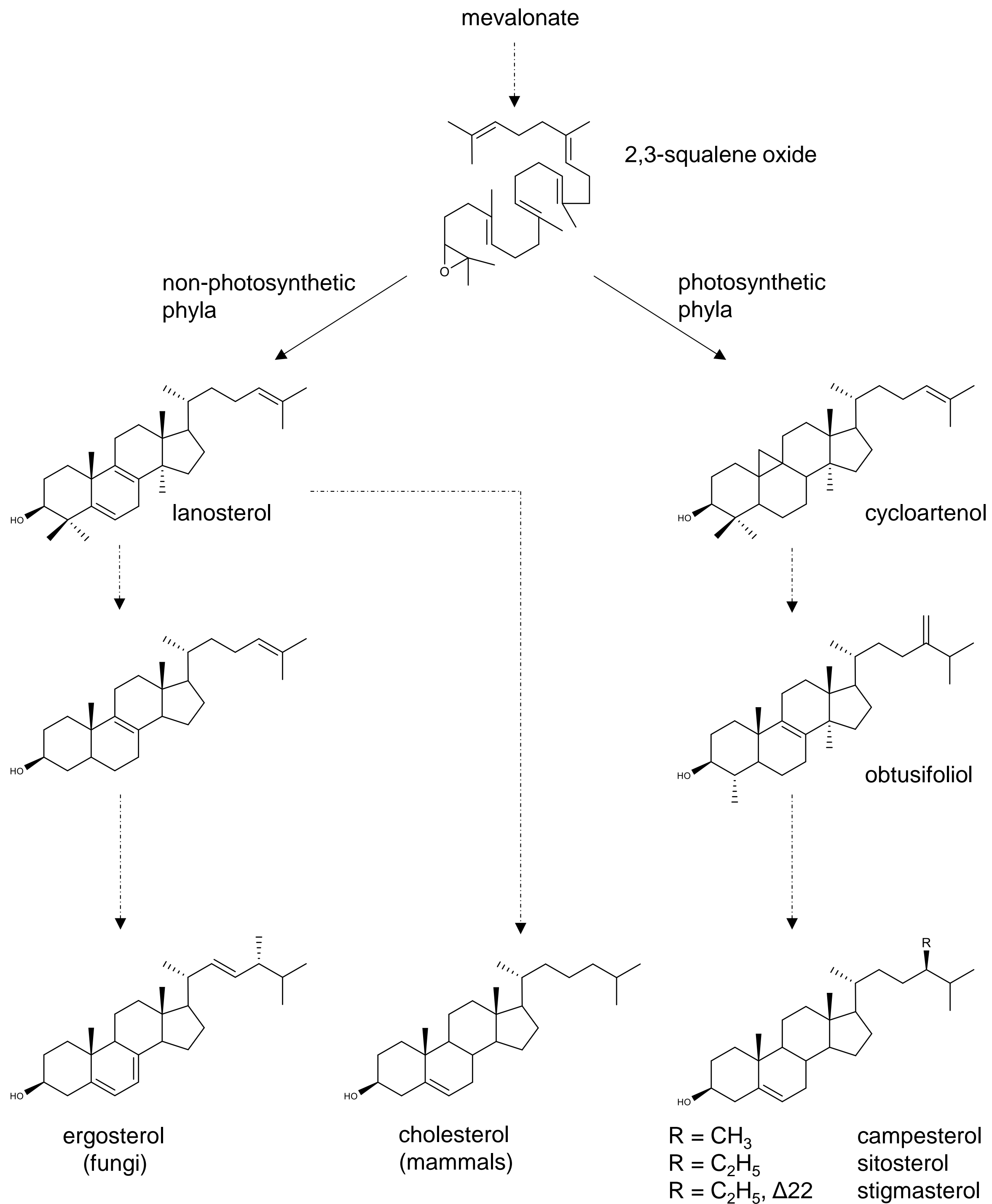

Figure 3. Simplified scheme of sterol biosynthesis pathways in fungi, mammals and plants. Dashed lines indicate multiple steps.

physical state of the lipid bilayer and phospholipids (Boutté and Grebe, 2009). In addition, sterols play an important role in cellular and developmental processes in plants as precursors of plant steroid hormones and further secondary metabolites, such as glycoalkaloids, cardenolides, and saponins (Hartmann, 1998; Piironen *et al.*, 2000). Moreover, sterols are involved in

membrane-associated metabolic processes by e.g. regulating the activity of the plasma membrane H^+-ATPase (Grandmougin-Ferjani *et al.*, 1997). Some studies also reported a participation of phytosterols in plant defence responses against various types of stress both biotic and abiotic (Devarenne *et al.*, 2002; Griebel and Zeier, 2010).

In plants, the hydroxyl group at C_3 of free sterols may also be esterified with various fatty acids or phenolic acids, and may be involved in an ether bond *β*-linked to the 1´ position of carbohydrates (Piironen *et al.*, 2000). Other biological functions than those described for free sterols are attributed to the resulting steryl fatty acid esters, steryl phenolic acid esters, and steryl glycosides or acetylated steryl glycosides (Figure 4).

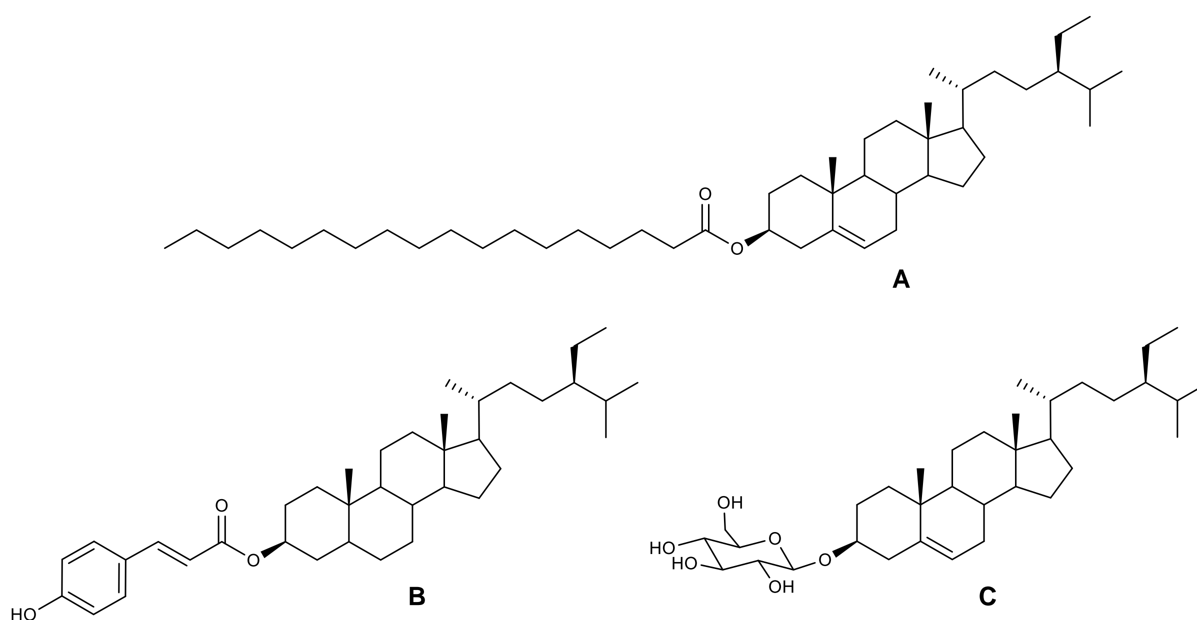

Figure 4. Representative structures of phytosteryl/-stanyl conjugates: (**A**) sitosteryl stearate, (**B**) *trans*-sitostanyl *p*-coumarate, and (**C**) sitosteryl-*β*-D-glucopyranoside.

The esterified fatty acids in steryl fatty acid esters usually exhibit 12-22 carbon atoms, with palmitic (C_{16}) and stearic acid (C_{18}) being the predominant fatty acids. Their roles are assumed to include mainly storage and transport functions; this is supported by their presence in lipid bodies in the cytosol and in soluble forms in droplets (Wojciechowski, 1991; Dyas and Goad, 1993). Furthermore, the acylation of sterols is considered as a regulating mechanism to maintain the physiological levels of sterols and stanols in cell membranes, whereby several acyl donors including phospholipids, diacylglycerols or triacylglycerols were suggested (Dyas and Goad, 1993; Schaller, 2004; Chen *et al.*, 2007). Steryl and stanyl phenolic acid esters were only reported to occur in cereals (Moreau *et al.*, 2002; Esche *et al.*, 2012; Mandak and Nystroem, 2012). Their biological functions are not yet well understood; however, their role in plants may

be associated with their demonstrated antioxidant properties (Juliano *et al.*, 2005; Nystroem *et al.*, 2005; Tan and Shahidi, 2013). In their function as structural components, steryl glycosides and acylated steryl glycosides co-occur with free sterols in various plant-cell membranes (Grille *et al.*, 2010).

2.1.2. Metabolism of Sterols

In humans, cholesterol can originate from dietary intake as well as from *de novo* synthesis, whereas phytosterols only derive from the consumption of plant-derived foods (Salen *et al.*, 1970; McLean *et al.*, 2012). This means that cholesterol biosynthesis is influenced by the dietary intake and cellular requirements based on complex feedback mechanisms (McLean *et al.*, 2012). The western-type diet contains on average approximately 300 mg of cholesterol per day, which is generally recognized to be important for the serum cholesterol homoeostasis. Likewise, similar amounts of plant sterols and roughly 50 mg of plant stanols are consumed every day (Olkkonen *et al.*, 2017). Both dietary cholesterol and plant sterols can occur either in their free form or esterified to fatty acids. To become available for intestinal absorption, the fatty acid esters are hydrolysed by the pancreatic cholesterol esterase, which was also shown to accept phytosteryl/-stanyl esters as substrates (Moreau and Hicks, 2004). Several *in vitro* and *in vivo* studies indicated an influence of both the phytosterol/-stanol and the fatty acid moieties on intestinal hydrolysis rates (Brown *et al.*, 2010b; Ash *et al.*, 2011; Lubinus *et al.*, 2013). A schematic representation of subsequent major pathways of the metabolism of sterols in humans is depicted in Figure 5. In a first step (Figure 5A), sterols are incorporated in micellar forms, enabling the interaction of the sterol-laden micelles with the intestinal brush border membrane and, thus, facilitating the uptake of sterols by enterocytes (Brufau *et al.*, 2008). The Niemann-Pick C1-like protein 1 (NPC1L1) mediates the uptake of sterols and stanols, but at a lower efficiency for plant sterols and stanols than for cholesterol (Othman *et al.*, 2013; Olkkonen *et al.*, 2017). In enterocytes, the absorbed sterols may be esterified with fatty acids by acyl CoA cholesteryl acyltransferase 2 (ACAT 2). However, plant sterols were shown to be poor substrates for ACAT 2, therefore the majority of plant sterols remains in their free form (Lin *et al.*, 2010). The ABCG5 and ABCG8 transporters form a membrane-associated pump, which recycles non-esterified sterols and stanols from enterocytes back into the intestinal lumen.

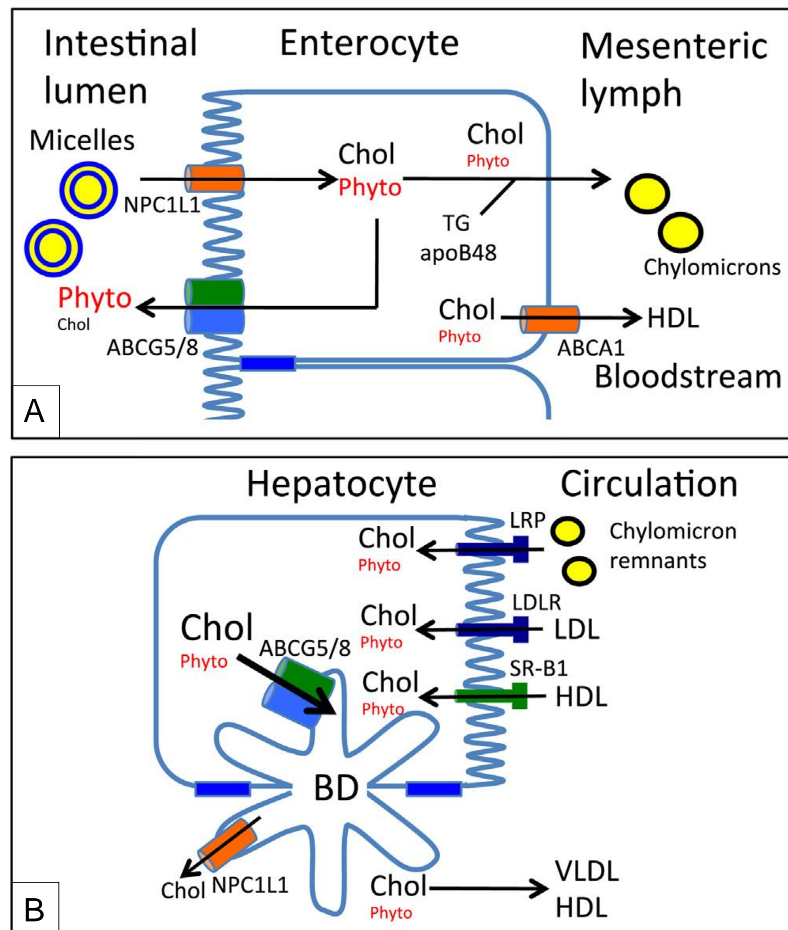

Figure 5. Overview of major pathways in the absorption and intracellular traffic of cholesterol (Chol) and plant sterols (Phyto) in (A) enterocytes (intestine) and (B) hepatocytes (liver) (adapted from Olkkonen *et al*., 2017). ABC, ATP-binding cassette transporter; apoB48, apolipoprotein-B48; BD, bile duct; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDLR, LDL receptor; LRP, LDL receptor-related protein; NPC1L1, Niemann-Pick C1-like protein 1; SR-B1, scavenger receptor class B, type 1; TG, triacylglycerides; very low-density lipoprotein, VLDL.

These types of transporters also play a role at the hepatocyte plasma membrane domain facing bile ducts (BD), by removing plant sterols and stanols that have already entered hepatocytes, into bile and subsequently in the intestine (Sabeva *et al.*, 2011; Olkkonen *et al.*, 2017) (Figure 5B). The esterified sterols in enterocytes, along with triacylglycerides and apoprotein B48 (apoB48), are secreted as chylomicrons into the mesenteric lymph and into the bloodstream via high-density lipoprotein (HDL), and are taken up by the liver via lipoprotein receptors (LRP/SR-B1). In hepatocytes, absorbed sterols and *de novo* synthesized cholesterol are either stored or partly excreted into bile ducts, and incorporated into very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) particles for secretion into the circulation (Figure 5B). Via hydrolysis of lipid constituents, VLDL particles in the circulation are transformed into smaller LDL particles; a majority of LDL particles is, in turn, reinternalized into the liver.

Likewise, most of the HDL formed in the liver and in the small intestine returns to the liver (Elshourbagy *et al.*, 2014; Olkkonen *et al.*, 2017).

Considerable quantitative differences between the intestinal absorption of phytosterols/-stanols and cholesterol were reported. In humans, about 29-80 % of dietary cholesterol is absorbed, whereas significantly lower percentages were determined for phytosterols/-stanols, ranging from 0.04 % for sitostanol up to 16 % for campesterol (Salen *et al.*, 1970; Heinemann *et al.*, 1993; Bosner *et al.*, 1999; Ostlund *et al.*, 2002; Sudhop *et al.*, 2002). Lower intestinal absorption of phytosterols/-stanols may be explained by an increased hydrophobicity of phytosterols/-stanols compared to cholesterol caused by the presence of an additional methyl or ethyl group in the side chain on C_{24} and by the fully saturated structure of stanols, and by the low affinity of phytosterols/-stanols towards ACAT 2 catalysed esterification in enterocytes (Heinemann *et al.*, 1993; Lin *et al.*, 2010).

2.1.3. Cholesterol-lowering Properties of Phytosterols/-stanols

In the early 1950s, the dietary intake of sitosterol was reported to lower blood cholesterol levels in humans, indicating cholesterol-lowering effects of phytosterols for the first time (Pollak, 1953a). These findings laid the foundation for the application of phytosterols/-stanols and their esters in the treatment of hypercholesterolemia (Pollak, 1953b; Thompson and Grundy, 2005). A high plasma level of low-density lipoprotein (LDL)-cholesterol was identified as a major risk factor for the development of cardiovascular diseases, the prevalent cause of death in developed countries (McLean *et al.*, 2012). In the meantime, the LDL-cholesterol-lowering effect upon dietary intake of phytosterols/-stanols is well-established by numerous meta-analyses (Katan *et al.*, 2003; AbuMweis *et al.*, 2008; Demonty *et al.*, 2009; Ras *et al.*, 2014). However, the exact mechanisms of action are still subject of current research and not yet fully understood.

The impaired micellar solubilisation of intestinal cholesterol in the presence of phytosterols/-stanols is generally recognized to contribute to the cholesterol-lowering properties of phytosterols/-stanols (Trautwein *et al.*, 2003; Mel'nikov *et al.*, 2004; Plat *et al.*, 2015). Due to their higher hydrophobicity, phytosterols/-stanols exhibit a higher affinity towards the incorporation into micelles than cholesterol, leading to a decrease of cholesterol solubilisation in the intestine, a requirement for its absorption in enterocytes. Among plant sterols, stigmasterol exhibited the highest potential to exclude cholesterol from the incorporation into micelles in model bile. However, phytostanols are generally considered to be more potent than phytosterols in the replacement of cholesterol in micelles (Brown *et al.*, 2010a; De Smet *et al.*,

2012). However, the findings of studies reporting equal efficacies in LDL-cholesterol-lowering effects after the consumption of the same amount of phytosterols/-stanols as a whole or divided over the day, are difficult to explain with the impaired micellar solubilisation of cholesterol (Plat *et al.*, 2000; Matvienko *et al.*, 2002). A co-crystallization of phytosterols/-stanols with cholesterol leading to poorly absorbable mixed crystals during food lipolysis, was proposed as another possible mechanism (Trautwein *et al.*, 2003; Rozner *et al.*, 2009). However, due to the high solubility of free sterols/stanols in products of fat hydrolysis, this mechanism is not assumed to largely affect the intestinal absorption of cholesterol (Mel'nikov *et al.*, 2004). Due to their structural relationship to cholesterol, phytosterols/-stanols may also exert effects as competitive inhibitors on the NPC1L1 transporter and acyl CoA cholesteryl acyltransferase 2 (ACAT 2), resulting in less absorption of cholesterol into enterocytes and less esterification of cholesterol in enterocytes (Trautwein *et al.*, 2003; De Smet *et al.*, 2012). In addition, phytosterols/-stanols are assumed to be involved in the regulation of genes related to cholesterol metabolism. Phytosterols/-stanols were shown to be potent liver X receptor (LXR) ligands, which are linked to an increased ABCAG5/G8 activity (De Smet *et al.*, 2012; Hoang *et al.*, 2012); they were also shown to induce the expression of cholesteryl-7-α-hydroxylase (Cyp7α1), which regulates the synthesis of bile acids and thus, indicating another potential mechanism of cholesterol reduction (Davis *et al.*, 2002). It is known that the activation of sterol regulatory element-binding proteins (SREBPs), master regulators of lipid homeostasis, is tightly controlled by a sterol-sensing domain (SSD). In a cholesterol-depleted state, SREBP activation upregulates the expression of genes involved in cholesterol synthesis. A recent study reported that phytosterols are also being sensed by SSD, thus preventing the activation of the nuclear receptors and consequent upregulation of genes associated with cholesterol metabolism (Danesi *et al.*, 2016). As dietary phytosterols/-stanols may be present in esterified form, an essential precondition for all the proposed cholesterol-lowering mechanisms is the intestinal hydrolysis of phytosteryl/-stanyl esters (De Smet *et al.*, 2012; Carden *et al.*, 2015).

As soon as phytosterols are causing a reduced cholesterol absorption, the endogenous cholesterol biosynthesis is being upregulated as well as the expression and activity of LDL-receptors. This results in a reduction of serum LDL-cholesterol levels despite the increase of cholesterol biosynthesis (Plat and Mensink, 2005; Brufau *et al.*, 2008).

2.1.4. Enrichment of Foods with Phytosteryl/-stanyl Fatty Acid Esters

Owing to their cholesterol-lowering properties, phytosterols/-stanols have been used for the enrichment of a broad range of foods, including mainly margarines, yoghurt-type products, milk/soy-based drinks, cheese-type products, dressings and edible oils (EFSA, 2008). The percentages of phytosterols/-stanols in enriched foods differ considerably, ranging from 0.3 % in milk-type products to 8 % in margarines (Barnsteiner *et al.*, 2012). In order to improve the intestinal solubility and the solubility in the mostly fat-based food matrices, phytosterols/-stanols are not added in their free forms but rather as fatty acid esters (Moreau *et al.*, 2002; Trautwein *et al.*, 2003; MacKay and Jones, 2011). Phytosterols/-stanols and their fatty acid esters are classified as "*novel food ingredients*" since their dietary intake via enriched foods is eight- to twelvefold higher than that expected from a traditional diet. Therefore, in the European Union, foods enriched with phytosteryl/-stanyl fatty acid esters fall in the scope of the Regulation (EC) No. 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients (EU, 1997) that was amended and replaced by Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 (EU, 2015).

Meanwhile, several meta-analyses clearly demonstrated the cholesterol-lowering effects upon dietary intake of foods enriched with phytosteryl/-stanyl fatty acid esters. The daily dietary intake of 2-3 g phytosterols/-stanols was reported to reduce total and LDL-cholesterol levels in hypercholesterolemic patients by approximately 9-12 % (Katan *et al.*, 2003; AbuMweis *et al.*, 2008; Demonty *et al.*, 2009; Talati *et al.*, 2010; Ras *et al.*, 2014). In contrast, in a population of healthy volunteers with no or only mild hypercholesterolemia supplied with a daily amount of 3 g plant sterols, total serum cholesterol and LDL-cholesterol were not affected (Weingaertner *et al.*, 2017). Daily doses higher than 3 g of phytosterols/-stanols did not result in additional beneficial effects, but might even trigger undesired reactions, such as the reduction of serum concentrations of *β*-carotene, *α*-carotene and lycopene (Plat and Mensink, 2005; Demonty *et al.*, 2009). Therefore, foods enriched with phytosterols/-stanols need to be labelled with the information that the consumption of 3 g phytosterols/-stanols per day should not be exceeded (EC, 2004).

As discussed in chapter 2.1.3, the intestinal hydrolysis of phytosteryl/-stanyl fatty acid esters was identified as an essential precondition, enabling phytosteryl/-stanyl fatty acid esters to exert their cholesterol-lowering properties. In both *in vitro* and *in vivo* studies, intestinal hydrolysis rates of phytosteryl/-stanyl fatty acid esters were shown to be influenced by the sterol/stanol

and fatty acid moieties, indicating possible structural dependencies on the cholesterol-lowering efficiencies (Brown *et al.*, 2010b; Lubinus *et al.*, 2013). This is, however, not reflected by the available meta-analyses, as phytosteryl and phytostanyl fatty acid esters generally exhibited similar cholesterol-lowering potencies (Moreau *et al.*, 2002; Ras *et al.*, 2014). Likewise, the influence of the fatty acid moieties on the cholesterol-lowering properties of phytosteryl fatty acid esters was demonstrated to be low in hamsters supplemented with phytosteryl esters derived from sunflower and canola oil (Liu *et al.*, 2015).

2.2. Oxidation of Sterols

2.2.1. Cholesterol Oxidation Products

Oxidation reactions of cholesterol have already been studied for years. The so-called oxysterols are oxidised derivatives of cholesterol (Bjorkhem and Diczfalusy, 2002; Olkkonen *et al.*, 2017). In healthy mammals, the most abundant oxysterols are formed in *vivo* by enzymatic cholesterol oxidation in various tissues where they are involved in the regulation of the cholesterol metabolism and the permeability of membranes (Sottero *et al.*, 2009; Olkkonen *et al.*, 2017) (Figure 6). Under pathophysiological conditions involving oxidative stress, such as hyperlipidemia, atherosclerosis, obesity, insulin resistance, and advanced kidney disease, oxysterols may also arise from in *vivo* autoxidation leading to increasing oxysterol serum concentrations (Olkkonen and Lehto, 2004; van Reyk *et al.*, 2006; Alemany *et al.*, 2014). Finally, oxysterols may be derived from processed foods and subsequent dietary intake (Leonarduzzi *et al.*, 2002). Owing to the structural resemblance of cholesterol and phytosterols, the demonstrated biological effects of oxysterols, and the use of phytosterols as active ingredients in foods, increasing scientific efforts on phytosterol oxidation products have been undertaken in recent years.

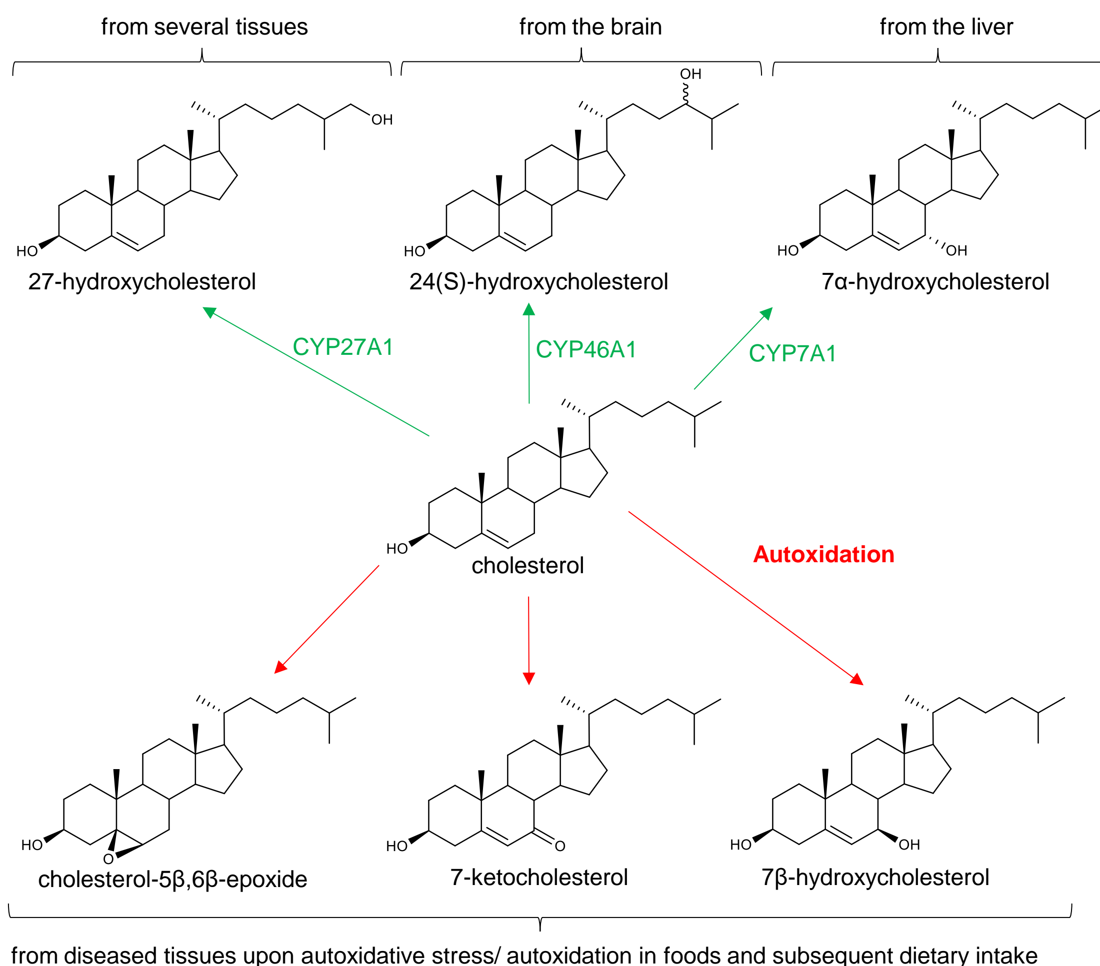

Figure 6. Generation and origin of some abundant cholesterol oxidation products in mammals. The species in the top row are enzymatically formed via cholesterol hydroxylases belonging to the cytochrome P450 family (green arrows). The species depicted at the bottom arise from non-enzymatic oxidation (autoxidation) *in vivo* or derive from cholesterol autoxidation in foods and subsequent dietary intake (red arrows) (adapted from Olkkonen *et* al., 2017).

2.2.2. Phytosterol Oxidation Products

2.2.2.1. Enzymatic Oxidation of Phytosterols

Similar to the enzymatic *in vivo* oxidation of cholesterol, several *in vitro* studies indicated a potential endogenous, enzymatic formation of phytosterols. Both oxidations in the sterol and in the side chain of *β*-sitosterol were reported in rat liver mitochondria, but conversion rates of *β*-sitosterol were low compared to cholesterol (Aringer and Eneroth, 1973; Aringer and Eneroth, 1974; Aringer *et al.*, 1976). Nonetheless, similar formation rates were observed for the side chain hydroxylation of campesterol and cholesterol (Aringer *et al.*, 1976). However, studies in this field are rather limited. Moreover, it is widely acknowledged that the main routes of phytosterol oxidations proceed via non-enzymatic autoxidation (Garcia-Llatas and Rodriguez-Estrada, 2011).

2.2.2.2. Autoxidation of Phytosterols

In accordance with the reactivity of other unsaturated lipid molecules, non-enzymatic oxidation of phytosterols may generally be accomplished via photooxidation and autoxidation (Dutta, 2004; Otaegui-Arrazola *et al.*, 2010). However, the current knowledge on photooxidation reactions of plant sterols, which commonly require the presence of photosensitizers to convert triplet oxygen ($^3O_2$) into highly reactive singlet oxygen ($^1O_2$), is rather scarce. Therefore, autoxidation by triplet oxygen is considered to be the major non-enzymatic oxidation mechanism of phytosterols (Otaegui-Arrazola *et al.*, 2010; Garcia-Llatas and Rodriguez-Estrada, 2011). Most of the knowledge on oxidation mechanisms of phytosterols refers back to studies on the structurally related cholesterol (Smith, 1987; 1996). In accordance with the knowledge about other unsaturated lipids, autoxidation of phytosterols proceeds via a free radical mechanism, which is catalysed by the presence of heat, light, air, water and transition metals (Porter *et al.*, 1995; Garcia-Llatas and Rodriguez-Estrada, 2011). The reaction starts with the abstraction of a reactive hydrogen radical in allylic position at C_7 in the B-ring of Δ5-sterols, followed by the reaction of triplet oxygen with the resulting radical (Figure 7). After the transfer of a hydrogen radical of another sterol molecule to the instable 7-peroxy radical, a more stable 7-hydroperoxide molecule and a new sterol radical, which can initiate a new autoxidation circle, are yielded (Smith, 1987; Porter *et al.*, 1995; Garcia-Llatas and Rodriguez-Estrada, 2011).

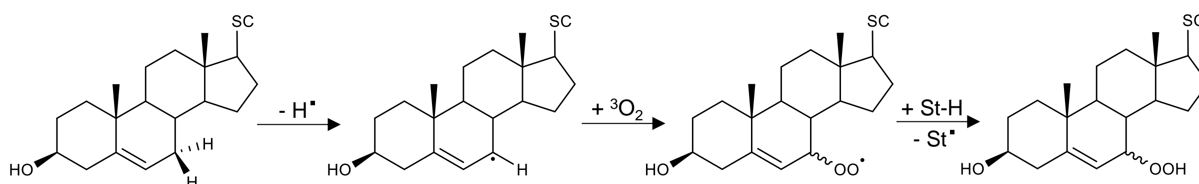

Figure 7. Formation sequence of primarily formed 7-hydroperoxysterols in the course of autoxidation of sterols; [SC], sterol side chain; [St-H], sterol molecule; [St•], sterol radical.

In the subsequent course of autoxidation, the primary 7-hydroperoxysterols (**1**) are readily decomposed into secondary oxidation products: stereoisomers of 7-hydroxysterols (**2**) and 7-ketosterols (**3**) (Figure 8). In addition, stereoisomers of 5,6-epoxysterols (**4**) can emerge from a biomolecular reaction mechanism involving a non-oxidized sterol molecule and a hydroperoxy radical. Under acidic conditions, 5,6-epoxysterols can be further hydrolysed yielding the corresponding triols (**5**) (Smith, 1987; Otaegui-Arrazola *et al.*, 2010; Garcia-Llatas and Rodriguez-Estrada, 2011). These polar secondary oxidation products are considered as the principal oxidation products and thus, are widely termed as “phytosterol oxidation products”

(POPs). These secondary oxidation products may also be degraded to tertiary oxidation products. Amongst monomeric degradation products, mid-polar oxidation products, such as 7-ketodienes/-trienes (**6**) were described upon liberation of the hydroxyl group at C_3 (Menéndez-Carreño *et al.*, 2010). In addition, the formation of non-polar and mid-polar dimeric, oligomeric and polymeric compounds (**7**) was demonstrated upon thermo-oxidation of sterols, and possible structures of dimers, such as disteryl ethers, were elucidated (Rudzinska *et al.*, 2010; Sosinska *et al.*, 2013; Sosinska *et al.*, 2014).

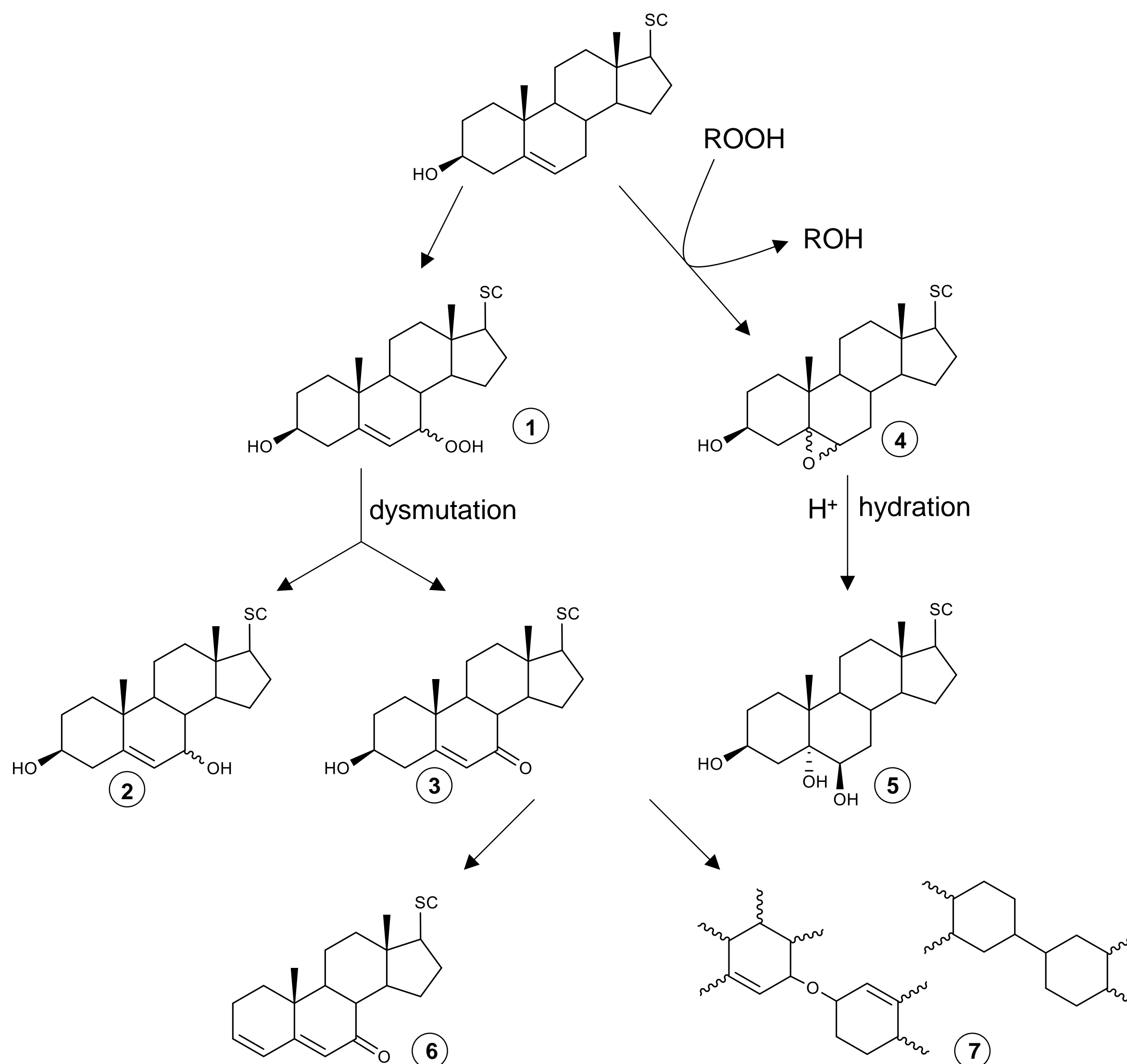

Figure 8. Schematic illustration of major sterol autoxidation pathways. Primary oxidation products: 7*α*/*β*-hydroperoxysterols (**1**); secondary oxidation products: 7*α*/*β*-hydroxy- (**2**), 7-keto- (**3**), 5,6*α*/*β*-epoxysterols (**4**), and 3*β*,5*α*,6*β*-triols (**5**); tertiary oxidation products: dienes/trienes (**6**), and dimers/oligomers/polymers (**7**).

Besides the described sterol ring oxidation products, oxidations may also occur in the side chain of sterols, but to a less pronounced effect. The formation of hydroperoxides in the first stage of

autoxidation is preferred at the tertiary C-atoms on the positions 20, 24 and 25; for sterols exhibiting an unsaturation between C_{22} and C_{23}, such as stigmasterol, the allylic positions at C_{20} and C_{24} may be most susceptible to the formation of hydroperoxides. The subsequent degradation of hydroperoxides finally yields secondary side-chain oxidation products (Figure 9) (Smith, 1987; Johnsson *et al.*, 2003; Johnsson and Dutta, 2003; Garcia-Llatas and Rodriguez-Estrada, 2011). However, knowledge on side-chain oxidation of phytosterols, particularly in quantitative regards, is still rather limited. Moreover, the formation of a series of volatile compounds was demonstrated upon thermo-oxidation of phytosterols, however, quantitative data indicated only small contributions to the degradations of phytosterols (Rudzinska *et al.*, 2009; Raczyk *et al.*, 2017b).

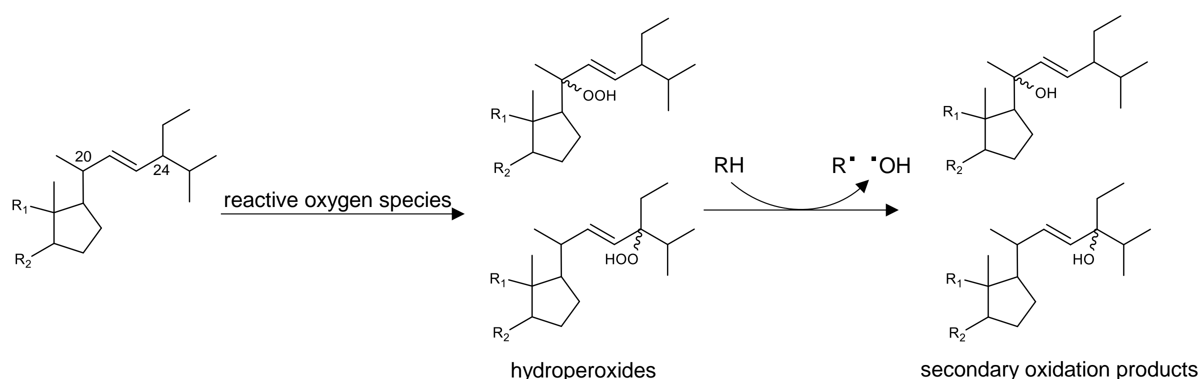

Figure 9. Schematic illustration of the formation of hydroperoxides and secondary oxidation products in the side-chain of sterols in the course of autoxidation, exemplarily shown for the side-chain of stigmasterol (modified from Garcia-Llatas and Rodriguez-Estrada, 2011).

Despite all the elucidated oxidation products of phytosterols, quantitative determinations showed distinct differences between the formations of those oxidation products and the losses of intact phytosterols (Menéndez-Carreño *et al.*, 2010; Rudzinska *et al.*, 2010; Scholz *et al.*, 2016). However, there is no study available covering the quantification of all known oxidation products of phytosterols at the same time. In the most comprehensive study available, non-polar, mid-polar and polar oxidation products as well as dimers and oligomers accounted for in total approximately 70 % of degraded stigmasterol upon prolonged heating (Menéndez-Carreño *et al.*, 2010). In another holistic study on cholesterol oxidation products only up to 25 % of thermally degraded cholesterol was explained by a series of oxidation products, comprising volatiles as well as monomeric and oligomeric secondary and tertiary oxidation products (Derewiaka and Molinska, 2015). These studies indicate that large parts of oxidation products are still unknown and remain to be elucidated. Very recently, the formation of substantial amounts of novel cholesterol hydroperoxides at the positions C_4 and C_6 upon autoxidation of

cholesterol in chlorobenzene was revealed and thus, the dogma of the exclusive formation of the 7-hydroperoxide was proven to be wrong (Zielinski and Pratt, 2016). These findings may pave the way for the discovery of novel sterol oxidation products in the future.

Although phytostanols, the saturated analogues of phytosterols, are considered to exhibit a much lower susceptibility to oxidations than the C_5-C_6 unsaturated phytosterols, several ring-oxidised phytostanol structures were identified (Soupas *et al.*, 2004b; Soupas *et al.*, 2005). However, the oxidation of saturated phytostanols requires high temperatures, which decreases the regioselectivity and thus leads to a broader spectrum of ring oxidation products comprising not only oxygenated species at C_7, but also at C_6 or C_{15} (Soupas *et al.*, 2004b; Frankel, 2005). In a recent study, various heat-treatments with a margarine enriched with phytosteryl/-stanyl fatty acid esters yielded by a factor of 10-20 more phytosterol oxidation products than the same heat-treatments carried out with a margarine only containing phytostanyl fatty acid esters. This study clearly demonstrated the different susceptibilities to oxidations of phytosterols and phytostanols (Scholz *et al.*, 2016).

2.2.2.3. Parameters Influencing the Oxidation of Phytosterols

Although the autoxidation of phytosterols is a complex process and dependent on many interacting parameters, several crucial factors were identified. The degree of phytosterol oxidations is commonly evaluated on the basis of degradations of sterols and/or formations of primary oxidation products (hydroperoxides), and/or secondary phytosterol oxidation products (POPs) (Lehtonen *et al.*, 2011b; Lin *et al.*, 2016b; Barriuso *et al.*, 2017).

2.2.2.3.1. Temperature and Time

Since heating is known to be a lipid oxidation inducer, it is conceivable that the temperature and the period of time are inversely proportional to the degradation of sterols and the formation of POPs (Otaegui-Arrazola *et al.*, 2010; Barriuso *et al.*, 2017). However, excessive heating may also lead to degradations of POPs, once maximum concentrations were reached (Menéndez-Carreño *et al.*, 2010; Barriuso *et al.*, 2012; Lin *et al.*, 2017). By contrast, amounts of tertiary dimeric or oligomeric compounds were reported to increase with prolonged heating times, as their formation competes with that of secondary POPs (Lehtonen *et al.*, 2012a). Moreover, higher amounts of phytosterols subjected to a heating-treatment were reported to hamper the heat transfer and to reduce the surface-to-volume ratio and therefore, slow down the oxidation process (Lampi *et al.*, 2002).

2.2.2.3.2. Type of Sterol

As determined by the transformation of individual phytosterols into phytosterol oxidation products, oxidation susceptibilities were found to depend on the type of phytosterol, both heated in pure form and incorporated in a lipid matrix (campesterol > sitosterol > stigmasterol) (Barriuso *et al.*, 2012; Barriuso *et al.*, 2016a; Lin *et al.*, 2017).

2.2.2.3.3. Esterification

For the enrichment of foods, phytosterols are incorporated into a food matrix in form of their fatty acid esters. Several studies reported distinct differences between oxidized free phytosterols and phytosteryl fatty acid esters with regards to the qualitative and quantitative profiles of secondary phytosterol oxidation products. Upon prolonged heating at 100 °C, higher amounts of POPs were found for phytosteryl fatty acid esters compared to free phytosterols (Soupas *et al.*, 2005; Lehtonen *et al.*, 2011a), whereas only slightly higher POP amounts were detected for free phytosterols at a heating temperature of 180 °C (Soupas *et al.*, 2005). As the fatty acid moieties, particularly of unsaturated fatty acids, are also susceptible to oxidations, one possible explanation for the higher reactivity of phytosteryl fatty acid esters at moderate temperatures is the interaction of the steryl and fatty acid moieties at an early stage of autoxidation. The quantitative determination of primary hydroperoxides in both steryl and fatty acid moieties upon prolonged heating of cholesteryl fatty acid esters at 100 °C, revealed that increasing unsaturation in the fatty acid moieties leads to higher formation rates of hydroperoxides both in the fatty acid and sterol moieties (Lehtonen *et al.*, 2011b). This effect may be caused by either intra- or intermolecular radical propagation and seems to be particularly significant at moderate temperatures (Smith, 1987). Higher formation rates of tertiary dimeric or oligomeric oxidation products with increasing unsaturation in the fatty acid moieties of cholesteryl fatty acid esters may also be explained by a promoted formation of primary hydroperoxides at an early stage of autoxidation (Lehtonen *et al.*, 2012a).

2.2.2.3.4. Matrix Effects

Pure sterols are oxidized to a greater extent than sterols incorporated in a saturated or unsaturated lipid matrix (Xu *et al.*, 2009; Otaegui-Arrazola *et al.*, 2010). Comparing saturated and unsaturated lipid matrices, less formation of POPs was reported at high temperatures (>140 °C) in unsaturated matrices, whereas POP formation was higher in unsaturated matrices at moderate temperatures (<140 °C) (Soupas *et al.*, 2004a; Barriuso *et al.*, 2016a). The mechanism

behind this observations still remains largely unclear. Further, the presence of antioxidants, e.g. from plant extracts, in the food matrix may have a protective effect on the oxidation of sterols (Xu *et al.*, 2009; Barriuso *et al.*, 2015; Barriuso *et al.*, 2016b). Finally, POP contents strongly depend on the level of phytosterol-enrichment in foods (Lin *et al.*, 2016b; Lin *et al.*, 2017).

2.2.2.4. Phytosterol Oxidation Products in Foods

Phytosterols/-stanols and their fatty acid esters may be present in foods as naturally occurring ingredients and/or as a result of additional enrichment. Data on phytosterol oxidation products are available for their occurrence in various non-enriched and enriched foods, including foods that were subjected to specific treatments, such as long-term storage, microwave heating and various thermal processes (e.g. pan-frying or oven-heating). A selection of previous studies is shown in Table 1. Reflecting the market dominance of phytosterol/-stanol-enriched margarines and a wide range of possible (heating) applications, most of the studies investigated POP contents in (liquid) margarines and edible oils, but POP contents were also determined in milk, milk products and chocolate. However, comparisons between individual studies are difficult, as the formation of phytosterol oxidation products is affected by numerous factors (cf. 2.2.2.3), and the analytical methods used for POP analysis are not standardized. Only a few comprehensive studies are available, which allow comparisons of POP contents depending on the food matrices and/or different (heat) treatments (Scholz *et al.*, 2016; Lin *et al.*, 2017). Higher initial levels of phytosterols/-stanols in margarines and oils were shown to result in higher POP contents, particularly when non-enriched and enriched foods were compared (Conchillo *et al.*, 2005; Lin *et al.*, 2016a; Lin *et al.*, 2017). In a recent study, POP contents in enriched margarines were determined upon various heating treatments that may reflect heating conditions in the home. A PSE-enriched margarine yielded POP contents by a factor of approximately 10-20 higher than those yielded upon the same heating treatments of a PAE-enriched margarine (Scholz *et al.*, 2016). POP contents were also strongly depended on the treatment of the enriched margarines; POP contents differed by a factor of approximately 5-7 between the treatment that yielded lowest (microwave) and highest (oven-casserole) POP contents (Scholz *et al.*, 2016).

Recently, the occurrence of POPs was investigated in a spectrum of processed foods that were prepared (cooked or baked) with both non-enriched and enriched margarines (Lin *et al.*, 2016a). The POP contents found in the processed foods are presented in Table 2. The POP contents strongly depended on the type of food, its processing and the type of margarine. Foods prepared with non-enriched margarine showed lower POP contents than the corresponding foods

prepared with enriched margarine. However, most of the POPs formed during the frying experiments were found in the residual fats and not in the processed foods. Low POP contents upon baking experiments were explained by a lower exposure of phytosterols to oxygen, as the dough had a high volume-to-surface ratio and the phytosterols were incorporated in the dough, whereas during stir- or shallow-frying the fat was present as a thin layer in the pan with better access to oxygen (Lin *et al.*, 2016a). However, these potential effects were not further investigated. For all cooking and baking experiments with non-enriched margarine, the oxidation rates were higher compared to the corresponding foods prepared with enriched margarine. The reasons for this effect remained unclear. It was hypothesized that an increase in phytosterol levels might lead to a lower ratio of reactive oxygen species (ROS) to phytosterols, which might, in turn, decrease the likelihood of ROS to be in contact with phytosterols (Ansorena *et al.*, 2013; Lin *et al.*, 2016a).

Table 1. Selected examples of POP contents in various non-enriched and enriched foods.

reference	type of food	level of enrichment[a]	treatment	POP content [mg/kg]	ORP[b] [%]
Conchillo *et al.* (2005)	margarine	non-enriched	-	13	0.4
	margarine	PSE (≙ 6 % PS)	-	46	0.07
Johnsson and Dutta (2006)	margarine	n.a.[c]	-	12	n.a.
Lin *et al.* (2016a)	margarine	non-enriched	-	4	0.3
	margarine	PSE/PAE (≙ 7.5 % PS)	-	11	0.01
Grandgirad *et al.* (2004)	margarine	PSE (≙ 8 % PS)	-	68	0.09
Rudzińska *et al.* (2014)	margarine	PAE/PSE (≙ 7.9 PS)	-	255[d]	0.32
	margarine	PAE/PSE (≙ 7.9 % PS)	storage (6 weeks, 4 °C)	354[d]	0.45
	margarine	PAE/PSE (≙ 7.9 % PS)	storage (6 weeks, 20 °C)	734[d]	0.93
Lin *et al.* (2017)	sunflower oil	non-enriched	oven-heating (150-210 °C, 0-16 min)	65-124	0.1-2.3
	rapeseed oil	non-enriched	oven-heating (150-210 °C, 0-16 min)	14-168	0.1-2.3
	brick margarine	non-enriched	oven-heating (150-210 °C, 0-16 min)	16-75	0.1-1.8
	liquid margarine	non-enriched	oven-heating (150-210 °C, 0-16 min)	37-96	0.1-1.4
	soft margarine	PSE (≙ 7.5 % PS)	oven-heating (150-210 °C, 0-16 min)	131-891	0.1-0.9
	liquid margarine	PSE (≙ 7.6 % PS)	oven-heating (150-210 °C, 0-16 min)	115-1505	0.1-1.4
Julien-David *et al.* (2014)	margarine	PSE (≙ 10.5 % PS)	oven-heating (140-200 °C, 0-120 min)	2-5036	0.01-4.80
Scholz *et al.* (2015b)	margarine	PSE (≙ 7.5 % PS)	oven-heating (180 °C, 0-150 min)	373-8311	0.49-11.08
Scholz *et al.* (2016)	margarine	PSE (≙ 7.5 % PS)	microwave (800 W, 4 min)	780	1.04
			pan-frying (200 °C, 9 min)	1079	1.44
			oven-bottle (200 °C, 20 min)	2903	3.87
			oven-casserole (200 °C, 20 min)	5109	6.81
Scholz *et al.* (2016)	margarine	PAE (≙ 8 % PS)	microwave (800 W, 4 min)	43[e]	0.04
			pan-frying (200 °C, 9 min)	137[e]	0.08
			oven-bottle (200 °C, 20 min)	105[e]	0.07
			oven-casserole (200 °C, 20 min)	226[e]	0.11
Soupas *et al.* (2007)	liquid margarine	PAE (≙ 5 % PS)	pan-frying (180 °C, 10 min)	63	0.1
	liquid margarine	PSE (≙ 5 % PS)	pan-frying (180 °C, 10 min)	668	1.3
Botelho *et al.* (2014)	dark chocolate	PSE (≙ 6.9 % PS)	-	69	0.1
	dark chocolate	PSE (≙ 6.9 % PS)	storage (30 °C, 5 months)	71	0.1

Table 1. Continued.

reference	type of food	level of enrichment	treatment	POP content [mg/kg]	ORP [%]
González-Larena *et al.* (2015)	milk beverage	PSE/PAE (≙ 0.67 % PS)	storage (37 °C, 6 months)	651	0.12
		PSE/PAE (≙ 0.67 % PS)	storage (24 °C, 6 months)	702	0.14
Soupas *et al.* (2006)	pasteurized milk	PSE (≙ 0.5 % PS)	storage (4 °C, 6 months)	2.4	0.11
	milk powder	PSE (≙ 7 % PS)	storage (38 °C, 12 months)	33.9	0.07
Menéndez-Carreño et al. (2008)	milk	PSE/PAE (≙ 0.3 % PS)	oven (65 °C, 24 h)	3.1	0.10
	milk	PSE/PAE (≙ 0.3 % PS)	microwave (900 W, 2 min)	3.9	0.13
	milk	PSE/PAE (≙ 0.3 % PS)	electrical heating (15 min)	3.4	0.11

[a] Abbreviations: PSE, phytosteryl fatty acid esters; PAE, phytostanyl fatty acid esters; PS, free phytosterols/-stanols.
[b] Oxidation rate: percentage correlation of POP content and the initial phytosterol content.
[c] Not available.
[d] Only phytosterol oxidation products were determined
[e] Both phytostanol and phytosterol oxidation products were determined.

Table 2. POP contents in various processed foods cooked or baked with a non-enriched or a PSE-enriched margarine (Lin *et al.*, 2016).

processed food	treatment[a,b]	non-enriched margarine		enriched margarine (≙ 7.5 % PS)	
		POPs [mg/kg]	ORP[c] [%]	POPs [mg/kg]	ORP[c] [%]
green beans	stir-frying (200 °C/ 6 min)	4.1	2.69	16.8	0.21
cabbage	stir-frying (249-260 °C/ 6 min)	5.5	2.32	55.8	0.50
chicken	stir-frying (228-241 °C/ 6 min)	4.5	3.66	96.6	2.20
egg	shallow-frying (168-171 °C/ 9 min)	3.3	1.86	6.1	0.28
onions	shallow-frying (161-162 °C/ 11 min)	5.0	1.17	15.4	0.10
codfish	shallow-frying (173-175 °C/ 11 min)	1.6	2.30	8.7	0.93
fish fingers	shallow-frying (185-189 °C/ 13 min)	7.7	4.82	32.1	0.43
pork fillet	shallow-frying (177 °C/ 13 min)	0.4	3.37	5.0	1.64
beef steak	shallow-frying (169-171 °C/ 15 min)	8.7	9.82	67.5	2.34
salmon	shallow-frying (193-194 °C/ 15 min)	4.7	5.45	9.5	1.60
potatoes	shallow-frying (195-199 °C/ 20 min)	4.8	8.05	98.3	3.45
minced meat	shallow-frying (169-171 °C/ 23 min)	5.9	6.19	15.2	1.22
beef	stewing (84-85 °C/ 95 min)[d]	5.9	5.05	10.3	0.27
beef	roasting (140 °C/ 35 min)[d]	3.0	5.09	7.1	0.41
codfish	microwave cooking (600 W/ 5 min)	0.4	0.51	0.7	0.02
cookies	baking (170 °C/ 12 min)	6.6	1.67	11.3	0.06
muffins/cupcakes	baking (140 °C/ 25 min)	2.4	2.04	4.3	0.08
banana bread	baking (155 °C/ 60 min)	1.5	0.94	3.4	0.05
sponge cake	baking (155 °C/ 60 min)	4.2	1.08	12	0.07

[a] Heating times are given as the sum of preheating and main cooking; for baking processes only the baking times are given.
[b] Temperature ranges are given, when the product temperature differed between treatments with non-enriched and enriched margarine.
[c] Oxidation rate: percentage correlation of POP content and the initial phytosterol content. For the calculation, the POP content of the residual fat was taken into account (data not shown); baking did not yield residual fats.
[d] Beef stewing and roasting had a prior shallowing-frying step of 4 and 9 min, respectively.

2.2.2.5. Biological Aspects Related to Phytosterol Oxidation Products

2.2.2.5.1. Absorption and Plasma Levels in Humans

First observations of phytosterol oxidation products in human serum were reported in a study with phytosterolemic patients (Plat *et al.*, 2001). Subsequently, numerous quantitative studies reported the occurrence of POPs in the plasma of healthy subjects (Grandgirard *et al.*, 2004; Husche *et al.*, 2011; Menendez-Carreno *et al.*, 2012; Baumgartner *et al.*, 2013). In two of those studies, both based on isotope dilution GC/MS analysis, similar concentration ranges of individual phytosterol oxidation products of 0.07 – 3.01 ng/mL (Husche *et al.*, 2011) serum and 0.09 – 2.49 ng/mL serum (Baumgartner *et al.*, 2013) were determined in 16 and 43 healthy volunteers, respectively. However, the determination of plasma POP concentrations after consumption of 3 g phytosterols/-stanols per day via a margarine over a period of 28 days, offered contradictory results regarding the concentration of 7β-hydroxysitosterol; in one study the concentration of 7β-hydroxysitosterol rose by 87 % to a serum concentration of 2.2 ng/mL (Husche *et al.*, 2011), whereas only a negligible increase in the plasma concentration of 7β-hydroxysitosterol was observed in the second study (Baumgartner *et al.*, 2013). The reasons for this contrasting results remained unexplained.

2.2.2.5.2. Potential Effect on Cholesterol Uptake and Metabolism

Some studies investigated the ability of phytosterol oxidation products to alter the expression of several cholesterol metabolizing proteins and enzymes. Under particular observation was the NPC1L1 protein, a transporter protein involved in the intestinal uptake and absorption of cholesterol (O'Callaghan *et al.*, 2014). However, the treatment of Caco-2 cells with either 7-ketosterol or 7-ketostigmasterol did not result in a significantly higher expression of NPC1L1 protein compared to control cells (Alemany *et al.*, 2013). Likewise, no increased expression of NPC1L1 protein was found in hamsters fed with β-sitosterol oxides over a period of six weeks (Liang *et al.*, 2011). By contrast, a reduced expression of ABCG5 protein, which controls the efflux of cholesterol from the enterocyte, was observed in hamsters fed with POPs. In consequence, the absorption of cholesterol in enterocytes may be facilitated. In the *in vitro* study with Caco-2 cells, decreased expression of both efflux transporters, ABCG5 and ABCG8, was found (Alemany *et al.*, 2013; O'Callaghan *et al.*, 2014).

2.2.2.5.3. Atherosclerosis

Several studies investigated whether POPs, similar to COPs, are involved in the development of atherosclerotic plaques. Tomoyori *et al.* did not observe an increase of atherosclerotic lesions in apolipoprotein E-deficient mice fed with a diet enriched with POPs after 9 weeks. It was concluded that POPs are not involved in the development of atherosclerosis (Tomoyori *et al.*, 2004). Similar observations were made in another study following a 35 week study with LDLR +/- mice, a model for human mild hypercholesterolemia, fed with a POP-enriched diet; however, the proportion of severe arteriosclerotic lesions was increased (Plat *et al.*, 2014). Yang *et al.* found that sitosterol oxidation products attenuate vasorelaxation, a marker of vascular health, in isolated rat aorta. The authors interpreted this observation as an indicator for the impairment of the physiological aorta function caused by POPs (Yang *et al.*, 2013). In a more recent study, Weingärtner *et al.* investigated potential vascular effects of daily intraperitoneal injections of cholesterol, sitosterol, 7β-hydroxycholesterol and 7β-hydroxysitosterol in ApoE -/- mice for four weeks. Increased plasma levels were only found for 7β-hydroxysitosterol that were associated with an increased production of reactive oxygen species (ROS), but did not affect endothelial progenitor cells, endothelial function or early atherosclerosis (Weingaertner *et al.*, 2015). Luister *et al.* found that patients with aortic stenosis and concomitant coronary artery disease (CAD) are characterized by an increased deposition of plant sterols, but not any cholesterol in aortic valve tissues, compared to patients with aortic stenosis but no concomitant CAD. Furthermore, the patients with concomitant CAD exhibited increased oxyphytosterol concentrations in plasma and aortic cusps. These observations led to the conclusion that phytosterol oxidation products might originate from local endogenous oxidation processes (Luister *et al.*, 2015).

2.2.2.5.4. Inflammatory Processes

Inflammatory processes are characterized by elevated levels of inflammatory cytokines and are involved in various diseases, including Alzheimer’s disease and cardiovascular disease (O'Callaghan *et al.*, 2014). Vejux *et al.* reported that the treatment of U937 cells with 7β-hydroxysitosterol did not affect the secretion of pro-inflammatory cytokines, monocyte chemotactic protein-1 (MCP-1) or interleukin-8 (IL-8) (Vejux *et al.*, 2012). Similarly, Plat *et al.* found that the serum concentrations of MCP-1 and tumor necrosis factor-α (TNF-α) were not affected by a POP-enriched diet in mice (Plat *et al.*, 2014). However, increasing concentrations of pro-inflammatory cytokines, TNF-α and IL-8 were observed in human colon adenocarcinoma (Caco-2) cells upon treatment with 7-ketostigmasterol (Alemany *et al.*, 2013).

In a follow-up study, proteome changes in Caco-2 cells caused by the exposure to 7-ketostigmasterol were investigated. Incubation of Caco-2 cells with 7-ketostigmasterol led to an increase of proteins associated with inflammatory responses, cell proliferation and cell signal processes, but proteins involved in immune responses and cell survival processes were decreased (Laparra *et al.*, 2015).

2.2.2.5.5. Cytotoxicity

It was shown that higher concentrations of POPs are required to cause similar cytotoxic effects in cell cultures as described for COPs (Adcox *et al.*, 2001). This was confirmed by Vejux *et al.*, who did not find COP-like effects for POPs in U937 cells at similar concentration levels as used for COPs (Vejux *et al.*, 2012). Progress in the synthesis of pure phytosterol oxidation products allowed to study potential cytotoxic effects of individual POPs. In numerous studies, POPs were shown to exert cytotoxic effects in various cell lines; apoptosis was also reported among the discussed mechanisms of actions (Maguire *et al.*, 2003; O'Callaghan *et al.*, 2010; Kenny *et al.*, 2012; O'Callaghan *et al.*, 2013; Gao *et al.*, 2015). The cytotoxic potential was shown to depend not only on the type of oxygenation in the sterol ring, but also on the structural properties of the phytosterol side chain (Gao *et al.*, 2015).

2.2.2.5.6. Impact of Oxidations of the Sterol Ring on the Hydrolysis of Phytosteryl Fatty Acid Esters

The intestinal hydrolysis of the ester bond of phytosteryl fatty acid esters was identified as an essential precondition for the cholesterol-lowering properties of phytosterols. A mechanistic *in vitro* study revealed that the oxidation of sitosteryl oleate to 7-ketositosteryl oleate increased the affinity towards pancreatic cholesterol esterase and led to higher conversion rates compared to non-oxidized sitosteryl oleate (Julien-David *et al.*, 2009). This study may indicate that oxidative modifications in the sterol ring of phytosteryl fatty acid esters influence the intestinal hydrolysis of phytosteryl fatty acid esters.

2.3. Oxidation of Esterified Fatty Acids

As described earlier, phytosterols/-stanols are added to foods as their fatty acid esters, and the type of fatty acid was shown to affect oxidations in the sterol ring in the course of autoxidation (Lehtonen *et al.*, 2011a). However, the current knowledge on oxidative modifications in the fatty acid moieties upon autoxidation of steryl fatty acid esters is still rather limited. By contrast,

the endogenous occurrence of cholesteryl esters oxidized in the fatty acid moieties (OxCE) was demonstrated, and their involvement in pathophysiological processes was discussed in several studies (Leitinger, 2003; Hutchins *et al.*, 2011; Ravandi *et al.*, 2014; Choi *et al.*, 2017). Nevertheless, the vast majority of knowledge about oxidation reactions of ester-bound fatty acids was generated from autoxidation-studies of fatty acid methyl/ethyl esters and glycerol-bound fatty acids, such as triacylglycerides (Velasco *et al.*, 2004; Frankel, 2005; Dobarganes and Márquez-Ruiz, 2007; Berdeaux *et al.*, 2012).

2.3.1. Autoxidation of Esterified Fatty Acids

Non-enzymatic oxidations of esterified fatty acids may proceed via autoxidation or photooxidation. However, particularly in the presence of heat, autoxidation is considered to be the most common oxidation mechanism of ester-bound fatty acids (Frankel, 2005). While sterols are assumed to provide only one possible position (C_7) for hydroperoxide formation (cf. 2.2.2.2), autoxidation of esterified fatty acids may result in complex mixtures of hydroperoxides, strongly depending on the degree of unsaturation of the respective fatty acids. The degree of unsaturation not only determines the regioselectivity of hydroperoxide formations, but also the oxidation rates of hydroperoxides (Figure 10).

The classical mechanism of the free radical oxidation of oleates comprises the abstraction of allylic hydrogen atoms at C_8 or C_{11} to produce two delocalized radicals, which may further react to hydroperoxides at the carbons 8, 9, 10 and 11, in equal amounts (Figure 10A). Linoleates, by contrast, are 40 times more reactive than oleates owing to a bis-allylic methylene group on C_{11} which allows rapid hydrogen abstraction. The resulting pentadienyl radical intermediate is effectively stabilized by resonance and reacts to the conjugated diene 9- and 13-hydroperoxides in the presence of oxygen (Figure 10B) (Holman and Elmer, 1947; Frankel, 2005). For this reason, the 11-hydroperoxide is only formed in negligible amounts (Berdeaux *et al.*, 1999). The mono-allylic positions on C_8 and C_{14} of linoleates also react only to a minor extent, leading to hydroperoxides on the positions 8, 10, 12 and 14; the proportion of these side products is approximately 4 % (Belitz *et al.*, 2008).

Under thermal conditions, the primary hydroperoxides are quickly decomposed to alkoxyl radicals, which may undergo homolytic β-scission to form aldehydes 8 (route I), alkyl and olefinic radicals (route II) (Figure 11). The alkyl radicals may further react with a hydrogen or a hydroxyl radical to form hydrocarbons and alcohols, respectively (Figure 11A).

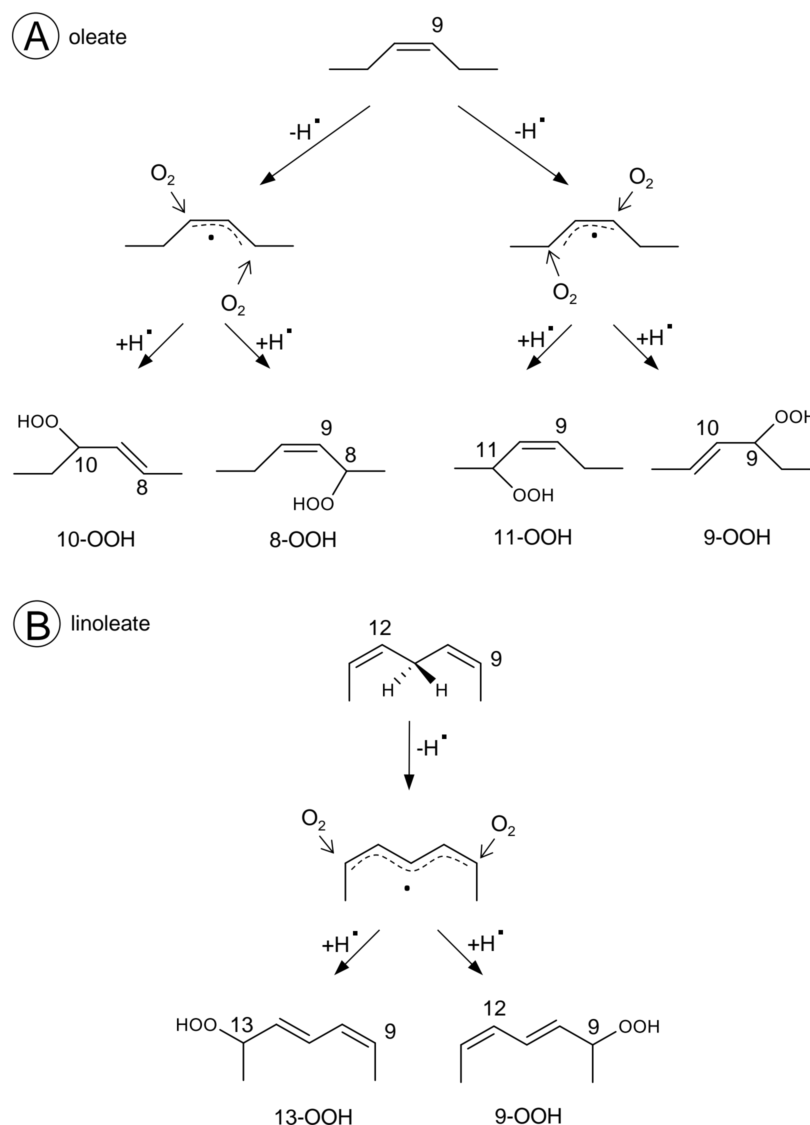

Figure 10. Schematic representation of the formation of the principal hydroperoxides in the course of (A) oleate and (B) linoleate autoxidation (modified from Frankel, 2005).

Similarly, reactions of olefinic radicals with a hydrogen or hydroxyl radical lead to the formation of olefins and 1-enols, which tautomerize into aldehydes (Figure 11B) (Frankel, 2005). Tables 3 and 4, respectively, give an overview of the principal ester-bound degradation products of oleate and linoleate monohydroperoxides upon homolytic β-scission.

Upon thermo-oxidation of glycerol-bound unsaturated fatty acids and subsequent transesterification into FAME, octanoates and 9-oxononanoates were identified as the major short-chain compounds, whereas lower amounts of heptanoates and 8-oxooctanoates were reported (Berdeaux *et al.*, 2002; Velasco *et al.*, 2004; Velasco *et al.*, 2005). Similar observations were made for unsaturated fatty acid methyl esters (FAME) (Berdeaux *et al.*, 1999).

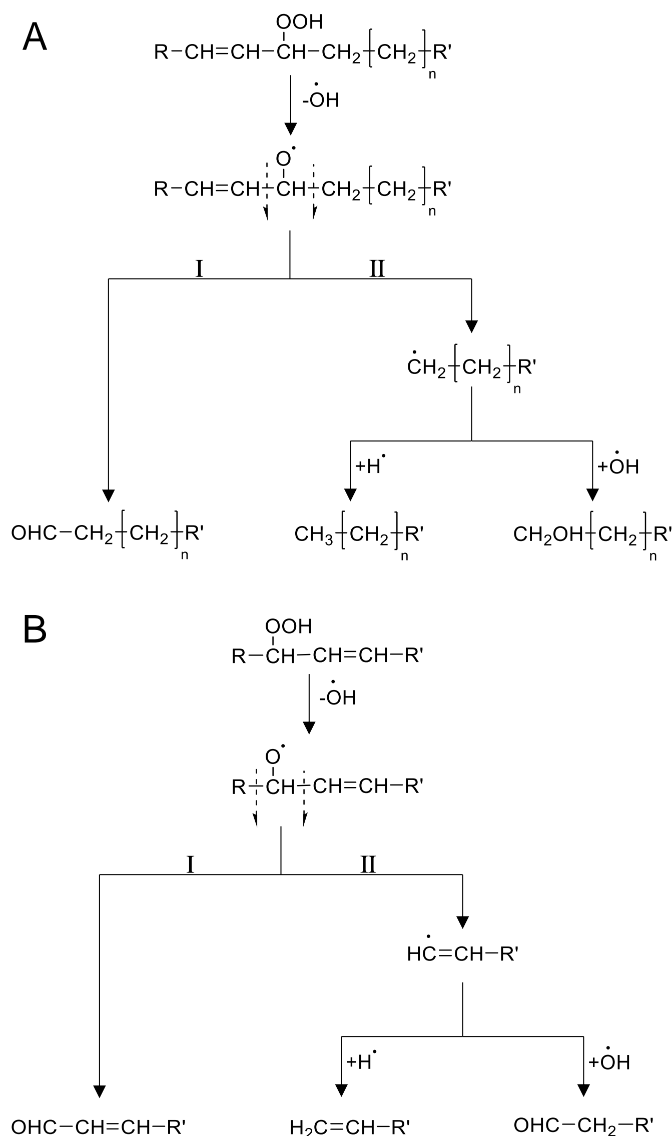

Figure 11. Schematic representation of principal decomposition pathways of monohydroperoxides at the positions (A) 8 and 9, and (B) 10, 11, 12, 13 and 14 of oleates and linoleates by homolytic β-scission. Only degradation products comprising an ester-bound fatty acid part are illustrated; R= non-ester-bound fatty acid part, R`= ester-bound fatty acid part (modified from Frankel, 2005).

Apart from short-chain oxidation products, further reactions of hydroperoxides were shown to yield oxidized compounds of molecular weights similar to that of the esterified starting acid. This class of oxidation products comprises various esterified fatty acids containing hydroxyl, epoxy and keto groups (Velasco *et al.*, 2002; Frankel, 2005; Dobarganes and Márquez-Ruiz, 2007; Marmesat *et al.*, 2008; Berdeaux *et al.*, 2012).

Table 3. Short-chain ester-bound compounds formed by *β*-scission of oleate monohydroperoxides.

oleate	degradation products of monohydroperoxides[a]		
position OOH	route I	route II[b]	
		+ H$^{\cdot}$	+ OH$^{\cdot}$
8-OOH	8-oxooctanoate	heptanoate	7-hydroxyheptanoate
9-OOH	9-oxononanoate	octanoate	8-hydroxyoctanoate
10-OOH	10-oxodec-8-enoate	non-8-enoate	9-oxononanoate
11-OOH	11-oxoundec-9-enoate	dec-9-enoate	10-oxodecanoate

[a] Degradation products comprising an ester-bound fatty acid part.
[b] Radicals produced via route II (Figure 11) react with OH$^{\cdot}$ or H$^{\cdot}$.

In addition, polar and nonpolar dimeric structures were identified upon heating of FAME, particularly at high temperatures (> 200 °C) (Dobarganes and Márquez-Ruiz, 2007). Their formation pathways are complex and may be based on: (i) the combination of two allylic radicals to dehydrodimers, (ii) the intermolecular addition of an allylic radical and an unsaturated molecule, and (iii) the formation of cyclic dimers by intramolecular addition of an intermediate dimeric radical to a double bond in the same molecule. The monomers may thereby be connected via C-C or C-O-C linkages. However, structural information of, in particular, polar dimers are still limited owing to the complex analysis of a large number of possible structures. Moreover, structures of higher oligomers have not yet been elucidated due to the increasing complexity of possible structures and limitations in the availability of analytical methods (Figge, 1971; Frankel, 2005; Dobarganes and Márquez-Ruiz, 2007).

Table 4. Short-chain ester-bound compounds formed by *β*-scission of linoleate monohydroperoxides.

linoleate	degradation products of monohydroperoxides[a]		
position OOH	route I	route II[b]	
		+ H$^{\cdot}$	+ OH$^{\cdot}$
8-OOH	8-oxooctanoate	heptanoate	7-hydroxyheptanoate
9-OOH[c]	9-oxononanoate	octanoate	8-hydroxyoctanoate
10-OOH	10-oxodec-8-enoate	non-8-enoate	9-oxononanoate
11-OOH	11-oxoundec-9-enoate	dec-9-enoate	10-oxodecanoate
12-OOH	12-oxododec-9-enoate	undec-9-enoate	11-hydroxyundec-9-enoate
13-OOH[c]	13-oxotrideca-9,11-dienoate	dodeca-9,11-dienoate	12-oxododec-9-enoate
14-OOH	14-oxotetradeca-9,12-dienoate	trideca-9,12-dienoate	13-oxotridec-9-enoate

[a] Degradation products comprising an ester-bound fatty acid part.
[b] Radicals produced via route II (Figure 11) react with OH$^{\cdot}$ or H$^{\cdot}$.
[c] Predominant hydroperoxides formed in the course of linoleate autoxidation.

2.3.2. Oxidation Products of Steryl Fatty Acid Esters

2.3.2.1. Autoxidation of Steryl Fatty Acid Esters

In two studies (Lehtonen *et al.*, 2011b; Lehtonen *et al.*, 2012b), the formation of primary hydroperoxides in the fatty acid moieties of cholesteryl stearate/oleate/linoleate and stigmasteryl oleate in pure form and incorporated in a lipid matrix has been investigated upon prolonged heating at 100 °C (Table 5). Similar hydroperoxide patterns were observed as described earlier for FAME and TAG (cf. 2.3.1). Increasing unsaturation in the fatty acid moieties led to higher formation rates of primary hydroperoxides and shorter induction periods. Moreover, the hydroperoxides started to decompose earlier. Incorporated in a saturated lipid matrix, the degradation of stigmasteryl oleate hydroperoxides started significantly earlier than for the corresponding cholesteryl oleate hydroperoxides, indicating different oxidation behaviors depending on the sterol side chain (Lehtonen *et al.*, 2012b). However, very little is known about the structures of secondary oxidation products formed upon autoxidation of steryl fatty acid esters. Only one study reported the formation of the oxidized fatty acid 9,10-dihydroxystearic acid, esterified to sitosterol and sitosterol oxidation products, formed from sitosteryl oleate upon heating of a margarine enriched with phytosteryl fatty acid esters (Table 5). Only little amounts of predominantly sitosteryl 9,10-dihydroxystearate were determined compared to the oleates at various temperatures (Julien-David *et al.*, 2014). Moreover, significant amounts of tertiary oxidation products (oligomers) were reported upon prolonged heating of cholesteryl fatty acid esters at 100 and 140 °C (Table 5). Increased temperature and unsaturation in the fatty acid moieties was shown to promote the formation rates of oligomers (Lehtonen *et al.*, 2012a; Lehtonen *et al.*, 2012b). As structural elucidations of oligomers were not achieved, the exact role of oxidized fatty acid moieties in the formation of oligomers remains largely unknown.

2.3.2.2. Chemical Oxidations of Cholesteryl Fatty Acid Esters

Whereas the current knowledge about autoxidation products of steryl fatty acid esters is still limited, more information on chemical and endogenous oxidations of cholesteryl fatty acid esters are available. In a series of studies (Kamido *et al.*, 1992a; Kamido *et al.*, 1992b; Kamido *et al.*, 1993; 1995), Kamido *et* al. demonstrated the susceptibility of cholesterol-bound unsaturated fatty acids to oxidations using oxidizing reagents, such as osmium tetroxide, *tert*-butyl hydroperoxide and cooper ions. *In vitro* oxidations of cholesteryl linoleate and arachidonate in pure form and contained in human LDL/HDL revealed the occurrence of

aldehydes still esterified to cholesterol, generally termed as "core-aldehydes". Cholesteryl 9-oxononanoate/8-oxooctanoate were identified as the major core-aldehydes resulting from linoleate oxidation, whereas cholesteryl 5-oxovalerate was most abundant upon arachidonate oxidation.

Table 5. Primary, Secondary and Tertiary Oxidation Products Formed Upon Thermo-oxidations of Steryl Fatty Acid Esters.

reference	thermal treatment	sample	oxidation products	analytical method	content range
primary oxidation products (hydroperoxides)					
Lehtonen *et al.* (2011a)	100 °C 0-4 d	Chol-C18:0 Chol-C18:1 Chol-C18:2	ROOH	HPLC-ELSD	nd[a] 0-32 mg/g[b] 0-26 mg/g
Lehtonen *et al.* (2012a)	100 °C 0-5 d	Chol-C18:0 Chol-C18:1 Stig-C18:1 Chol-C18:2 (in tripalmitin matrix)	ROOH	SPE/ HPLC-ELSD	nd 0-113 mg/g[b] 0-44 mg/g 0-77 mg/g
secondary oxidation products					
Julien-David *et al.* (2014)	140/170/200 °C 0-120 min	Margarine (12.5 % PSE)	sitosteryl 9,10-dihydroxystearate	LC-MS	0-15.2 µg/g[c]
tertiary oxidation products (oligomers)					
Lehtonen *et al.* (2012b)	100/140 °C 0-5 d	Chol-C18:0 Chol-C18:1 Chol-C18:2	oligomers	SEC-RI/MS	0-428 mg/g[b] 0-569 mg/g 0-679 mg/g
Lehtonen *et al.* (2012a)	100 °C 0-5 d	Chol-C18:0 Chol-C18:1 Stig-C18:1 Chol-C18:2 (in tripalmitin matrix)	oligomers	SEC-RI	nd 0-185 mg/g[b] 0-304 mg/g 0-413 mg/g

[a] Not detected.
[b] Values expressed as mg of oxidation products per g of steryl fatty acid esters present before thermo-oxidation.
[c] Values expressed as µg of oxidation product per g of PSE-enriched margarine subjected to thermo-oxidations; the maximum value corresponds to approximately 0.01 % of oxidized sitosteryl oleate present in the margarine.

Besides these major oxidation products, minor proportions of C_4, C_6, C_7 and C_{10} core aldehydes were reported. Upon cooper-catalyzed oxidations of human plasma lipoproteins, the major core aldehydes C_5, C_8 and C_9 esterified to cholesterol and/or 7-ketocholesterol were estimated to account for at least 1-2 % of degraded cholesteryl linoleate and arachidonate (Kamido *et al.*,

1995). In a more recent *in vitro* study, cholesteryl 9-oxononanoate was confirmed as the major oxidation product of cholesteryl oleate/linoleate upon ozone-catalyzed oxidation of human LDL (Miyoshi *et al.*, 2013).

2.3.2.3. Endogenous Formation of Oxidized Cholesteryl Fatty Acid Esters

Several studies reported the occurrence of cholesteryl ester core aldehydes in advanced human atherosclerotic lesions and thus, indicating their formation *in vivo* (Hoppe *et al.*, 1997; Karten *et al.*, 1998; Hutchins *et al.*, 2011). In the most comprehensive study available, both cholesteryl ester core aldehydes (C_3-C_9) and numerous cholesteryl esters containing oxidized fatty acids of original chain length were identified in human atheromata. The most abundant cholesteryl ester was cholesteryl linoleate of which on average 23 % were present in oxidized form (Hutchins *et al.*, 2011). The analysis of lipid extracts obtained from advanced human atherosclerotic lesions revealed cholesterol 9-oxononanoate as the major core aldehyde at mean concentrations of approximately 29 µmol/mol cholesterol (Karten *et al.*, 1998). The analysis of oxidized lipids extracted from human atherosclerotic lesions indicated that lipoxygenase may contribute significantly to cholesteryl ester oxidations, however, the available data are not conclusive (Kuehn *et al.*, 1992; Kuehn *et al.*, 1994; Folcik *et al.*, 1995; Hutchins *et al.*, 2011). As human vascular diseases develop over decades, a strong participation of lipoxygenase in the initial phase of cholesteryl ester oxidations in human atheromata, followed by nonenzymatic mechanisms, was proposed (Hutchins *et al.*, 2011).

2.3.2.4. Biological Aspects Related to Oxidized Steryl Fatty Acid Esters

2.3.2.4.1. Atherosclerosis

Cholesteryl fatty acid esters oxidized in the fatty acid moieties (oxCE) were found to be major components in oxidized LDL, which is considered as a major causative factor for the development of atherosclerosis (Steinberg, 2009). Several mechanisms regarding the role of oxCE in the pathogenesis of atherosclerosis were proposed, however, the available data are limited (Leitinger, 2003; Choi *et al.*, 2017). Cholesteryl ester core aldehydes were shown to be significantly less hydrolyzed by mouse peritoneal macrophages compared to intact cholesteryl linoleate (CL) at pH 4. Further, core aldehydes formed complexes with serum proteins, and purified cholesteryl 9-oxononanoate was shown to react with the ε-amino group of lysine (Hoppe *et al.*, 1997; Kawai *et al.*, 2003). It was concluded that core aldehydes, yielded from extensively oxidized CL, are not adequately hydrolyzed by lysosomal acid cholesteryl ester

hydrolase (ACEH) and may form insoluble lipid-protein complexes, so-called ceroids, which are characteristic for atherosclerotic plaques (Hoppe *et al.*, 1997). Conflicting results were reported in another study; 13-hydroxylinoleic acid, representative for oxidized CL, was hydrolyzed more effectively by cholesteryl hydrolases of murine macrophages at neutral and acid pH than non-oxidized CL. Therefore, the intracellular oxidation of cholesteryl esters was suggested to represent an anti-atherogenic process (Belkner *et al.*, 2000). Moreover, oxCE, such as cholesteryl 9-oxononanoate and cholesteryl linoleate hydroperoxides, were shown to stimulate endothelial cells to specifically bind monocytes via the extracellular signal-regulated kinase 1/2 pathway. As monocyte adhesion to the endothelium is a crucial initial event in atherogenesis, oxCE may play an important role as mediators in the pathogenesis of atherosclerosis (Huber *et al.*, 2002). Another possible mechanism is the up-regulation of the expression and the synthesis of the pro-fibrogenic cytokine transforming growth factor β1 (TGFβ1), which was shown to be caused by cholesteryl 9-oxononanoate in cultured macrophages. This mechanism suggests that LDL lipid oxidation may be a powerful source of pro-fibrogenic stimuli (Sottero *et al.*, 2005; Gargiulo *et al.*, 2009).

2.3.2.4.2. Impact of Oxidations of the Fatty Acid Moieties on the Hydrolysis of Phytosteryl Fatty Acid Esters

The intestinal hydrolysis of the ester bond of phytosteryl fatty acid esters was identified as an essential precondition for the cholesterol-lowering properties of phytosterols upon dietary uptake. A mechanistic *in vitro* study revealed that the oxidation of sitosteryl oleate to sitosteryl 9,10-dihydroxystearate lowered the affinity towards pancreatic cholesterol esterase resulting in an almost complete loss of hydrolysis. Moreover, the hydrolysis of non-oxidized sitosteryl oleate was found to be significantly decreased in the presence of sitosteryl 9,10-dihydroxystearate, suggesting the 9,10-dihydroxystearate to be a non-competitive inhibitor (Julien-David *et al.*, 2009). This study may indicate that oxidative modifications in the fatty acid moieties of phytosteryl fatty acid esters influence the intestinal hydrolysis of phytosteryl fatty acid esters.

2.4. Analytical Methods

2.4.1. Analysis of Phytosterol Oxidation Products

Most of the methodologies described for the analysis of phytosterol oxidation products refer to the analysis of polar secondary oxidation products (POPs) in their free from, i.e. after cleavage

of the ester-bond, if phytosterols were present in esterified form. The described analytical methods commonly comprise 7-keto, 7-hydroxy, 5,6-epoxy and triol-derivatives of various phytosterols. With sitosterol being the major phytosterol in both natural and enriched foods, sitosterol oxides are the most abundant oxides in foods, but depending on the phytosterol composition in foods also minor proportions of campesterol, stigmasterol and brassicasterol oxides may be found (Scholz *et al.*, 2015a; Lin *et al.*, 2016b). The analysis of a potentially great number of phytosterol oxidation products generally occurring at low concentrations in complex food matrices is challenging. As the analysis of phytosterol oxidation products is not standardized, several analytical methodologies are available. However, all of these methodologies are characterized by specific consecutive procedures. When POPs are determined in complex food matrices, lipid extraction is performed in a first step. The lipids are subjected to saponification or transesterification to remove the quantitatively dominating triacylglycerides and to liberate the oxidized sterol moieites from the esterified fatty acids. To avoid interferences during chromatographic analysis, POPs are commonly separated from the quantitatively dominating non-oxidized phytosterols via column chromatography, thin-layer chromatography (TLC) or solid-phase extraction (SPE). Before the individual POPs are separated and analyzed via GC-FID/MS or HPLC-UV/DAD/MS, the free hydroxyl group of POPs on C_3 is derivatized to increase the volatility of POPs for the gas chromatographic analysis and to increase the detector response for the HPLC analysis (Dutta, 2004; Guardiola *et al.*, 2004; Vanmierlo *et al.*, 2013).

2.4.1.1. Lipid Extraction From Foods

In a recent study, several methodologies for the lipid extraction from different complex food matrices were evaluated for the subsequent POP analysis (Menéndez-Carreño *et al.*, 2016). The evaluation was mainly based on the lipid extraction yields that were likewise considered to reflect the extraction efficiency of POPs. Therefore, lipid extraction from meat and fish samples was done with mixtures of chloroform and methanol at room temperature according to Folch *et al.* and Bligh and Dyer, respectively (Folch-Pi *et al.*, 1957; Bligh and Dyer, 1959). Vegetables and potatoes were lyophilized before the lipid extraction was performed with a binary solvent consisting of *n*-hexane and *iso*-propanol, as originally described by Hara and Radin (Hara and Radin, 1978). Bakery products were subjected to acidic hydrolysis in a Soxhlet extractor prior to lipid extraction to release the phytosterols from phytosteryl glycosides (Toivo *et al.*, 2000). However, in acidic aqueous media 5,6-epoxysterols are known to react to the corresponding triols via epoxide ring opening (Giuffrida *et al.*, 2004; Poirot and Silvente-Poirot, 2013).

Therefore, procedures comprising acidic media and heat should be used with caution. For the analysis of eggs, the samples were freeze-dried and hydrolyzed under acidic conditions, before the lipid fraction was extracted in a Soxhlet extractor using a solvent system consisting of diethyl ether and petroleum ether.

2.4.1.2. On-line LC-GC Analysis

Recently, a novel approach based on liquid chromatography coupled on-line to gas chromatography (on-line LC-GC) was established for the analysis of POPs in foods, using heated PSE enriched margarine as an example (Scholz *et al.*, 2015b). Transesterification of the heated margarine with sodium methoxide was preferred over saponification, as complete transesterification was achieved under mild conditions and short reaction times. Therefore, the risk of artifact formations and degradations of POPs was reduced. After acetylation of the sample with acetic anhydride, the non-oxidized acetylated phytosterols were preseparated from the acetylated POPs via LC. The two LC fractions containing the acetylated (i) 5,6-epoxy- and 7-hydroxyphytosterols and (ii) 7-ketophytosterols were transferred on-line to the GC for the analysis of individual POPs. This allowed the analysis of 5,6-epoxy-, 7-hydroxy- and 7-ketophytosterols within two analytical runs. The acetylated POPs were identified via on-line LC-GC coupled to a mass spectrometer, quantifications were performed with a flame ionization detector that enabled quantifications over a broad concentration range. The LC-GC on-line coupling combined the preconcentration of POPs and their analysis into one analytical step that can be performed in a closed, automatized system and therefore, risks concerning sample losses and undesired reactions can be minimized (Scholz *et al.*, 2015b). Figure 12 shows a schematic representation of the used on-line LC-GC system equipped with a temperature programmable multimode inlet (MMI). The multimode inlet is a type of programmable temperature vaporizer (PTV) interface that allows the injection of large volumes of HPLC eluent into the GC. In the solvent vent mode, the chosen HPLC fraction is injected into the GC inlet at a temperature above the boiling point of the solvent. The analytes accumulate in the inlet liner while the solvent is removed via a gas flow to split vent. After evaporation of the solvent, the inlet is heated up quickly to transfer the analytes to the GC column. The carrier gas is supplied via a pneumatics control module (PCM) that enables a pressure controlled transfer by controlling the pressure in the transfer line and thus, a pushing back of the solvent vapors into the transfer line is prevented. For purge and clean-up purposes, the inlet may be switched to a purge mode that causes any retaining material in the inlet liner to be removed to waste (Scholz *et al.*, 2015b).

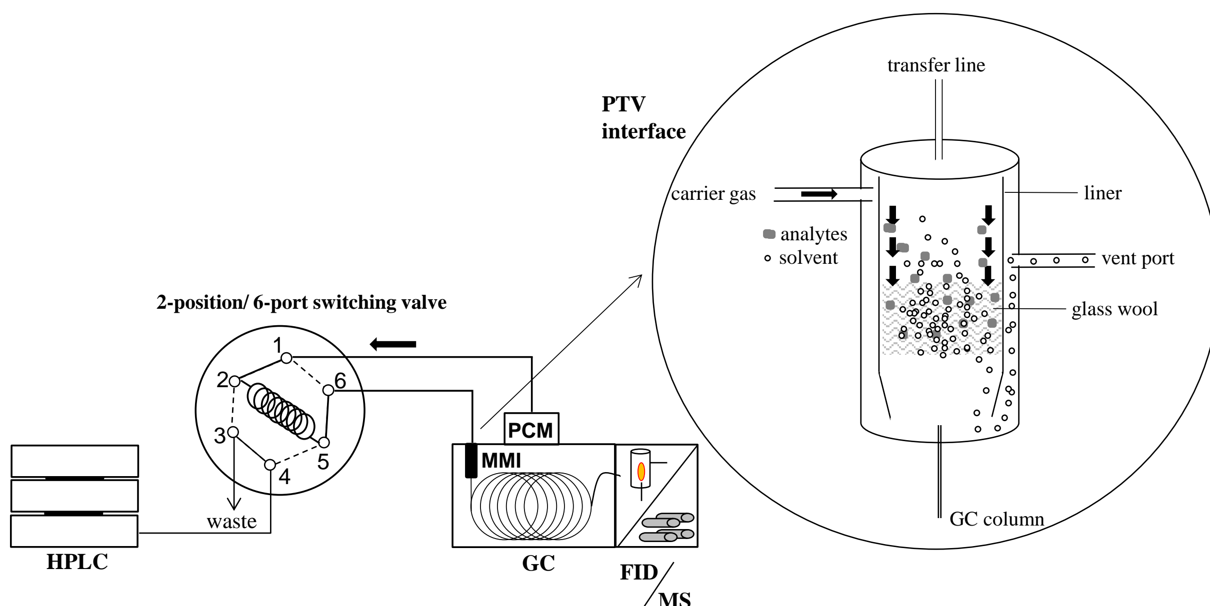

Figure 12. Scheme of the on-line LC-GC system (transfer mode): the chosen HPLC fraction is transferred from the HPLC to the GC via a 2-position/6-port switching valve. The removal of the HPLC solvent is performed in a temperature programmable multimode inlet in the PTV solvent vent mode. The transfer line between the valve and the inlet is pressure controlled by a second line controlled by the pneumatics control module (PCM) (modified from Scholz *et al.*, 2015b).

2.4.2. Analysis of Acyl Chain Oxidation Products

2.4.2.1. Oxidized Steryl Fatty Acid Esters

For the analysis of steryl fatty acid hydroperoxides, the hydroperoxides were separated from non-oxidized steryl fatty acid esters and, if required, from the lipid matrix by silica solid-phase extraction. The concentrated hydroperoxides were identified by high performance liquid chromatography coupled to a mass spectrometer, and an evaporative light scattering detector was used for quantifications (HPLC-ELSD) (Lehtonen *et al.*, 2011b; Lehtonen *et al.*, 2012b).

Probably owing to the fact that secondary oxidation products of oxidized steryl fatty acid esters show relatively high molecular masses, mainly analytical methods based on liquid chromatography were used. Cholesteryl fatty acid core aldehydes formed upon *in vitro* oxidations of unsaturated cholesteryl fatty acid esters and human LDL were derivatized to 2,4-dinitrophenylhydrazones (DNPH), preconcentrated via thin-layer chromatography and analyzed via HPLC-MS, or analyzed via GC-MS as methoxime derivatives (Kamido *et al.*, 1992a; Kamido *et al.*, 1992b; Kamido *et al.*, 1993; 1995). Similar LC-based methods were also applied for the analysis of various oxidized cholesteryl fatty acid esters extracted from atherosclerotic lesions (Hoppe *et al.*, 1997; Hutchins *et al.*, 2011). However, most of these studies are limited to the identification of oxidized cholesteryl fatty acid esters, reliable

quantitations of these oxidation products were not achieved. For the quantitative analysis of cholesteryl 9-oxononanoate (9-ONC) in oxidized human LDL and atherosclerotic lesions, 9-ONC was extracted from the samples and transformed into a fluorescent decahydroacridine derivative that was analyzed via HPLC coupled with fluorescence detection (Karten *et al.*, 1998). In the only study available for phytosteryl fatty acid esters, Julien-David *et al.* directly subjected heated PSE-enriched margarine to reversed phase HPLC-MS/MS for the quantitative analysis of oxidized sitosterols and sitosterol esterified to 9,10-dihydroxystearic acid. Very high concentrations of oxidized margarine had to be injected to detect these minor oxidation products (Julien-David *et al.*, 2014).

2.4.2.2. Oxidized Triacylglycerides

The heating of fats and oils may result in a very complex mixture of oxidized triacylglycerides. To reduce the complexity, quantitative analyses of individual oxidized acyl moieties are commonly done after conversion of the oxidized triacylglycerides into FAME. This enables the collective analysis of fatty acid methyl esters, which exhibit the same oxidized acyl moiety. On the other hand, the structural information of the original triacylglycerides are lost and methylation procedures may lead to artifact formations (Christie, 1998; Dobarganes and Márquez-Ruiz, 2007). Investigations for suitable methylation procedures for the quantitation of major short-chain glycerol-bound oxidation products revealed potassium hydroxide in methanol and sodium methoxide in *tert*-MTBE to be suitable methylation reagents, showing good repeatability and recovery of the target compounds (Berdeaux *et al.*, 1999). For the analysis of oxidized acyl moieties of original chain lengths, the methylation with sodium methoxide was considered particularly efficient (Velasco *et al.*, 2002; Velasco *et al.*, 2005; Marmesat *et al.*, 2008). In several studies, the nonpolar FAME were separated by silica column chromatography to preconcentrate the oxidized FAME and to exclude other interfering compounds, before the oxidized FAME were analyzed via GC-based methods (Kamal-Eldin *et al.*, 1997; Velasco *et al.*, 2002; Marmesat *et al.*, 2008; Berdeaux *et al.*, 2012). However, the quantitative analysis of specific oxidized FAME remains difficult due to the vast amount of possible oxidation products (Dobarganes and Márquez-Ruiz, 2007). For the analysis of oxidized long-chain keto or hydroxy FAME an additional hydrogenation step was required prior to GC analysis to reduce the vast spectrum of possible oxidation products (Marmesat *et al.*, 2008). Apart from these methodologies, approaches based on normal phase and reversed phase HPLC were developed for the separation and identification of oxidations in the fatty acid moieties of intact triacylglycerides (Neff and Byrdwell, 1998; Byrdwell and Neff, 2004;

Giuffrida *et al.*, 2004; Dobarganes and Márquez-Ruiz, 2007). However, the spectrum of identified oxidation products was rather limited.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Chemical	Supplier
beta-Sitosterol (plant sterol/stanol mixture): 75% sitosterol, 12% sitostanol, 10% campesterol, 2% campestanol and 1% others	Acros Organics, Morris Plains, NJ, USA
Stigmastanol (plant stanol mixture): 98 % sitostanol and 2 % campestanol	Santa Cruz Biotechnology, Dallas, TX, USA
Linoleic acid (≥ 99%)	Sigma-Aldrich, Steinheim, Germany
Oleic acid (≥ 99%)	Sigma-Aldrich, Steinheim, Germany
Pyridine (anhydrous, ≥ 99.8%)	Sigma-Aldrich, Steinheim, Germany
Cholesterol (95%)	Sigma-Aldrich, Steinheim, Germany
Cholesteryl palmitate (≥ 97%)	Sigma-Aldrich, Steinheim, Germany
5,6*β*-Epoxycholesterol (≥ 95%)	Sigma-Aldrich, Steinheim, Germany
7-Ketocholesterol (≥ 90%)	Sigma-Aldrich, Steinheim, Germany
Lactic acid (≥ 90%)	Sigma-Aldrich, Steinheim, Germany
Acetic anhydride (≥ 99%)	Sigma-Aldrich, Steinheim, Germany
BSTFA/TMCS (99:1)	Supelco, Bellefonte, PA, USA
Methyl *tert*-butyl ether	Evonik Industries AG, Essen, Germany
Cyclohexane (analytical grade)	Evonik Industries AG, Essen, Germany
n-Hexane (HiPerSolv Chromanorm)	VWR International, Darmstadt, Germany
Chloroform (AnalaR Normapur)	VWR International, Darmstadt, Germany
Octanoic acid (≥ 98%)	Sigma-Aldrich, Steinheim, Germany
Heptanoic acid (≥ 96%)	Sigma-Aldrich, Steinheim, Germany
Trimethylsilyl diazomethane (2 M in hexane)	Sigma-Aldrich, Steinheim, Germany
3-Chloroperbenzoic acid (≥ 77%)	Sigma-Aldrich, Steinheim, Germany
Cyclooctanone (≥ 98%)	Sigma-Aldrich, Steinheim, Germany
Cycloheptanone (≥ 98%)	Sigma-Aldrich, Steinheim, Germany
2-Iodoxybenzoic acid (45 wt. %)	Sigma-Aldrich, Steinheim, Germany
Sodium borohydride (≥ 96%)	Sigma-Aldrich, Steinheim, Germany
4-Toluenesulfonic acid monohydrate (≥ 98 %)	Sigma-Aldrich, Steinheim, Germany
Pyridinium chlorochromate (≥ 98%)	Sigma-Aldrich, Steinheim, Germany
Dicyclohexylcarbodiimide (1 M in dichloromethane, >99%)	Fluka Analytical, Steinheim, Germany
4-(Dimethylamino)pyridine (puriss.)	Fluka Analytical, Steinheim, Germany
Aleuritic acid (9,10,16-trihydroxypalmitic acid, ≥ 94%)	Santa Cruz Biotechnology, Heidelberg, Germany
Dichloromethane (p.A., 95 %)	Sigma-Aldrich, Steinheim, Germany

Diethyl ether (extra pure)	Sigma-Aldrich, Steinheim, Germany
Methanol (HiPerSolv Chromanorm)	VWR International, Darmstadt, Germany
Magnesium sulfate (anhydrous)	Sigma-Aldrich, Steinheim, Germany
Sodium chloride (p.a.)	Sigma-Aldrich, Steinheim, Germany
Sodium thiosulfate (purum, p.a.)	Sigma-Aldrich, Steinheim, Germany
Sodium methoxide (30 % in methanol)	Merck, Darmstadt, Germany
iso-Propanol (HiPerSolv, Chromanorm)	VWR International, Darmstadt, Germany
5α-Cholestane (≥ 97%)	Sigma-Aldrich, Steinheim, Germany
Potassium hydroxide (≥85 %)	Sigma-Aldrich, Steinheim, Germany
n-Heptane (HiPerSolv Chromanorm)	VWR International, Darmstadt, Germany

3.1.2. Mixture of Phytosteryl/-stanyl Fatty Acid Esters

Vegapure® 95E (phytosteryl/-stanyl fatty acid ester mixture): 2.7 % free sterols/stanols and 96.5 % phytosteryl/phytostanyl esters. The profile of esters was as follows: 44.5 % sitosteryl-18:2, 18.8 % sitosteryl-18:1, 11.0 % campesteryl-18:2, 4.6 % campesteryl-18:1, 4.5 % sitostanyl-18:2, 4.2 % sitosteryl-16:0, 2.3 % sitosteryl-18:0, 1.9 % brassicasteryl-18:2, 1.9 % sitostanyl-18:1, 1.0 % campesteryl-16:0, 0.8 % brassicasteryl-18:1, 0.6 % campestanyl-18:2, 0.6 % stigmasteryl-18:1, 0.6 % campesteryl-18:0, 0.4 % sitostanyl-16:0, 0.4 % sitosteryl-22:0, 0.3 % campestanyl-18:1, 0.3 % stigmasteryl-18:1, 0.2 % sitostanyl-18:0, 0.2 % brassicasteryl-16:1, 0.1 % sitosteryl-20:0, 0.1 % brassicasteryl-18:0, 0.1 % campesteryl-22:0, 0.1 % sitosteryl-16:1, 0.1 % sitosteryl-18:3, 0.1 % sitosteryl-20:1, 0.1 % campestanyl-16:0, 0.1 % stigmasteryl-16:0, 0.04 % sitostanyl-22:0, 0.03 % campesteryl-20:0, 0.03 % campestanyl-18:0, 0.03 % stigmasteryl-18:0, 0.02 % campesteryl-16:1, 0.02 % campesteryl-18:3, 0.02 % campesteryl-20:1, 0.01 % campestanyl-22:0, and 0.01 % sitostanyl-20:0). The mixture was provided by Cognis GmbH (Illertissen, Germany).

3.1.3. Liquid Margarine

The liquid margarine “Becel Cuisine” (Unilever, London, UK) was purchased in a local supermarket in Freising, Germany; ingredients: 82 wt. % vegetable oils (sunflower, rapeseed, linseed, hardened rapeseed, in variable proportions), water, potassium chloride, emulsifier (soya lecithin), acidity regulator (potassium citrate, citric acid), vitamins (A, D, E), flavors, coloring agents (carotenes). The margarine was labelled as shown in Table 6.

Table 6. Labelling of the liquid margarine

	per 100 ml (≙ 90 g)	per 10 ml (2 teaspoons)	GDA* per 10 ml
energy	2700 kJ/ 660 kcal	270 kJ/ 65 kcal	3%
total fat	74 g	7 g	10%
saturated fatty acids	8 g	0.8 g	4%
monounsaturated fatty acids	31 g	3 g	
polyunsaturated fatty acids	34 g	3.5 g	
total carbohydrates	< 0.5 g	<0.5 g	<1%
sugars	< 0.5 g	<0.5 g	<1%
proteins	< 0.5 g	<0.5 g	<1%
salt	< 0.013 g	<0.013 g	<1%
vitamin E	34 mg (283%)**	3.4 mg (28%)**	
vitamin A	720 µg (90%)**	72 µg (9%)**	
vitamin D	6.8 µg (136%)**	0.68 µg (14%)**	
omega-3 fatty acids	6.2 g	0.62 g	
omega-6 fatty acids	28 g	2.8 g	

* Guideline Daily Amount for an average adult person (8400 kJ/2000 kcal).
** Percentages based on recommended daily intakes.

3.1.4. Baking Ingredients

Wheat flour (type 405)	Rosenmühle GmbH, Ergolding, Germany
Sugar	Südzucker AG, Mannheim, Germany
Baking powder “Backin”	Dr. Oetker, Bielefeld, Germany

3.2. Methods

3.2.1. Analysis of Phytosterol Oxidation Products in Heated Foods

3.2.1.1. Enrichment of the Liquid Margarine

The phytosteryl/-stanyl fatty acid ester mixture “Vegapure 95 E” (6.25 g) was added to 43.75 g of the liquid margarine “Becel Cuisine” (cf. 3.1.3) in a plastic vessel (100 ml). The vessel with the sample material was flushed with nitrogen, closed and stirred at room temperature for 1 h with a magnetic stirrer. For final homogenisation, the product was treated with an Ultra-Turrax homogenisator (T25, Janke & Kunkel, Germany) for 10 minutes. After the enrichment step, the liquid margarine had a phytosteryl/-stanyl fatty acid ester content of 12.5 wt. % and a fat content of 84.25 %. The dry matter of the liquid margarine was 83.1 % before and 85.5 % after the PSE-enrichment step, as determined via the sea sand method. The production of the enriched liquid margarine was performed with different samples of the commercially available liquid margarine.

3.2.1.2. Dough Production

A dough was prepared with non-enriched and enriched liquid margarine, respectively, according to the following protocol: 4.2 g baking powder was mixed with 50 g wheat flour (type 405), and 15 g non-enriched or enriched liquid margarine was added to the mixture. A sugar solution, consisting of 8 g sugar in 25 g tap water, was given to the mixture, and a dough was formed (100.5 ± 0.5 g).

3.2.1.3. Heating Treatments

3.2.1.3.1. Pan-frying

Non-enriched (1 g, accuracy of ± 0.1 mg) and enriched liquid margarine (1 g and 3 g, accuracy of ± 0.1 mg, respectively), were heated in a Teflon-coated frying pan (Tefal, Ø 12 cm, ground surface 77.88 cm^2) on an electric plate (Bauknecht, München, Germany). The liquid margarine was placed in the middle of the frying pan and the electric plate was set to its maximum level “6” for 2 min. After 2 min the plate was turned down to level “2” and switched off after exactly 540 s. The temperature of the liquid margarine was recorded every 30 s with an infrared thermometer (MeasuPro IRT20) in the course of the heating procedure. The frying pan was removed from the plate and was cooled down to room temperature. The heated sample was extracted from the frying pan with 5 + 4 mL of chloroform, and the solvent was removed in vacuum and under nitrogen stream.

3.2.1.3.2. Oven-Heating in a Vial

Non-enriched (1 g, accuracy of ± 0.1 mg) and enriched liquid margarine (0.5 g and 1 g), respectively, were weighed into a 12 ml glass vial. The resulting surface of liquid margarine in contact to air was 2.01 cm^2. The samples were heated in a drying cabinet at 200 ± 2 °C for 20 min. The temperature was adjusted and controlled by a thermometer placed within the drying cabinet. After heating, the samples were cooled down to room temperature.

3.2.1.3.3. Oven-Heating in a Casserole

Non-enriched and enriched liquid margarine (2.91 g, accuracy of ± 0.1 mg), respectively, were evenly spread in a household ceramic casserole (total surface of 546.25 cm^2) with a brush. A household oven (Bauknecht, München, Germany) was pre-heated for 30 min at 200 °C in circulating air mode before the casserole was heated at 200 °C for 20 min. After heating, the casserole was cooled down to room temperature. The oxidized sample was extracted from the

casserole with 3×20 mL of chloroform, and the solvent was removed in vacuum and under nitrogen stream.

Each pan-frying, vial or casserole experiment was carried out twice.

3.2.1.3.4. Muffin Baking

For muffin baking, the dough (100.5 ± 0.5 g), containing non-enriched or enriched liquid margarine was transferred into a muffin baking tin. The resulting surface of dough in contact with air was 43 cm^2. The dough was baked at 200 °C for 20 min in an oven (Bauknecht, München, Germany) that was pre-heated for 30 min at 200 °C. Directly after heating, the surface temperature of the muffin was determined with an infrared thermometer (MeasuPro IRT20). After cooling down to room temperature the muffin was weighed (88.96 ± 0.33 g).

3.2.1.3.5. Cake Baking

For cake baking, the dough (100.5 ± 0.5 g), containing enriched liquid margarine, was transferred into a cake tin. The resulting surface of dough in contact with air was 254 cm^2. Cake baking and surface temperature measurement were performed as described for the muffin baking. After cooling down to room temperature the cake was weighed (72.3 ± 0.4 g).

3.2.1.3.6. Cookie Baking

For the preparation of a cookie, a piece (13.87 ± 0.03 g) of the initially prepared dough, containing enriched liquid margarine, was cut out with a cookie cutter (i.d. 6 cm). The resulting surface of dough in contact with air was 27 cm^2. The dough was baked at 200 °C for 20 min and 40 min, respectively. After cooling down to room temperature the cookies were weighed: 20 min (10.01 ± 0.1 g); 40 min (9.1 ± 0.1 g). The surface temperature measurement was performed as described for muffin and cake baking.

All bakery products were produced in triplicate.

3.2.1.4. Lipid Extraction from Bakery Products

For the lipid extraction, the muffin and the cake, respectively, were divided into four approximately equal pieces, and an aliquot of the muffin (23.70 ± 2.36 g) or the cake (24.39 ± 0.84 g) was ground in a ceramic bowl in the presence of 50 g sea sand before lipid extraction. The more brittle cookies (20 min, 10.01 ± 0.1 g; 40 min, 9.1 ± 0.1 g) were ground without sea sand. Lipid extraction was performed under continuous stirring with 100 mL of chloroform for 2 h at room temperature in a closed flask wrapped with aluminium foil. Subsequently, the crude

extract was filtered through a fritted glass funnel under vacuum. The filter cake was washed with 20 mL of chloroform. The extract was evaporated under reduced pressure and under nitrogen stream until the weight of the lipid extract remained constant. Additionally, a lipid extraction of a muffin baked with enriched liquid margarine was performed with 200 mL of chloroform.

3.2.1.5. Sample Preparation for POP Analysis

For the analysis of POPs in the non-heated, non-enriched or enriched liquid margarine, 250 mg of sample material (accuracy of ± 0.1 mg) was directly weighed into 12 mL vials and subjected to further analysis. For analyses of heated margarines, 210 mg of heated lipid matrix, corresponding to approx. 250 mg of originally used margarine, was weighed into 12 mL vials. The internal standards 5,6*β*-epoxycholesterol (IS_1) and 7-ketocholesterol (IS_2) were added in the range of 6.1–212.4 µg and 5-339.2 µg, respectively (for details see Table 32); the amounts of internal standards were adapted on the basis of preliminary tests. After removal of the solvent under a gentle nitrogen stream, 1 mL of MTBE and 2 mL of 10 % sodium methoxide (in methanol) were added to the samples (Scholz *et al.*, 2015b). The mixture was vortexed and allowed to stand for 1 h at room temperature in the dark. After transesterification, 5 mL of bi-distilled water and 3 mL of lactic acid were added to the mixture and the phytosterol oxidation products were extracted twice with 5 mL and 4 mL of chloroform. The combined organic phases were washed with 10 mL of saturated sodium hydrogen carbonate and 10 mL of bi-distilled water. The organic extract was evaporated to dryness under a gentle nitrogen stream and acetylated with 2 mL pyridine and 200 µL of acetic anhydride for 12 h at room temperature in the dark. The reagents were removed via a gentle stream of nitrogen, the residue was dissolved in 0.5–3 mL of *n*-hexane/MTBE/*iso*-propanol (80:20:0.3, v/v/v) and subjected to on-line LC–GC analysis.

3.2.1.6. Recovery Rates for POP Analysis

Non-enriched liquid margarine (250 mg) was spiked with defined amounts of 5,6*α*-epoxysitosterol, 7*β*-hydroxycholesterol and 7-ketositosterol, representing the three different chemical classes under study, at three different levels in the range of 3.5-310 µg in triplicate (for details see Table 8). The samples were taken through the entire preparation process as described for POP sample preparation (cf. 3.2.1.5).

3.2.1.7. Recoveries of POPs Extracted from Backery Products

A muffin with enriched liquid margarine as well as a muffin without any liquid margarine were baked on the same day (cf. 3.2.1.3.4). Lipid extraction from the muffin, containing liquid margarine, was performed, and POP contents were determined (cf. 3.2.1.4/3.2.1.5). Subsequently, the lipid extract was incorporated into the muffin that was baked without margarine. Afterwards, the incorporated lipids were reextracted from the muffin (cf. 3.2.1.4) and subsequent POP analysis was carried out (cf. 3.2.1.5).

3.2.1.8. On-Line LC-GC Analysis

The on-line LC-GC analysis was performed according to previously established parameters (Scholz *et al.*, 2015b). The on-line LC-GC system consisted of a 1220 Infinity LC which was coupled to a 7890A GC equipped with an FID via a 1200 Infinity Series 2-position/6-port switching valve (Agilent Technologies, Waldbronn, Germany). The valve was fitted with a 250 µL sample loop. LC separations were carried out on a 250 x 2 mm, 5 µm, Eurospher-100 Si column (Knauer, Berlin, Germany) at 30 °C using *n*-hexane/MTBE/*iso*-propanol (80:20:0.3, v/v/v) as eluent at 0.2 mL/min. The injection volume was 1 µL and UV detection was performed at 206 nm. The transfer valve switched for transfer 1 (5,6-epoxy- and 7-hydroxysterols) 6.7 min after injection and for transfer 2 (7-ketosterols) 8.1 min after injection. Evaporation of the solvent was performed via the temperature programmable multimode inlet (MMI) in the programmable temperature vaporizer (PTV) solvent vent mode. The injector was equipped with a completely deactivated liner (5190-2295 Ultra Inert Liner; universal, low pressure drop, glass wool, Agilent Technologies, Waldbronn, Germany). The injector temperature was set to 50 °C hold for 0.625 min and vent flow was adjusted to 900 mL/min with a vent pressure of 4 psi until 0.625 min. The inlet was then heated with 900 °C/min to 300 °C and the analytes were transferred to the GC column. The purge flow to the split vent for clean-up was started at 0.625 min with 2.5 mL/min. The stainless steel transfer line installed between the valve and the inlet was pressure-controlled by a second line controlled by the pneumatics control module (PCM). The pressure was set to 5 psi for 0.3 min followed by a ramp of 10 psi/min until 20 psi. GC separations were carried out on a 15 m x 0.25 mm i.d. fused silica capillary column coated with a film of 0.1 µm Rtx-200MS trifluoropropylmethyl polysiloxane (Restek GmbH, Bad Homburg, Germany). Hydrogen was used as carrier gas with a constant flow rate of 1.5 mL/min. The oven temperature program was as follows: initial temperature, 40 °C (2 min), programmed at 100 °C/min to 100 °C, then at 15 °C/min to 310 °C (5 min). The detector

temperature was set to 340 °C. Nitrogen was used as makeup gas with a flow rate of 25 mL/min. Data acquisition was performed by ChemStation software.
Identification of the analytes was performed by on-line LC-GC/MS. The GC part was coupled via a transfer line to a 5975C inert mass spectrometer with triple axis detector (Agilent Technologies, Waldbronn, Germany). Mass spectra were obtained by positive electron impact ionization at 70 eV in the scan mode at unit resolution from 50 to 700 Da. The interface was heated to 280 °C, the ion source to 250 °C, and the quadrupole to 150 °C. GC separations were performed on a 15 m x 0.25 mm i.d., 0.1 µm film, Rtx200-MS fused silica capillary column (Restek, Bad Homburg, Germany). The GC conditions were as described for on-line LC-GC-FID analysis. Data acquisition was performed by MSD Productivity ChemStation. The phytosterol oxidation products were quantitated via on-line LC-GC/FID using a response factor (Rf) of 1.0 for 5,6-epoxy- and 7-ketophytosterols and an Rf of 1.2 for hydroxyphytosterols.

3.2.1.9. Analysis of Intact Phytosterols

Determination of intact phytosterols was carried out as previously described (Menéndez-Carreño *et al.*, 2016). For analyses of non-heated liquid margarines, 60 mg (accuracy of ± 0.1 mg) of the liquid margarine was weighed into a 12 mL glass vial. For heated liquid margarines, 50 mg of the heated lipid matrix, corresponding to approx. 60 mg of originally used liquid margarine, was weighed in a 12 mL glass vial. Subsequently, 250 µL of the internal standard 5*α*-cholestane (2 mg/mL in *n*-hexane) was added, the solvent was evaporated under a gentle nitrogen stream, and the sample was saponified for 90 min at 90 °C after the addition of 7 mL of potassium hydroxide (2 M in 96 % ethanol). After cooling down to room temperature, 5 mL of demineralised water and 5 mL of *n*-heptane was added. The upper organic phase was withdrawn and the extraction was repeated with 2 × 5 mL *n*-heptane. The combined organic phase was evaporated in vacuum and the sample was dissolved in 1 mL of *n*-hexane. An aliquot of 100 µL was evaporated under nitrogen stream und silylated with 100 µL of pyridine and 100 µL of BSTFA/TMCS for 20 min at 80 °C. The sample was dried under nitrogen stream and dissolved in 100 µL (non-enriched liquid margarine) or 500 µl (enriched liquid margarine) *n*-hexane for GC-analysis (cf. 3.2.2.3).

3.2.2. Analysis of Oxidized Phytosteryl/-stanyl Fatty Acid Esters

3.2.2.1. Identification of Acyl Chain Oxidation Products

3.2.2.1.1. Synthesis of Phytosteryl/-stanyl Linoleates

The synthesis of the mixture of phytosteryl/-stanyl linoleates was carried out according to a previously described protocol (Barnsteiner *et al.*, 2012). Briefly, 100 mg of the mixture of phytosterols/-stanols ("*β*-sitosterol") and 170 mg linoleic acid were heated in a sealed, nitrogen-flushed 2 mL glass vial for 25 h at 180 °C. The excess of acid was removed with 2.5 mL potassium hydroxide (1 M), and the esters were extracted with 3 × 2.5 mL hexane/MTBE (3:2, v/v). Final purification on a 500 mg silica solid phase extraction (SPE) column (Supelco) with cyclohexane as an eluent resulted in 47 mg of a mixture of phytosteryl/-stanyl linoleates (yield 29%) as a colorless oil. The phytosterol/phytostanol distribution of the mixture corresponded to that of the "*β*-sitosterol" sample used for esterification (cf. 3.1.1). The purity of the linoleates determined by ^{1}H NMR and GC-FID was ≥ 98%.

3.2.2.1.2. Synthesis of Phytosteryl/-stanyl Octanoates and Heptanoates

Phytosteryl/-stanyl octanoates and heptanoates were synthesized according to the same procedure as described for the linoleates. The reaction of the phytosterol/-stanyl mixture "*β*-sitosterol" (100 mg) and octanoic acid/heptanoic acid (90 mg) resulted in 50 mg phytosteryl/-stanyl octanoates (yield 38%) and 61 mg phytosteryl/-stanyl heptanoates (yield 48%), respectively, as white solids. The sterol/stanol distributions corresponded to that of the "*β*-sitosterol" sample used for esterification. No impurities were observed in ^{1}H NMR and GC-FID analyses. The mass spectra of synthesized octanoates were in agreement with previously published spectra (Barnsteiner *et al.*, 2012).

3.2.2.1.3. Synthesis of 9-Oxononanoic acid

9-Oxononanoic acid was synthesized according to a previously described method via diol cleavage of aleuritic acid (Reuter and Salomon, 1978). Briefly, 6 g potassium periodate in 300 mL sulfuric acid (1 M) was quickly added to 8 g aleuritic acid in methanol/water (200 + 200 mL) at 40 °C. After stirring for 10 min, the mixture was cooled down to 15 °C in a methanol/ice bath and immediately extracted with 2 x 400 mL diethyl ether. The combined organic layers were extracted with 2 x 100 mL saturated sodium hydrogen carbonate solution and the combined aqueous layers were acidified with concentrated hydrochloric acid. The aqueous

solution was extracted with 2 x 100 mL diethyl ether, and the combined ether layers were washed with 2 x 100 mL of an aqueous sodium chloride solution (10 %) and dried over magnesium sulfate. After removal of the solvent under a nitrogen stream, 3.2 g of a colorless oil was obtained, which was used for the subsequent esterification step.

3.2.2.1.4. Synthesis of Phytosteryl/-stanyl 9-Oxononanoates

The esterification was carried out as previously described for the synthesis of cholesteryl 9-oxononanoate (Boechzelt *et al.*, 1998). 9-Oxononanoic acid (200 mg), DMAP (5.6 mg) and the phytosterol/-stanyl mixture "*β*-sitosterol" (195 mg) were dissolved in 4 mL dichloromethane, 1 mL DCC solution was added and the mixture was stirred for 24 h at room temperature. The mixture was filtered in the cold, dissolved in 10 mL dichloromethane, and the solution was subsequently washed with 25 mL hydrochloric acid (0.5 M) and 25 mL saturated sodium hydrogen carbonate solution. The organic layer was dried over magnesium sulfate and filtered. For final purification, the esterified product was dissolved in 5 mL cyclohexane. The sample was loaded onto a 500 mg silica SPE column, pre-conditioned with 6 mL cyclohexane. The esters were eluted with 15 mL heptane/MTBE (98:2, v/v). After evaporation of the solvent, 203 mg of a mixture of phytosteryl/-stanyl 9-oxononanoates was obtained as a white solid. The phytosterol/-stanol distribution of the mixture corresponded to that of the "*β*-sitosterol" sample used for esterification. The purity of the oxo-esters was determined to be ≥ 92% by ^{1}H NMR and GC-FID.

3.2.2.1.5. Synthesis of 8-Oxooctanoic Acid and 7-Oxoheptanoic Acid

8-Oxooctanoic acid and 7-oxoheptanoic acid were synthesized on the basis of a previously published method via Baeyer-Villiger reaction of the corresponding cycloalkanones, lactone opening and subsequent oxidation of the hydroxyacids (Rajabi *et al.*, 2014). Cyclooctanone and cycloheptanone (2.079 and 1.392 g, respectively) were dissolved in 10 mL dichloromethane and *m*-CPBA (2.152 and 1.392 g, respectively) was added. After stirring for 6 d at room temperature, the reaction was quenched by the addition of 500 µL saturated sodium thiosulfate solution. The organic layer was washed with 3 x 20 mL saturated sodium hydrogen carbonate solution, 2 x 20 mL of an aqueous sodium chloride solution (10 %), dried over magnesium sulfate, and filtered. After evaporation of the solvent under nitrogen, the following colorless liquids (purities determined by GC) were obtained: 2-oxonanone (723 mg; 20 % lactone and 80 % educt) and 2-oxocanone (563 mg; 21 % lactone and 79 % educt). For lactone opening, the reaction products were dissolved in 2 mL dioxane, treated with 15 mL sodium hydroxide (3 M)

and stirred over night at room temperature. The mixtures were washed with 15 mL ethyl acetate and the pH was adjusted to 3.5 with hydrochloric acid (25 %). The mixtures were extracted with 2 x 20 mL ethyl acetate, washed with 2 x 20 mL saturated sodium hydrogen carbonate solution, dried over magnesium sulfate, filtered and evaporated under nitrogen stream. 8-Hydroxyoctanoic acid (168 mg) and 7-hydroxyheptanoic acid (130 mg) were obtained as white solids. After silylation, GC-purities of ≥ 90% were determined for both acids. 8-Hydroxyoctanoic acid and 7-hydroxyheptanoic acid were dissolved in dimethyl sulfoxide (3.7 and 3.2 mL, respectively), before 2-iodoxybenzoic acid (463 and 440 mg, respectively) was added. The mixtures were stirred at room temperature for 4 h, the reactions were quenched with distilled water, and the mixtures were filtered. After extraction with 2 × 20 mL ethyl acetate, drying over magnesium sulfate, filtering and evaporation under nitrogen stream, 165 mg 8-oxooctanoic acid and 125 mg 7-oxoheptanoic acid were obtained as white solids.

3.2.2.1.6. Synthesis of Phytosteryl/-stanyl 8-Oxooctanoates and 7-Oxoheptanoates

Esterifications of the phytosterol/-stanyl mixture "*β*-sitosterol" with 8-oxooctanoic acid and 7-oxoheptanoic acid were performed as described for the 9-oxononanoates (cf. 3.2.2.1.4). After esterification, the esters were purified by silica SPE as described for the 9-oxononanoates, but the esters were eluted with MTBE. After purification, 43.5 mg of a mixture of phytosteryl/-stanyl 8-oxooctanoates and 26 mg of a mixture of phytosteryl/-stanyl 7-oxoheptanoates were obtained as colorless oils. The phytosterol/-stanol distribution of the mixture corresponded to that of the "*β*-sitosterol" sample used for esterification. The purities of the oxo-esters determined by ^{1}H NMR and GC-FID were ≥ 80% for 8-oxooctanoates and ≥ 70% for 7-oxoheptanoates.

3.2.2.1.7. Reduction of Oxo-esters to the Corresponding Hydroxy-esters

The oxo-esters were reduced to the hydroxy-esters according to a procedure described for the reduction of 9-oxononanoic acid (Kallury *et al.*, 1987). The oxo-esters (5-10 mg) were dissolved in 200 µL dioxane and cooled in an ice-bath. Sodium borohydride (5 mg) was added to the solution and stirred for 10 min at room temperature. Crushed ice was added to quench the reaction and the hydroxides were extracted with 2 × 3 mL hexane/MTBE (3:2, v/v). After drying over magnesium sulfate and solvent evaporation under nitrogen stream, 8.4 mg of a mixture of phytosteryl/-stanyl 8-hydroxyoctanoates was obtained as a white solid and 4.5 mg of a mixture of 7-hydroxyheptanoates as a colorless oil. The phytosterol/-stanol distributions of the mixtures corresponded to those of the oxo-esters used for reduction. The purities of the

hydroxy-esters determined by ^{1}H NMR and GC-FID (after silylation) were ≥ 82% for 8-hydroxyoctanoates and ≥ 80% for 7-hydroxyheptanoates.

3.2.2.1.8. Synthesis of Stigmasta-3,5-dien-7-one

"*β*-Sitosterol" (740 mg) was acetylated with 3 mL acetic acid anhydride in 30 mL pyridine at room temperature for 12 h. The solvent was removed under reduced pressure and sitosteryl acetate was used for the synthesis of 7-ketositosteryl acetate as described previously (Geoffroy *et al.*, 2008). Briefly, 7.4 g pyridinium chlorochromate was added to a suspension of 18 g Celite in 90 mL benzene, followed by the addition of sitosteryl acetate. The reaction mixture was refluxed for 24 h. After cooling down to room temperature, the reaction mixture was filtered on a fritted glass funnel, and the filtrate was carefully washed with 3 × 15 mL ethyl acetate. After removal of the solvents under reduced pressure, sitosteryl acetate (623 mg) was removed from the reaction mixture on a silica gel column (30 g) with ethyl acetate/hexane (5:95, v/v), followed by the elution of 7-ketositosteryl acetate (90 mg) with ethyl acetate/hexane (10:90, v/v). Then, an aliquot of 23 mg 7-ketositosteryl acetate and 52 mg *p*-toluenesulfonic acid, dissolved in 5 mL toluene, was refluxed for 2 h (Abramovitch and Micetich, 1962). The mixture was dissolved in diethyl ether and washed with an aqueous sodium carbonate solution and distilled water. After evaporation to dryness, 20 mg of stigmasta-3,5-dien-7-one was obtained as yellow oil (purity determined by GC: 75%).

3.2.2.1.9. Thermal Treatment of the Mixture of Phytosteryl/-stanyl Linoleates

The mixture of phytosteryl/-stanyl linoleates was weighed into a 2 mL brown glass vial without a lid (12 ± 0.2 mg) and oxidized in a heating block (VLM Metal, Bielefeld, Germany) at 180 °C for 40 min. After thermal treatment, the samples were cooled down to room temperature before undergoing further sample preparation.

3.2.2.1.10. Solid-phase Extraction (SPE)

The heated linoleates were dissolved in 3 mL cyclohexane, and the solution was loaded onto a Supelclean 500 mg silica SPE column (SUPELCO), that had been pre-conditioned with 6 mL *n*-hexane. The non-oxidized linoleates and non-polar oxidation products were eluted from the SPE column, attached to a vacuum chamber, with 21 mL cyclohexane at a flow rate of approximately 1 mL/min (fraction 1). Subsequently, the polar oxidation products were eluted with 6 mL MTBE (fraction 2). The solvents of both fractions were evaporated under a gentle

nitrogen stream. The residue of fraction 1 was dissolved in 1 mL hexane/MTBE (3:2, v/v) and subjected to GC-FID/GC-MS analysis. The residue of fraction 2 was silylated with 100 µL pyridine and 100 µL BSTFA/TMCS for 20 min at 80 °C. The silylation reagent was evaporated under a gentle nitrogen stream, the residue was dissolved in 500 µL hexane/MTBE (3:2, v/v) and subjected to GC-MS/FID analysis.

3.2.2.2. Quantitation of Acyl Chain Oxidation Products

3.2.2.2.1. Synthesis of Phytosteryl/-stanyl Oleates and Linoleates

The synthesis of phytosteryl/-stanyl oleates and linoleates was carried out as previously described (Barnsteiner *et al.*, 2012). Briefly, 100 mg of the phytosterol/-stanol sample "*β*-sitosterol" and of the phytostanol sample "stigmastanol", respectively, were treated as follows: 170 mg oleic or linoleic acid were placed in a sealed, nitrogen-flushed (in order to avoid oxidation) 2 mL glass vial and heated for 25 h at 180 °C. The excess of acid was removed with 2.5 mL potassium hydroxide (1 M), and the esters were extracted with 3 × 2.5 mL hexane/MTBE (3:2, v/v). Final purification on a 500 mg Supelclean silica SPE column from Supelco (Bellefonte, PA, USA) with cyclohexane as an eluent resulted in 107 mg phytosteryl/-stanyl oleates (yield = 65%), 94 mg phytostanyl oleates (yield = 58%), 79 mg phytosteryl/-stanyl linoleates (yield = 48%) and 72 mg phytostanyl linoleates (yield = 44%). The phytosterol/-stanol distributions corresponded to those of the phytosterol/-stanol samples used for esterification. The purity of the esters was determined by ^{1}H NMR and GC-FID and was ≥ 98% for all ester mixtures.

3.2.2.2.2. Synthesis of Oxidized Cholesteryl Esters as Internal Standards

Cholesteryl octanoate and cholesteryl 9-oxononanoate were synthesized via esterification with the respective acid, and cholesteryl 9-hydroxynonanoate was obtained by reduction of cholesteryl 9-oxononanoate with sodium borohydride as described for the corresponding sitosteryl esters (cf. 3.2.2.1.7). The NMR spectra of cholesteryl octanoate and cholesteryl 9-oxononanoate corresponded to previously published data (Cameron *et al.*, 1972; Boechzelt *et al.*, 1998). No impurities were observed for cholesteryl octanoate (GC-FID and ^{1}H NMR); cholesteryl 9-oxononanoate and cholesteryl 9-hydroxynonanoate had a purity of ≥ 92%.

3.2.2.2.3. Thermal Treatment of Phytosteryl/-stanyl Oleates and Linoleates

Each ester mixture (11 ± 0.2 mg) was weighed into a 2 mL brown glass vial without a lid and oxidized in a heating block (VLM Metal, Bielefeld, Germany) at 180 °C for 40 min. After cooling to room temperature, the sample was dissolved in *n*-hexane/MTBE (3:2, v/v) and transferred to a graduated flask with a final volume of 5 mL, which was used for the analysis of intact esters, ACOPs and POPs. For each ester mixture, the heating experiment was performed in triplicate (n=3).

For gravimetric determinations of nonpolar and polar compounds, heating experiments were repeated for each ester mixture in triplicate. The heated ester mixtures were directly subjected to solid-phase extraction (cf. 3.2.2.1.10). After evaporation of the solvents, the nonpolar und polar fractions were determined gravimetrically.

3.2.2.2.4. Quantitation of Nonpolar ACOPs and Non-oxidized Phytosteryl/-stanyl Oleates and Linoleates

An aliquot (0.5 mL) of the sample (5 mL) obtained after heat treatment was transferred into a 2 mL brown glass vial, and 20 µL cholesteryl octanoate (0.78 mg/mL) and 100 µL cholesteryl palmitate (2.5 mg/mL) were added as internal standards. After removal of the solvent under a gentle stream of nitrogen, the residue was dissolved in 250 µL hexane/MTBE (3:2, v/v). This sample was directly subjected to GC-FID/MS analysis for the determination of nonpolar oxidation products. The quantitation was performed relative to the internal standard cholesteryl octanoate using a response factor (R_f) of 1.0 as default value.

For the quantitation of non-oxidized oleates and linoleates, the samples were further diluted (between 1:3 and 1:8), depending on the amounts of non-oxidized esters remaining after thermal treatment. Subsequently, the samples were analyzed according to a previously established method based on GC-FID/MS and quantitated using cholesteryl palmitate as internal standard (Barnsteiner *et al.*, 2012).

3.2.2.2.5. Quantitation of Polar ACOPs

An aliquot (4 mL for phytostanyl ester mixtures and 2 mL for phytosteryl/-stanyl ester mixtures) of the sample (5 mL) obtained after heat treatment was transferred into a 12 mL glass vial. Subsequently, the following volumes of stock solutions of the internal standards cholesteryl 9-hydroxynonanoate (2.17 mg/mL) and cholesteryl 9-oxononanoate (1.03 mg/mL) were added: phytostanyl oleates (10 µL and 10 µL); phytostanyl linoleates (40 µL and 20 µL); phytosteryl/-

stanyl oleates (5 µL and 5 µL); phytosteryl/-stanyl linoleates (10 µL and 10 µL). After removal of the solvent under a gentle stream of nitrogen, the polar oxidation products were separated from non-oxidized esters by silica solid-phase extraction as described earlier (cf. 3.2.2.1.10). The residue of the polar MTBE fraction was silylated with 100 µL pyridine and 100 µL BSTFA/TMCS for 20 min at 80 °C. The silylation reagent was evaporated under a gentle nitrogen stream, the residue was dissolved in 100-400 µL hexane/MTBE (3:2, v/v) and subjected to GC-FID/MS analysis. The quantitation was done relative to the internal standards using a response factor (R_f) of 1.0 as default value.

3.2.2.2.6. Method Validation

The limits of detection (LOD) and quantification (LOQ) were determined according to the method of Vogelgesang and Hädrich (Vogelgesang and Hadrich, 1998) for sitosteryl octanoate, sitosteryl 9-hydroxynonanoate (silylated) and sitosteryl 9-oxononanoate, as representatives of the three classes of ACOPs covered by the employed methodology.
Linearity in the working range was validated by establishing five point calibration curves in the range from 3-73 µg/mL for cholesteryl octanoate and sitosteryl octanoate, 11-217 µg/mL for cholesteryl 9-hydroxynonanoate (silylated) and sitosteryl 9-hydroxynonanoate (silylated), and 10-191 µg/mL for cholesteryl 9-oxononanoate and sitosteryl 9-oxononanoate. The equations of the calibration curves were determined by linear regression analysis of the peak area versus the concentration of the analyte.
For the recovery of polar oxidation products, known amounts of sitosteryl 9-hydroxynonanoate in the range of 15-82 µg and of sitosteryl 9-oxononanoate in the range of 23-115 µg were spiked to 1.5 mg sitosteryl linoleate (non-heated) in triplicate. Subsequently, the samples were taken through the SPE-procedure as described above.
The reproducibility of the method including the thermo-oxidation and sample preparation step was tested by repeated analysis of heated phytostanyl linoleates on another day by a second operator in triplicate (n=3).

3.2.2.2.7. Quantitation of Phytosterol Oxidation Products

An aliquot (2 mL) of a sample (5 mL) obtained after heat treatment of *β*-sitosteryl oleate and linoleate sample, respectively, was transferred into a 12 mL glass vial. After adding 50 µL 5,6ß-epoxycholesterol (0.61 mg/mL) and 50 µL 7-ketocholesterol (0.5 mg/mL) as internal standards, the solvent was removed under a gentle stream of nitrogen. Transesterification, extraction of

phytosterol oxidation products and subsequent acetylation were performed as described earlier (cf. 3.2.1.5). The residue was dissolved in 0.3 mL of n-hexane/MTBE/isopropanol (80:20:0.3. v/v/v) and subjected to on-line LC-GC analysis (cf. 3.2.1.7).

According to the procedures described above, the synthesized phytosteryl/-stanyl oleates and linoleates were also analyzed regarding the presence of ACOPs and POPs before the thermal treatment.

3.2.2.2.8. Statistical Analysis

The software RStudio (version 0.99.903) was used for statistical analyses. Statistical differences were evaluated by one-way ANOVA and unpaired Student’s *t*-test, respectively. Levels of significance: $p < 0.001$, highly significant (***); $p < 0.01$, very significant (**); $p < 0.05$, significant (*). Statistically significant differences between means were identified by Tukey-Kramer test ($p < 0.05$).

3.2.2.3. GC-FID Analysis

GC-FID analyses (1 µL injection volume) were performed using a 6890N GC equipped with an FID (Agilent Technologies, Böblingen, Germany). The separations were carried out on a 30 m × 0.25 mm i.d., 0.1 µm film, Rtx-200MS fused silica capillary column (Restek, Bad Homburg, Germany). The temperature of the injector was set to 280 °C, and hydrogen was used as carrier gas with a constant flow rate of 1.5 mL/min. The split flow was set to 11 mL/min, resulting in a split ratio of 1:7.5. The oven temperature was programmed as follows: Initial temperature, 100 °C; programmed at 15 °C/min to 310 °C (2 min), then 1.5 °C/min to 340 °C (3 min). The FID temperature was set to 340 °C, and nitrogen was used as make-up gas with a flow rate of 25 mL/min. Data acquisition was performed by ChemStation B.04.03.

3.2.2.4. GC-MS Analysis

Mass spectra were recorded with a Finnigan Trace GC ultra-coupled with a Finnigan Trace DSQ mass spectrometer (Thermo Electro Corp., Austin, TX). Mass spectra were obtained by positive electron impact ionization at 70 eV in the scan mode at unit resolution from 40 to 750 Da. The interface was heated to 330 °C and the ion source to 250 °C. Helium was used as carrier gas with a constant flow rate of 1.0 mL/min. The other GC conditions were the same as

described for GC-FID analysis (cf. 3.2.2.3). Data acquisition was perfomed by Xcalibur 3.063 software.

3.2.2.5. NMR Spectroscopy

The compounds were dissolved in 0.5 mL deuterated chloroform. ^{1}H NMR and ^{13}C NMR spectra were recorded at 500 MHz and 126 MHz, respectively, with Avance-HD 500 spectrometers (Bruker, Billerica, MA) operating at 27 °C. ^{1}H-Detected experiments including two-dimensional COSY, NOESY, HSQC and HMBC were measured with an inverse $^{1}H/^{13}C$ probe head, direct ^{13}C-measurements were performed with a QNP $^{13}C/^{31}P/^{29}Si/^{19}F/^{1}H$ cryoprobe. All experiments were done in full automation using standard parameter sets of the TOPSPIN 3.2 software package (Bruker). ^{13}C NMR spectra were recorded in proton-decoupled mode. Data processing was done with the MestreNova software.

4. RESULTS AND DISCUSSION

4.1. Analysis of Phytosterol Oxidation Products in Foods

Cooking and baking applications with foods that contain phytosterols/-stanols or the respective fatty acid esters may lead to the formation of potentially harmful phytosterol oxidation products; this is of particular relevance for foods that were enriched with phytosteryl/-stanyl fatty acid esters due to their cholesterol-lowering properties (Lin *et al.*, 2016a). As discussed earlier (cf. 2.2.2.4), higher initial contents of phytosterols and their fatty acid esters in foods were shown to result in higher POP contents upon thermal treatments. However, the proportion of originally present phytosterols and their fatty acid esters being oxidized to phytosterol oxidation products upon thermal treatments was found to be lower for enriched foods compared to the corresponding non-enriched foods (Conchillo *et al.*, 2005; Lin *et al.*, 2016a; Lin *et al.*, 2017).

To increase the understanding of these observations, both a non-enriched and an enriched liquid margarine should be subjected to various heating treatments that may represent typical cooking applications in the home. The influence of the parameters (i) heat treatment, (ii) initial phytosterol/-stanol content, and (iii) amount of liquid margarine, on the formation of POPs should be investigated. The formation of POPs upon those heat treatments should be determined using a recently developed method based on on-line LC-GC (Scholz *et al.*, 2015b).

To investigate the formation of POPs in the course of baking applications, the non-enriched and the enriched liquid margarine should be used as an ingredient for the preparation of a dough. Subsequently, the dough should be processed to various bakery products. To investigate parameters that may influence the formation of POPs in the course of baking procedures, a muffin should be baked as a reference point. Under the same baking conditions, a cake should be produced to study (i) the influence of a significantly reduced volume-to-surface ratio, and a cookie to study (ii) the influence of a reduced amount of dough on the formation of POPs in the course of baking. In addition, (iii) the influence of the baking time on the formation of POPs should be investigated upon cookie baking.

4.1.1. Non-enriched and Enriched Liquid Margarine

For the preparation of the enriched liquid margarine, a mixture of phytosteryl/-stanyl fatty acid esters was added to the non-enriched liquid margarine to a final content of 12.5 wt.%

corresponding to 7.5 wt.% of phytosterols/-stanols. The degree of enrichment and the composition of phytosteryl/-stanyl fatty acid esters were comparable to those of margarines typically marketed in Europe (Lin *et al.*, 2016a; Scholz *et al.*, 2016). The experimentally determined contents of phytosterols/-stanols after cleavage of the ester bonds in the non-enriched and the enriched liquid margarine are given in Table 7.

Table 7. Contents of phytosterols/-stanols in the non-enriched and the enriched liquid margarine.

phytosterols/-stanols [mg/100g]	liquid margarine	
	non-enriched	enriched
brassicasterol	34.7 ± 1.5[a]	206.5 ± 3.5[a]
campesterol	142.8 ± 5.4	1410.8 ± 17.6
campestanol	19.8 ± 1.2	104.0 ± 4.7
stigmasterol	3.3 ± 0.2	57.8 ± 2.7
sitosterol	282.6 ± 17.1	5586.2 ± 90.8
sitostanol	17.6 ± 0.2	550.3 ± 18.0
others	33.7 ± 1.4	181.2 ± 92.8
total	534.5 ± 26.0	8096.7 ± 88.2

[a] Values represent the mean ± standard deviation (n=3).

The contents and distributions of phytosterols/-stanols determined in the non-enriched liquid margarine were typical for a mixture of oils containing high proportions of sunflower and rapeseed oil (Lin *et al.*, 2017). The content of phytosterols/-stanols determined in the enriched liquid margarine comprised the phytosterols/-stanols originally present in the non-enriched liquid margarine and the amount added during the enrichment step. An exemplary chromatogram of the analysis of phytosterols-stanols in the enriched liquid margarine is shown in Figure 13.

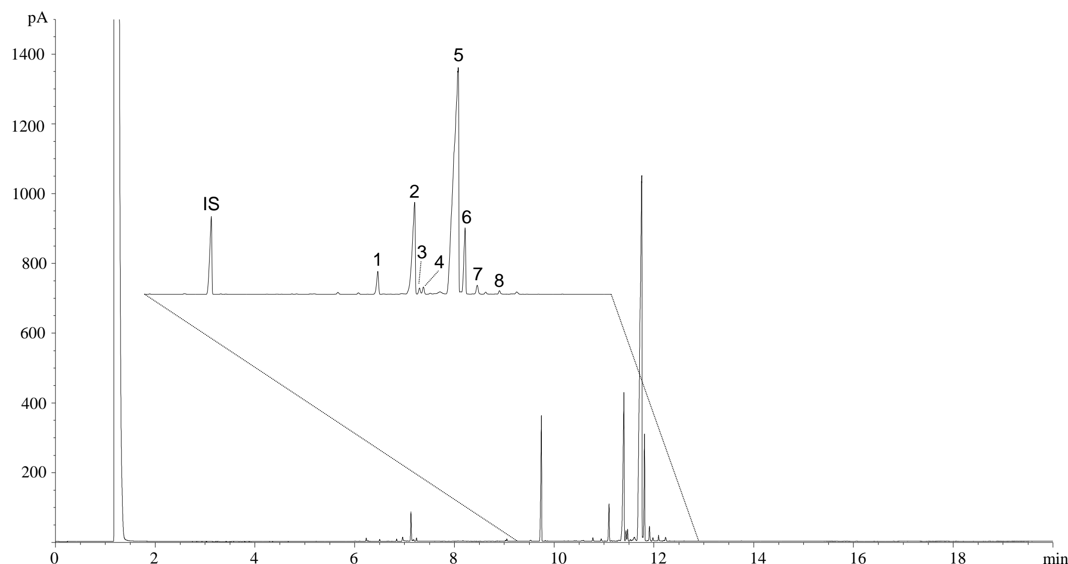

Figure 13. GC-FID analysis of phytosterols/-stanols in the enriched liquid margarine: IS, 5α-cholestane; 1, brassicasterol; 2, campesterol; 3, campestanol; 4, stigmasterol; 5, sitosterol; 6, sitostanol; 7/8, others (not identified).

4.1.2. POP Analysis in Liquid Margarines

A method based on on-line LC-GC that had been established and validated for the analysis of POPs in a margarine with a lipid content of 40 wt. % (Scholz *et al.*, 2015b) should be used for the analysis of POPs in the liquid margarines. However, following the described protocol for the extraction of POPs after transesterification, i.e. using chloroform and citric acid, no sharp phase separation between the aqueous and organic phase was achieved for the non-enriched and the enriched liquid margarine, probably due to the high lipid contents of 82 and 84.25 %, respectively. However, the combination of chloroform and lactic acid enabled a clear phase separation and thus, an efficient extraction of POPs.The applicability of the modified extraction procedure was confirmed by the determination of recovery rates of selected oxidized sterols, as representatives for the three classes of analytes, spiked to non-enriched liquid margarine. Good recovery rates ranging from 92-102 % with low standard deviations were obtained for the three classes of analytes at three spiking levels that covered a broad concentration range (Table 8).

Since sitosterol and campesterol were the quantitatively dominating phytosterols both in the non-enriched and the enriched liquid margarine (Table 7), sitosterol and campesterol oxides were analyzed via on-line LC-GC in the liquid margarines upon heating treatments. In the first LC-fraction 5,6-epoxysitosterols/-campesterols and 7-hydroxysitosterols/-campesterols were transferred on-line to the GC, the second LC-fraction comprised the 7-ketositosterols/-campesterols. Exemplary chromatograms of the analysis of phytosterol oxidation products in enriched liquid margarine are depicted in Figure 14.

Table 8. Recovery rates of reference compounds spiked to non-enriched liquid margarine.

	recovery [%]		
sterol oxidation product	level 1[a]	level 2[b]	level 3[c]
5,6*α*-epoxysitosterol	98.7 ± 1.9	99.7 ± 2.1	95.0 ± 0.8
7*ß*-hydroxycholesterol	101.0 ± 2.2	98.3 ± 3.7	101.7 ± 0.5
7-ketositosterol	96.1 ± 2.6	94.3 ± 0.5	92.3 ± 0.5

[a] Liquid margarine was spiked with 3.6 µg 5,6*α*-epoxysitosterol, 5.2 µg 7*ß*-hydroxycholesterol and 6.3 µg 7-ketostitosterol.
[b] Liquid margarine was spiked with 20 µg 5,6*α*-epoxysitosterol, 15 µg 7*ß*-hydroxycholesterol and 24 µg 7-ketositosterol.
[c] Liquid margarine was spiked with 305 µg 5,6*α*-epoxysitosterol, 310 µg 7*ß*-hydroxycholesterol and 310 µg 7-ketostitosterol.

4.1.3. POP Contents in Heated Liquid Margarines

4.1.3.1. Influence of the Heating Treatment

The contents of phytosterol oxidation products determined before and after various heating treatments on two different days in the non-enriched and the enriched liquid margarine are given in Table 9. In the untreated non-enriched liquid margarine samples POP contents of on average 13 mg/kg were determined, corresponding to an oxidation rate of 0.23 % that was in the range of previously reported oxidation rates for non-enriched margarines (cf. 2.2.2.4). In the untreated enriched liquid margarine samples on average 76 mg/kg of POPs were found, corresponding to an oxidation rate of 0.09 % that was comparable to previous studies (cf. 2.2.2.4).

Upon heat treatments performed with non-enriched and enriched liquid margarine, respectively, POP contents were in the following ascending order: pan-frying < vial (oven) < casserole (oven). Lowest POP contents were determined in pan-fried liquid margarines, as the heating time and the final temperature were lower compared to vial and casserole heating (Table 9). Despite the same time-temperature combination, heating of the liquid margarines in a casserole exceeded the amounts of POPs formed upon vial heating by a factor of approx. 5-7. This may be explained by a greater surface of liquid margarine in contact to air oxygen during casserole heating. The mass-to-surface ratio of the liquid margarines in the casserole (0.005 g/cm^2) was significantly lower than that in a vial (0.25-0.5 g/cm^2).

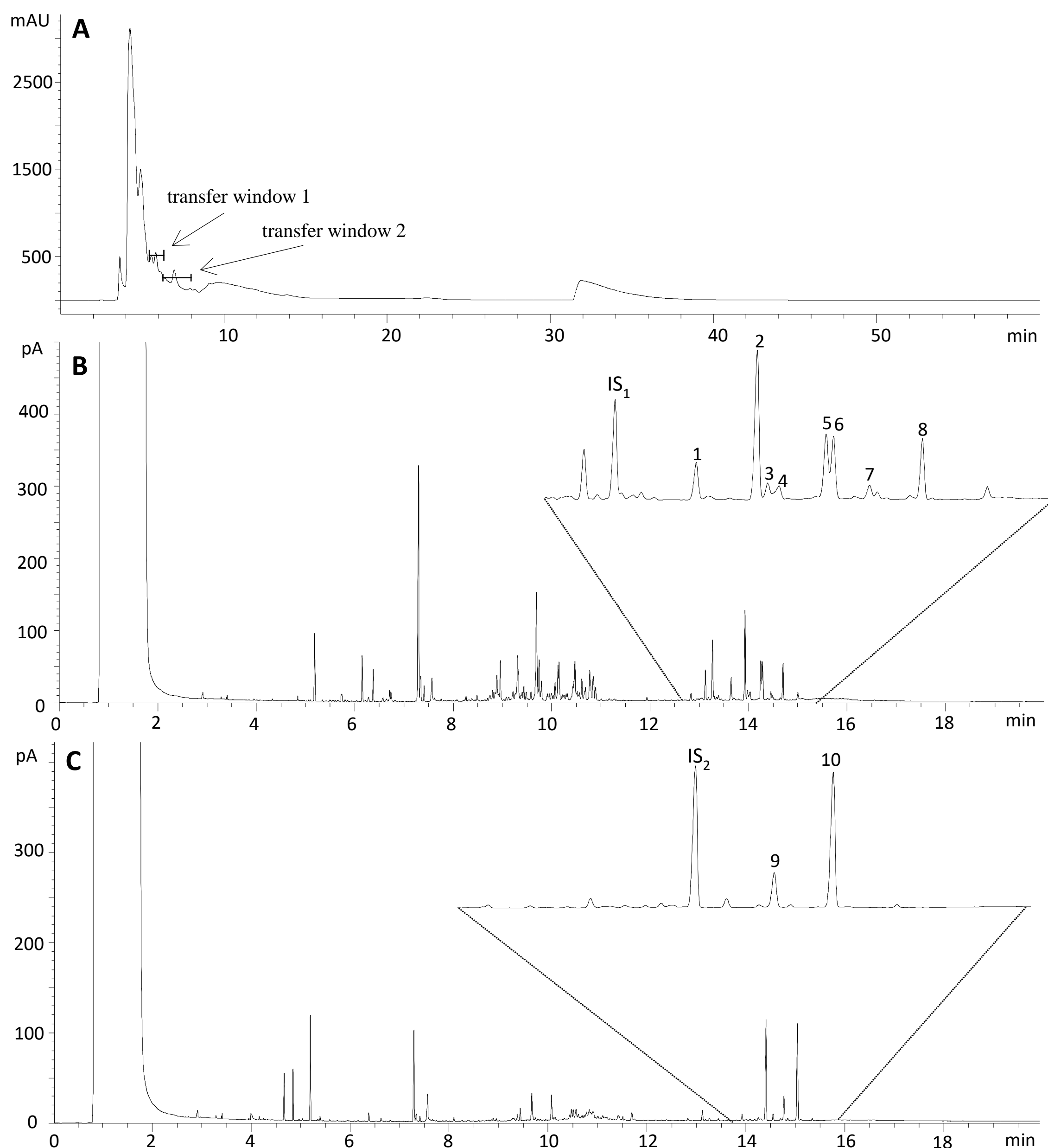

Figure 14. On-line LC-GC/FID analysis of acetylated phytosterol oxidation products in the enriched liquid margarine heated in a casserole in a domestic oven at 200 °C for 20 min. (A) LC-chromatogram (λ=206 nm); (B) GC/FID-chromatogram of the transferred LC-fraction 1: (1) 5,6*β*-epoxycampesterol, (2) 5,6*β*-epoxysitosterol, (3) 5,6*α*-epoxycampesterol, (4) 7*α*-hydroxycampesterol, (5) 5,6*α*-epoxysitosterol, (6) 7*α*-hydroxysitosterol, (7) 7*β*-hydroxycampesterol (8) 7*β*-hydroxysitosterol, and (IS_1) 5,6*β*-epoxycholesterol; (C) GC/FID-chromatogram of the transferred LC-fraction 2: (9) 7-ketocampesterol, (10) 7-ketositosterol, and (IS_2) 7-ketocholesterol.

4.1.3.2. Influence of the Initial Phytosterol Content

When the non-enriched and the enriched liquid margarine were subjected to the same heat treatment, i.e. pan-frying (1 g), vial heating (1 g) or casserole heating (2.91 g), higher POP contents were observed in the enriched liquid margarine compared to the non-enriched liquid margarine due to the increased availability of phytosterols for oxidations (Table 9).

Table 9. Phytosterol oxidation products determined in a non-enriched and in a phytosteryl/-stanyl fatty acid ester-enriched liquid margarine before and after various heat treatments.

heat treatment	liquid margarine [g]	margarine sample no.[a]	POPs before heating [mg/kg][b]	POPs after heating [mg/kg][b]	ORP [%][c]	lit. ORP [%][d]	rel. ORP$_1$[e]	rel. ORP$_2$[f]
frying pan								
194 °C/ 9 min[g]	1 (non-enriched)	2 2	11.1 ± 0.6 11.1 ± 0.6	34.6 ± 1.1 37.1 ± 1.6	0.47 ± 0.03	0.67 ± 0.02	2.47	
194 °C/ 9 min	1 (enriched)	1 1	99.9 ± 3.0 99.9 ± 3.0	244.9 ± 3.8 254.7 ± 6.7	0.19 ± 0.01	0.31 ± 0.01		1.46
182 °C/ 9 min	3 (enriched)	2 2	50.7 ± 4.2 50.7 ± 4.2	160.6 ± 4.5 150.5 ± 8.6	0.13 ± 0.01	0.19 ± 0.01		
vial (oven)								
200 °C/ 20 min	1 (non-enriched)	1 2	14.0 ± 0.6 11.1 ± 0.6	61.7 ± 1.0 62.5 ± 1.2	0.96 ± 0.03	1.16 ± 0.02	1.25	
200 °C/ 20 min	1 (enriched)	1 2	99.9 ± 3.0 50.7 ± 4.2	699.9 ± 3.5 692.9 ± 0.9	0.77 ± 0.03	0.86 ± 0.00		1.77
200 °C/ 20 min	0.5 (enriched)	1 2	99.9 ± 3.0 50.7 ± 4.2	1181.3 ± 38.6 1167.3 ± 24.8	1.36 ± 0.05	1.45 ± 0.04		
casserol (oven)								
200 °C/ 20 min	2.91 (non-enriched)	1 2	14.0 ± 0.6 11.1 ± 0.6	348.9 ± 9.9 332.9 ± 3.4	6.15 ± 0.20	6.38 ± 0.12	0.91	
200 °C/ 20 min	2.91 (enriched)	1 1	99.9 ± 3.0 99.9 ± 3.0	5415.2 ± 165.0 5777.2 ± 158.5	6.79 ± 0.30	6.91 ± 0.20		

[a] Two different bottles of the same non-enriched liquid margarine were used for the heating treatments and the production of enriched liquid margarine. Different POP contents were determined in the non-enriched and in the enriched liquid margarine samples before heating.
[b] Values represent duplicate experiments on different days. Each experiment was analyzed in triplicate.
[c] Oxidation rate of the heat treatment, calculated as average percentage of phytosterol oxidation products being formed in the course of the heat treatment on two days with respect to the initial phytosterol/-stanol content.
[d] Literature oxidation rate for the comparison with previous studies, calculated as average percentage of total phytosterol oxidation products after heat treatments on two days with respect to the initial phytosterol/-stanol content.
[e] Relative ORP$_1$ = ORP (non-enriched)/ ORP (enriched); calculated for the same heating treatments carried out with non-enriched and enriched liquid margarine.

[f] Relative ORP_2 = ORP (lower amount of the enriched liquid margarine)/ ORP (higher amount of the enriched liquid margarine); calculated for the same heating treatments carried out with different amounts of enriched liquid margarine.
[g] Final temperature/ duration of frying process; complete temperature profiles are shown in Figure 15.

Pan-frying (1 g) resulted in POP contents of on average 36 mg/kg in the non-enriched and 250 mg/kg in the enriched liquid margarine. Higher POP contents of 668 mg/kg have previously been determined in an enriched liquid margarine upon pan-frying for 10 min at 180 °C (Soupas *et al.*, 2007). However, in that study the frying pan had been preheated and thus, the liquid margarine was affected by the high temperature throughout the pan-frying procedure, whereas in this study the liquid margarine was put in a cold frying pan and successively heated (Figure 15). Higher POP contents of 1079 mg/kg have also been determined in an enriched margarine under comparable pan-frying conditions in a recent study (Scholz *et al.*, 2016). However, the fat content of the margarine used (40 %) was much lower compared to that of the enriched liquid margarine (84.25 %) used in this study, which may influence the formation of POPs.

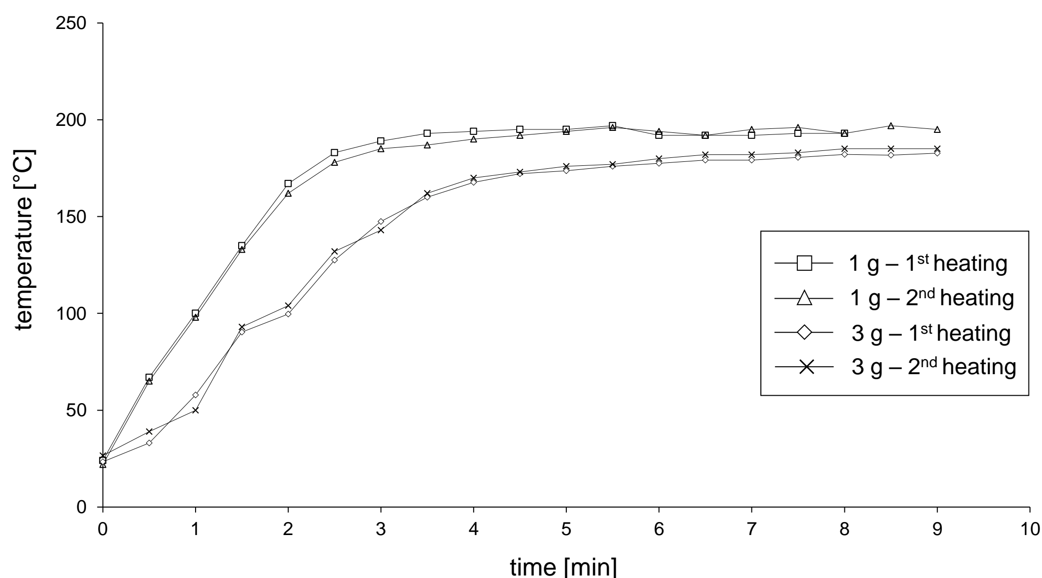

Figure 15. Temperature profiles of the enriched liquid margarine in the course of pan-frying of 1 g (upper curves) and 3 g (lower curves) of the enriched liquid margarine.

Vial heating (1 g) yielded POP contents of on average 62 mg/kg in the non-enriched and 696 mg/kg in the enriched liquid margarine. In a recent study, higher amounts of 96 mg/kg and 1505 mg/kg were determined for a comparable non-enriched and enriched liquid margarine, respectively, heated in a Petri-dish for 16 min at 210 °C (Lin *et al.*, 2017). However, the mass-to-surface ratio of the liquid margarines in the Petri-dish (approx. 0.16 g/cm^2) was smaller than in the vial (0.25-0.5 g/cm^2). Casserole heating led to the highest absolute differences of POP contents between the non-enriched and enriched liquid margarine (250 mg/kg and 5596 mg/kg, respectively). POP contents in the same order of magnitude were previously determined in a commercially available enriched margarine (60 % fat) upon casserole heating (Scholz *et al.*, 2016).

Although higher POP contents were obtained for the enriched liquid margarine compared to the non-enriched liquid margarine upon pan-frying (1 g) and vial heating (1 g), the oxidation rates (ORP) of phytosterols initially present in the margarines were higher for the non-enriched liquid margarine (Table 9). These findings corresponded to a previous study that reported consistently higher oxidation rates for a non-enriched margarine used for the preparation of foods compared to an enriched margarine (Lin *et al.*, 2016a). The relative ORP_1, expressing the ratio of the oxidation rate in the non-enriched to that of the enriched liquid margarine, was higher for pan-frying (2.47) compared to vial heating (1.25).

Interestingly, the oxidation rate in the non-enriched liquid margarine was slightly lower than in the enriched liquid margarine upon casserole heating, as reflected by a relative $ORP_1<1$ (0.91). Apparently, under the strong oxidizing conditions provided in the casserole, reflected in a very small mass-to-surface ratio (0.005 g/cm^2), the access of phytosterols to air oxygen was not the inhibitory factor that may prevent high proportions of phytosterols from oxidation in enriched margarines in the course of pan-frying or vial oven-heating. In a recent study, a non-enriched and an enriched liquid margarine that showed a mass-to-surface ratio of approx. 0.16 g/cm^2, were heated under occasional stirring in a Petri-dish. Apparently, these conditions provided sufficient access of the phytosterols to air oxygen resulting in comparable oxidation rates for the non-enriched and the enriched liquid margarine (Lin *et al.*, 2017).

4.1.3.3. Influence of the Amount of Margarine

As the mass-to-surface ratio of the liquid margarine was a crucial parameter for the oxidation of phytosterols, particularly in the enriched liquid margarine, pan-frying was repeated with an increased amount of enriched liquid margarine (1 g to 3 g) and vial heating with a reduced amount of the enriched liquid margarine (1 g to 0.5 g).

After being placed in the middle of the frying pan, the liquid margarine spread in the frying pan. The mass-to-surface ratio of 1 g liquid margarine in the frying pan was approx. 0.16 g/cm^2, and 0.25 g/cm^2 for 3 g of liquid margarine. This led to a slower temperature increase and a lower final temperature of the enriched liquid margarine in the course of pan-frying of 3 g of liquid margarine compared to 1 g of liquid margarine (Figure 15). These effects were reflected in lower POP contents in 3 g of enriched liquid margarine (155.6 mg/kg) compared to 1 g of enriched liquid margarine (249.8 mg/kg). The relative ORP_2 expresses the ratio of the oxidation rate of the lower amount of enriched liquid margarine to that of the higher amount of enriched liquid margarine. The ORP_2 of 1.46 was comparable with the factor of 1.59 that represents the

ratio of the mass-to-surface values of 3 g and 1 g of liquid margarine, and that may be expected under ideal conditions. Therefore, the mass-to-surface ratio was shown to be a good indicator for the formation of POPs in the enriched liquid margarine upon pan-frying.

As the surface of liquid margarine in contact to air oxygen remained unchanged for the reduced amount of liquid margarine in the vial, the mass-to-surface ratio was only affected by the reduced amount and decreased from 0.5 g/cm^2 (1 g) to 0.25 g/cm^2 (0.5 g). Therefore, only half of the original amount of liquid margarine was affected by the same heat impact in the oven. This resulted in an increase of POP contents from 696 mg/kg (1 g) to 1174 mg/kg (0.5 g). For vial heating, the ORP_2 of 1.77 was slightly below the factor of 2 that represents the ratio of the mass-to-surface values of 1 g and 0.5 g of liquid margarine, and that may be expected under ideal conditions. This deviation from ideal conditions may be explained by a mass-depended movement behaviour of the liquid margarine in the vial that is whirled up in the course of heating as a consequence of water evaporation.

In Figure 16 the percentage distributions of the three classes of phytosterol oxidation products in the non-enriched and in the enriched liquid margarine before and after heat treatments are depicted. In the untreated non-enriched liquid margarine, solely 7-ketosterols were detected corresponding to previous studies that only found 7-ketosterols in a non-enriched margarine (Lin *et al.*, 2016a) and a predominant proportion (> 90 %) of 7-ketosterols in a non-enriched liquid margarine (Lin *et al.*, 2017). Upon heat treatments, the proportions of 7-ketosterols decreased and 5,6-epoxysterols and 7-hydroxysterols were detected. Similar findings were recently reported upon heating of a non-enriched liquid margarine in a Petri-dish (Lin *et al.*, 2017). However, in that study the proportion of 7-ketosterols remained predominant after heating.

In the non-treated enriched liquid margarine, 7-ketosterols were more abundant than 5,6-epoxysterols and only a minor proportion of 7-hydroxysterols was observed. A similar distribution of POPs in an enriched liquid margarine was also reported previously (Lin *et al.*, 2017). Upon pan-frying of the enriched liquid margarine, POP distributions similar to that of untreated enriched liquid margarine were obtained. By contrast, heating treatments that yielded higher POP contents than pan-frying, i.e. vial and casserole heating, led to a distinct relative decrease of 7-ketosterols and relative increase of 5,6-epoxysterols and 7-hydroxysterols. Similar changes in the relative proportions of POP upon various heating treatments with an enriched margarine (Scholz *et al.*, 2016) and upon pan-frying of an enriched butter oil were reported (Soupas *et al.*, 2007). Surprisingly, heating of an enriched liquid margarine in a Petri-

dish hardly affected the initial relative proportion of 7-ketosterols in a recent study, only the proportions of 5,6-epoxysterols and 7-hydroxysterols were slightly changed (Lin *et al.*, 2017). However, several factors, such as the fat content of the margarines and the individual heating conditions, may contribute to the differences observed between studies.

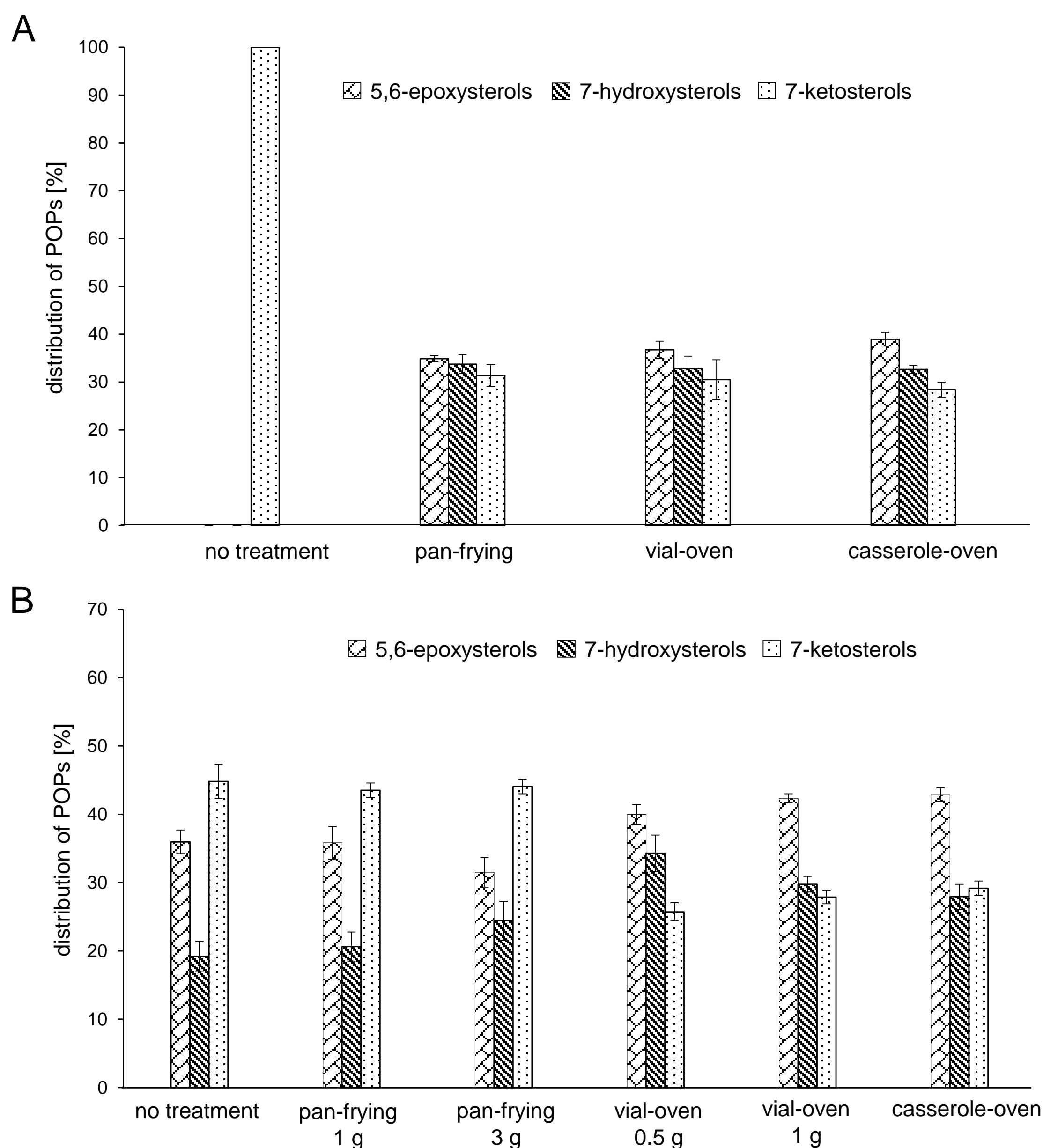

Figure 16. Percentage distributions of the three classes of phytosterol oxidation products determined in the (A) non-enriched and (B) enriched liquid margarine before and after heat treatments.

4.1.4. Baking with Non-enriched and Enriched Liquid Margarine

4.1.4.1. Lipid Extraction

For the analysis of POPs in bakery products, the lipids had to be extracted from the food matrix. Lipid extraction from bakery products was performed with chloroform that had been shown to be a suitable solvent for the extraction of POPs in the course of the sample preparation for POP analysis (Scholz *et al.*, 2015b). To limit the required volume of chloroform, only a quarter of a muffin and cake, respectively, was homogenized and subjected to lipid extraction. Due to their low weight, cookies were subjected to lipid extraction as a whole. Lipid extractions were performed with 100 mL of chloroform at room temperature with the extraction vessel being wrapped with aluminium foil to minimize potential effects of light on the stability of POPs. Acidic hydrolysis prior to lipid hydrolysis, known to be necessary to release phytosterols from phytosteryl glycosides (Toivo *et al.*, 2000), was not performed. Edible oils that occur in the non-enriched liquid margarine, i.e. sunflower oil and rapeseed oil, have been reported to show only little proportions (< 5 %) of phytosterols being present in glycosidic form (Lacoste *et al.*, 2009; Aguirre *et al.*, 2012), and the phytosterols/-stanols were added as fatty acid esters to the enriched liquid margarine. In addition, 5,6-epoxysterols have been shown to react to the corresponding triols via epoxide ring opening in acidic aqueous media (Giuffrida *et al.*, 2004; Poirot and Silvente-Poirot, 2013). This should particularly be avoided, as triols were not covered by the method used for POP analysis. The applied extraction method resulted in good lipid extraction yields ranging from 80-90 % for muffins, cakes and cookies under gentle conditions. Carrying out the lipid extraction with 200 mL of chloroform instead of 100 mL resulted in almost complete extraction (97.8 %) of the theoretical yield from a muffin prepared with enriched liquid margarine. However, the POP content was not affected by the volume of solvent; therefore, the lipid extract was considered to be homogenous, and all lipid extractions were performed with the lower volume of solvent.

As no reference compounds of oxidized phytosteryl fatty acid esters were available, individual oxidized phytosteryl fatty acid esters could not be spiked to bakery products to determine the recoveries of POPs in the course of the lipid extraction step. Therefore, a lipid extract that had been obtained from a muffin prepared with enriched liquid margarine and whose POP content was known, was spiked to a muffin that had been baked without liquid margarine. After lipid extraction of the muffin with the added lipid extract, POP levels were determined and compared to those of the muffin that had been baked with enriched liquid margarine. The advantage of

this approach was that recoveries were determined for a POP content and composition corresponding to that expected in the bakery products. Good recovery rates demonstrated the suitability of the lipid extraction procedure for the bakery products (Table 10).

Table 10. Recovery rates of phytosterol oxidation products in a lipid matrix extracted from a muffin.

phytosterol oxidation product	recovery [%][a]
5,6*β*-epoxycampesterol	102.0 ± 3.8
5,6*β*-epoxysitosterol	96.2 ± 4.4
5,6*α*-epoxycampesterol	96.6 ± 2.6
7*α*-hydroxycampesterol	99.9 ± 4.4
5,6*α*-epoxysitosterol	98.8 ± 3.1
7*α*-hydroxysitosterol	112.0 ± 1.6
7*β*-hydroxycampesterol	107.2 ± 2.2
7*β*-hydroxysitosterol	99.4 ± 1.3
7-ketocampesterol	102.3 ± 6.0
7-ketositosterol	105.3 ± 2.1
total	102.9 ± 2.0

[a] Values represent the mean ± standard deviation (n=3).

4.1.4.2. POP Contents in Bakery Products

4.1.4.2.1. Influence of the Initial Phytosterol Content

In accordance with the heat treatments of pure liquid margarines, higher POP contents (68.7 mg/kg) were found in the enriched liquid margarine that was heated in a muffin matrix compared to the non-enriched liquid margarine (35.5 mg/kg) (Table 11). However, most of the POPs found in the muffin that was prepared with enriched liquid margarine, were already present in the enriched liquid margarine and thus, were not formed in the course of muffin baking. Exemplary chromatograms of the analysis of POPs in enriched liquid margarine after muffin baking are depicted in Figure 17. The oxidation rates of non-enriched liquid margarine (0.59 %) and the enriched liquid margarine (0.01 %) also reflected only very little formation of POPs in the course of muffin baking. Under these low-oxidizing conditions, the rel. ORP_1 of 47 indicated a significantly higher percentage oxidation of phytosterols in the non-enriched liquid margarine than in the enriched liquid margarine. To allow the comparison with a previous study, an ORP derived from literature data (lit. ORP) was additionally calculated that takes not only the POPs into account that were formed in the course of the heating process, but the total POPs that were present after baking. As the POPs that had already been present before baking are included in that calculation, the lit. ORP values were higher (0.66 % in the non-enriched and 0.08 % in the enriched liquid margarine) than the oxidation rates calculated on the basis of

the POPs being formed in the course of baking. The same lit. ORP for an enriched margarine (0.08) and a higher lit. ORP for a non-enriched margarine (2.04) were recently determined upon muffin baking (Lin *et al.*, 2016a). However, different receipts, baking conditions, initial phytosterol contents in the margarine and in the dough, and initial POP levels in the margarine probably contribute to these differences. These parameters were also considered accountable for the higher muffin POP contents (0.59 and 1.2 mg/100 g) compared to those of the previous study (0.24 and 0.43 mg/100 g) (Lin *et al.*, 2016a).

Table 11. Phytosterol oxidation products in the non-enriched and in the enriched liquid margarine (LM) before and after heating in a muffin matrix.

heat treatment	liquid margarine	margarine sample[a]	POPs before heating [mg/kg LM][b]	POPs after heating [mg/kg LM][c]	POPs [mg/100 g muffin][d]	ORP [%][e]	lit. ORP [%][f]	rel. ORP_1[g]
baking (oven)								
		1	9.4 ± 0.5	38.1 ± 0.3				
200 °C/ 20 min	non-enriched	1	9.2 ± 0.6	34.4 ± 0.6	0.59 ± 0.04	0.47 ± 0.06	0.66 ± 0.05	
		2	12.0 ± 0.7	33.3 ± 0.1				47
		1	61.3 ± 1.9	69.5 ± 2.2				
200 °C/ 20 min	enriched	2	58.2 ± 5.2	70.9 ± 4.5	1.16 ± 0.06	0.01 ± 0.01	0.08 ± 0.00	
		2	58.2 ± 5.2	65.6 ± 2.2				

[a] Two different bottles of the same non-enriched liquid margarine were used for the heating treatments and the production of enriched liquid margarine.
[b] Values represent triplicate experiments on different days. Each experiment was analyzed in triplicate.
[c] Calculated as the POP content in the liquid margarine originally used for baking.
[d] Values represent the mean value ± standard deviation of three days of baking.
[e] Oxidation rate of the heat treatment, calculated as average percentage of phytosterol oxidation products being formed in the course of the heat treatment on three days with respect to the initial phytosterol/-stanol content.
[f] Literature oxidation rate for the comparison with previous studies, calculated as average percentage of total phytosterol oxidation products after heat treatments on three days with respect to the initial phytosterol/-stanol content.
[g] Relative ORP_1 = ORP (non-enriched)/ ORP (enriched).

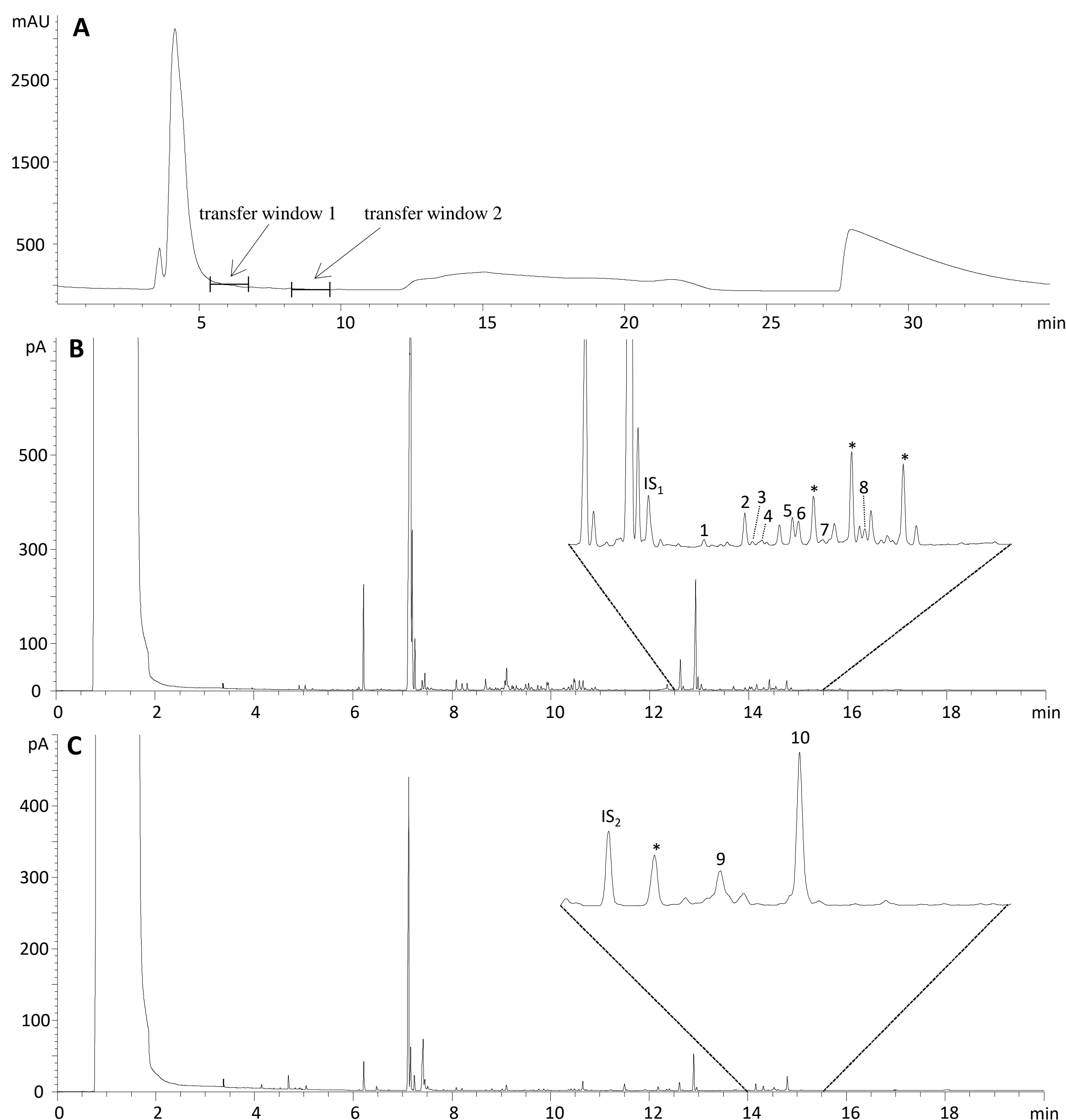

Figure 17. On-Line LC-GC/FID analysis of acetylated phytosterol oxidation products in the enriched liquid margarine heated in a muffin matrix in a domestic oven at 200 °C for 20 min. (A) LC-chromatogram (λ=206 nm); (B) GC/FID-chromatogram of the transferred LC-fraction 1: (1) 5,6*β*-epoxycampesterol, (2) 5,6*β*-epoxysitosterol, (3) 5,6*α*-epoxycampesterol, (4) 7*α*-hydroxycampesterol, (5) 5,6*α*-epoxysitosterol, (6) 7*α*-hydroxysitosterol, (7) 7*β*-hydroxycampesterol (8) 7*β*-hydroxysitosterol, and (IS_1) 5,6*β*-epoxycholesterol; (C) GC/FID-chromatogram of the transferred LC-fraction 2: (9) 7-ketocampesterol, (10) 7-ketositosterol, and (IS_2) 7-ketocholesterol. Matrix peaks originating from the wheat flour are labelled with an asterisk.

The low-oxidizing conditions in the course of muffin baking were also reflected in the phytosterol/-stanol contents before and after heating (Table 12). For both the non-enriched and the enriched liquid margarine, respectively, the phytosterol/-stanol contents before and after heating were not significantly different ($p \geq 0.05$).

Table 12. Phytosterols/-stanols in non-enriched and enriched liquid margarine (LM) before and after heating in a muffin matrix.

phytosterols/-stanols [mg/100g LM]	non-enriched LM		enriched LM	
	before heating	muffin	before heating	muffin
brassicasterol	34.7 ± 1.5[a]	35.1 ± 1.8	206.5 ± 3.5	203.1 ± 1.9
campesterol	142.8 ± 5.4	147.1 ± 5.9	1410.8 ± 17.6	1391.3 ± 40.5
campestanol	19.8 ± 1.2	17.8 ± 1.4	104.0 ± 4.7	108.8 ± 3.7
stigmasterol	3.3 ± 0.2	6.5 ± 0.2	57.8 ± 2.7	67.9 ± 1.1
sitosterol	282.6 ± 17.1	302.3 ± 6.2	5586.2 ± 90.8	5475.3 ± 137.3
sitostanol	17.6 ± 0.2	14.9 ± 0.0	550.3 ± 18.0	542.9 ± 11.0
others	33.7 ± 1.4	29.7 ± 2.0	181.2 ± 92.8	170.8 ± 33.8
total[b]	534.5 ± 26.0	553.4 ± 16.6	8096.7 ± 88.2	7960.1 ± 161.1

[a] Values represent the mean ± standard deviation (n=3).
[b] Analysis of variance was carried out with *t*-test. For the non-enriched and enriched LM, respectively, phytosterol/-stanol contents were not significantly different before and after heating in a muffin matrix on the following levels of significance: $p < 0.001$, highly significant (***); $p < 0.01$, very significant (**); $p < 0.05$, significant (*).

4.1.4.2.2. Influence of an Increased Dough Surface

To investigate a potential influence of the dough surface in contact to air oxygen on the formation of POPs in the course of baking, a cake was produced under the same conditions that were chosen for the muffin that was prepared with enriched liquid margarine. The dough in a cake tin only differed from the dough in a muffin tin in a larger surface of dough in contact to air oxygen. Therefore, the amount-to-surface ratio of the dough in the cake tin (0.4 g/cm^2) was significantly lower than in the muffin tin (2.3 g/cm^2) (Table 13). A lower weight of the cake (72.3 g) compared to the muffin (88.9 g) indicated higher water evaporation in the course of cake baking.

However, only a slightly higher POP content (79.9 mg/kg) was found in the enriched liquid margarine upon cake baking compared to muffin baking (68.7 mg/kg). This was reflected in a small oxidation rate of 0.03 % that hardly differed from that determined after muffin baking (0.01 %). The POP content in the cake (1.69 mg/100 g) was slightly higher compared to the POP content recently determined in a sponge cake (1.20 mg/100 g) (Lin *et al.*, 2016a); the reasons may be the same as discussed for muffins (cf. 4.1.4.2.1). Furthermore, the phytosterol/-stanol content in the enriched liquid margarine after baking did not significantly differ from that determined in the untreated enriched liquid margarine and after muffin baking (Table 13). This findings demonstrated that an increased surface of dough was not a crucial parameter for the oxidation of phytosterols under the applied conditions.

Table 13 Summary of baking procedures performed with enriched liquid margarine (LM).

	muffin[a]	cake[a]	cookie[a]	
heating conditions	200 °C/ 20 min	200 °C/ 20 min	200 °C/ 20 min	200 °C/ 40 min
amount of dough [g][b]	100.5 ± 0.5	100.5 ± 0.5	13.87 ± 0.03	
surface of dough [cm^2][c]	43	254	27	
amount/surface [g/cm^2]	2.3 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	
weight baked product [g]	88.9 ± 0.5	72.3 ± 0.4	10.0 ± 0.1	9.1 ± 0.1
POPs [mg/kg LM][d]	68.7 ± 3.6	79.9 ± 5.6	94.1 ± 4.5	8220.0 ± 879.1
POPs [mg/100 g bakery product]	1.16 ± 0.06	1.69 ± 0.18	1.96 ± 0.10	189.75 ± 22.37
ORP [%][e]	0.01 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	10.05 ± 1.08
lit. ORP [%][f]	0.08 ± 0.00	0.10 ± 0.00	0.12 ± 0.00	10.12 ± 1.09
phytosterols [mg/100 g LM][g]	7960.1 ± 161.1	7782.1 ± 157.4	8189.9 ± 207.8	5651.5 ± 292.1

[a] Muffin, cake and cookies were baked three times. Analyses of POPs and intact phytosterols were performed in triplicate.
[b] Amount of dough used for baking.
[c] Surface of dough in contact with air oxygen in the course of baking.
[d] Calculated as the POP content in enriched liquid margarine originally used for baking.
[e] Oxidation rate of the heat treatment, calculated as average percentage of phytosterol oxidation products being formed in the course of baking on three days with respect to the initial phytosterol/-stanol content.
[f] Literature oxidation rate for the comparison with previous studies, calculated as average percentage of total phytosterol oxidation products after baking on three days with respect to the initial phytosterol/-stanol content (Table 7).
[g] Content of intact phytosterols after baking.

4.1.4.2.3. Influence of a Reduced Amount of Dough

To investigate a potential influence of a reduced amount of dough on the formation of POPs in the course of baking, only a part (approx. 14 %) of the original dough was cut out with a cookie cutter and used for baking (Table 13). The baking conditions corresponded to those applied for muffin and cake baking. The amount-to-surface ratio of the cookie dough (0.5 g/cm^2) was in the same order of magnitude as for the cake dough (0.4 g/cm^2).

Despite a significantly reduced amount of dough used for cookie baking, only a slightly higher POP content was found in the enriched liquid margarine (94.1 mg/kg) compared to that determined upon cake baking (79.9 mg/kg). This was also reflected in comparable oxidation rates upon cookie (0.05 %) and cake baking (0.03 %). The phytosterol/-stanol content in the enriched liquid margarine after cookie baking was not significantly different from that before heating and upon muffin/cake baking. These findings pointed out that the amount of dough was not a crucial factor for the oxidation of phytosterols in the course of baking either. Therefore, the higher POP content in a cookie (1.96 mg/100 g) compared to that recently reported (1.13 mg/100 g) (Lin *et al.*, 2016a), may be rather explained by the baking conditions, i.e. temperature and time, the initial phytosterol content in the margarine and in the dough, respectively.

4.1.4.2.4. Influence of the Baking Time

As the surface and the amount of the dough were shown to be negligible parameters for the oxidation of phytosterols under the applied baking conditions, the baking time for the production of a cookie was increased to investigate a potential influence on the oxidation of phytosterols. The original baking conditions (200 °C/20 min) yielded bakery products that exhibited attributes that may be suitable for human consumption with regard to their color and texture. The prolongation of the baking time of a cookie from 20 to 40 min, yielded a cookie of burnt color and texture that did not reflect realistic baking conditions. However, the burnt cookie served as a model product to study the influence of the baking time on the oxidation of phytosterol in the course of baking.

Prolonged baking resulted in a lower cookie weight (9.1 g) compared to the original time (10.0 g) that indicated higher water evaporation upon prolonged heating. A significant POP content of 8220 mg/kg was determined in the enriched liquid margarine that was heated in the cookie matrix for 40 min at 200 °C. An exemplary chromatogram is given in Figure 18.

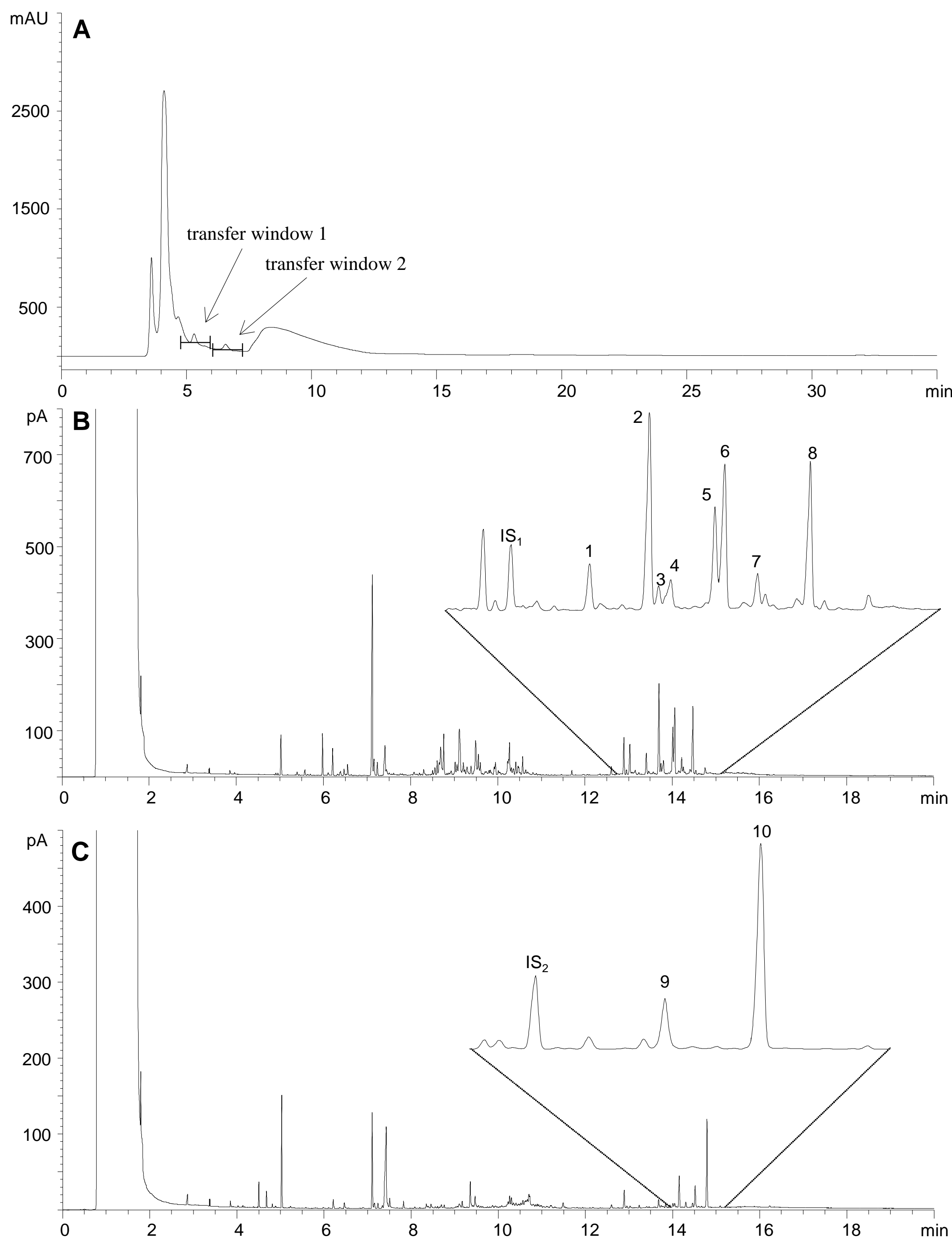

Figure 18. On-Line LC-GC/FID analysis of acetylated phytosterol oxidation products in the enriched liquid margarine heated in a cookie matrix in a domestic oven at 200 °C for 40 min. (A) LC-chromatogram (λ=206 nm); (B) GC/FID-chromatogram of the transferred LC-fraction 1: (1) 5,6*β*-epoxycampesterol, (2) 5,6*β*-epoxysitosterol, (3) 5,6*α*-epoxycampesterol, (4) 7*α*-hydroxycampesterol, (5) 5,6*α*-epoxysitosterol, (6) 7*α*-hydroxysitosterol, (7) 7*β*-hydroxycampesterol (8) 7*β*-hydroxysitosterol, and (IS_1) 5,6*β*-epoxycholesterol; (C) GC/FID-chromatogram of the transferred LC-fraction 2: (9) 7-ketocampesterol, (10) 7-ketositosterol, and (IS_2) 7-ketocholesterol.

Highly oxidizing conditions were also reflected in the oxidation rate (10.05 %), and in a significant reduction of intact phytosterols (31.3 %). The POP content of the burnt cookie (189.8 mg/100 g) exceeded that of the other bakery products by a factor of at least 95 (Table

13). These distinct differences were also reflected in the percentage distributions of POPs (Figure 19). Upon prolonged baking (40 min) of the enriched liquid margarine in a cookie matrix, a relative decrease of 7-ketosterols and increases of 5,6-epoxysterols and 7-hydroxysterols were observed, whereas the percentage distributions of POPs upon the other baking procedures were comparable to that in the untreated enriched liquid margarine.

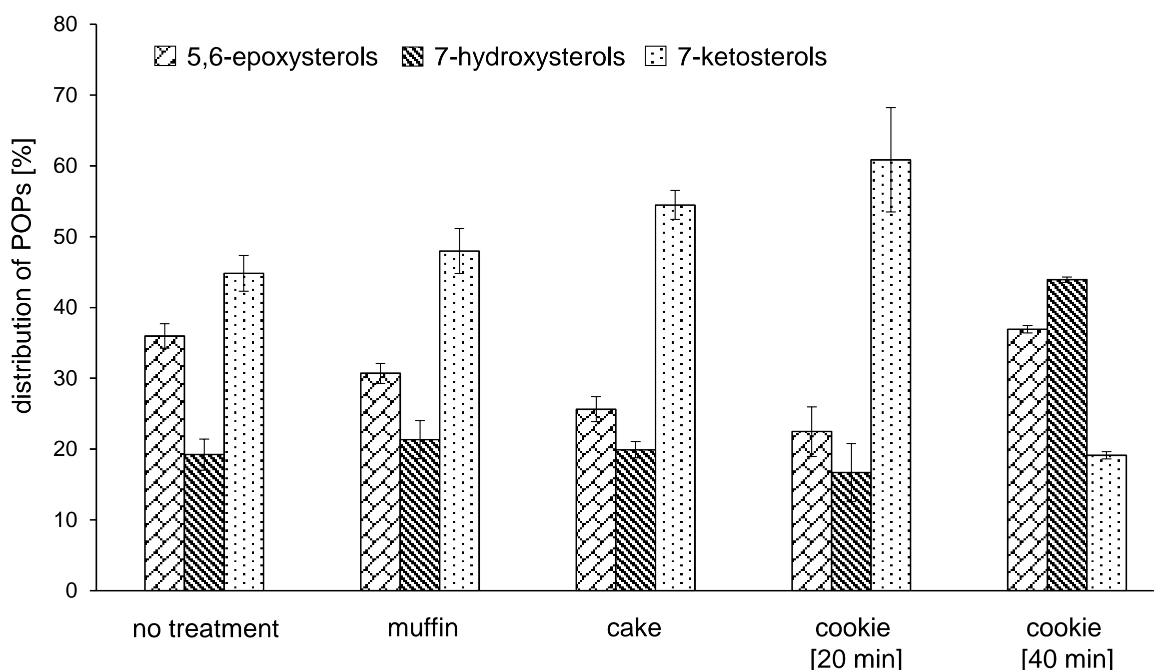

Figure 19. Percentage distributions of the three classes of phytosterol oxidation products determined in the enriched liquid margarine before and after baking procedures.

One possible explanation for the considerable increase of phytosterol oxidation products upon prolonged baking was the formation of porous, brittle structures in the dough that may have enabled better access of phytosterols to air oxygen. However, the visual examination of the cookies, both baked for 20 and 40 min, did not suggest substantial differences in their surface structures except for the coloring. Therefore, the temperature of the dough in the course of baking was considered as the essential parameter for the formation of phytosterol oxidation products. In Figure 20 the surface temperatures of the bakery products upon baking at 200 °C for 20 and 40 min, respectively, are depicted. The low surface temperatures observed upon muffin, cake and cookie baking at 200 °C for 20 min (< 130 °C) corresponded to only very low oxidation rates (Table 13). This was in accordance with a previously reported minimum temperature of approx. 150 °C that is required for sterol oxidations (Medina-Meza and Barnaba, 2013).

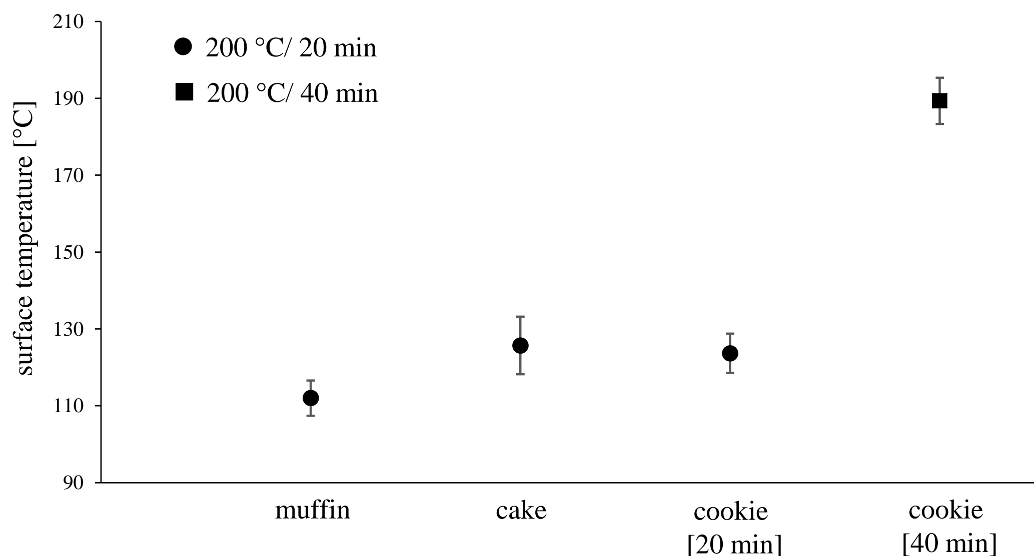

Figure 20. Surface temperatures of bakery products determined after baking at 200 °C. Values represent the mean ± standard deviation (n=3).

Moreover, even lower temperatures than those measured on the surface may be expected in the core of the dough in the course of baking. By contrast, a significantly higher surface temperature of 189 °C was measured on the surface of a cookie after 40 min. Obviously, sufficient energy was provided under these baking conditions for a substantial percentage of phytosterols to be oxidized. Among those phytosterols/-stanols that were degraded in the course of cake baking (40 min), phytosterol oxidation products accounted for 31.6 % (Figure 21), leaving the remaining percentage to others than polar secondary oxidation products (cf. 2.2.2.2).

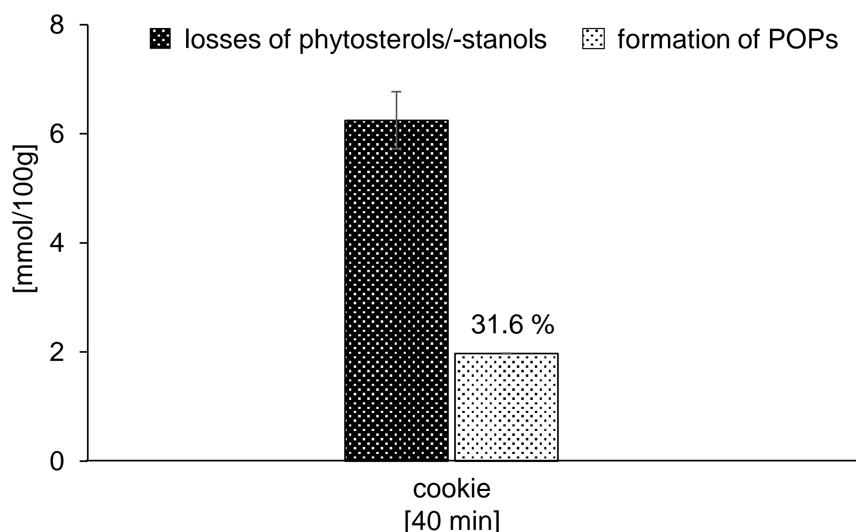

Figure 21. Comparison of the loss of phytosterols/-stanols and the formation of POPs in an enriched liquid margarine that was heated in a cookie matrix at 200 °C for 40 min. The percentage above the POP bar represents the contribution of POPs to the loss of phytosterols/-stanols.

In a recent study, 17-24 % of degraded phytosteryl/-stanyl fatty acid esters were explained by the formation of POPs upon various heat treatments with an enriched margarine (Scholz *et al.*, 2016). However, as phytosteryl/-stanyl fatty acid esters were directly analyzed without prior saponification to the respective free phytosterols/-stanols, less intact phytosteryl/-stanyl fatty acid esters might have been analyzed due to potentially co-occurring oxidations in the fatty acid moieties. The importance of this aspect was strengthened by a recent study demonstrating that the thermal degradation of the unsaturated fatty acid moieties of stigmasteryl fatty acid esters proceeded faster than the degradation of the sterol moiety (Raczyk *et al.*, 2017a).

4.1.5. Summary

A commercially available liquid margarine was enriched with a mixture of phytosteryl/-stanyl fatty acid esters. Both the non-enriched and the enriched liquid margarine were subjected to various heating treatments, and the POP contents were determined via a previously established method based on on-line LC-GC that was adapted for the POP analysis in liquid margarines. POP contents determined in the non-enriched and enriched liquid margarine, respectively, were in the following ascending order: pan-frying < vial (oven) < casserole (oven). Higher POP contents were found in the enriched liquid margarine compared to the non-enriched liquid margarine that was subjected to the same heat treatment. However, the proportion of initially present phytosterols/-stanols that were oxidized in the course of pan-frying and vial oven-heating was higher in the non-enriched than in the enriched liquid margarine. Only upon casserole oven-heating comparable oxidation rates were determined for the non-enriched and the enriched liquid margarine. This indicated that the access of phytosterols to air oxygen, reflected in a low mass-to-surface ratio, was less restricted in the course of casserole oven-heating compared to the other heat treatments. Moreover, a reduced amount of enriched liquid margarine subjected to vial oven-heating resulted in a lower mass-to-surface ratio and higher POP contents. Likewise, pan-frying of an increased amount of enriched liquid margarine resulted in a higher mass-to-surface ratio that led to less formation of POPs.

In addition, the non-enriched and enriched liquid margarine, respectively, were used as ingredient for the preparation of a dough that was used for muffin baking. Heating of the liquid margarines in a muffin matrix was demonstrated to be a low-oxidizing process, as reflected in negligible formations of POPs and unaltered phytosterol/-stanol contents. In addition, neither the increase of the dough surface nor the reduction of the amount of dough, as performed via cake and cookie baking, respectively, had a significant influence on the formation of POPs and

degradation of phytosterols/-stanols. By doubling the baking time, a burnt cookie was produced that served as a model to study the influence of the heating time on the formation of POPs. Very high POP levels and a significant reduction of phytosterols/-stanols in the burnt cookie suggested the temperature of the dough in the course of baking to be the crucial factor for the oxidation of phytosterols/-stanols.

4.2. Analysis of Oxidized Phytosteryl/-stanyl Fatty Acid Esters

4.2.1. Identification of Acyl Chain Oxidation Products upon Thermo-oxidation of Phytosteryl/-stanyl Linoleates

The identities of oxidation products of the phytosterol moieties (POPs) formed upon thermo-oxidations of phytosteryl fatty acid esters are well-known and their occurrence in enriched foods has been investigated (cf. 2.2.2.4). However, only 17-24 % of degraded phytosteryl/-stanyl fatty acid esters were explained by the formation of POPs upon various heat treatments with an enriched margarine in a recent study (Scholz *et al.*, 2016). Therefore, oxidations of the acyl moieties may account for further losses of phytosteryl/-stanyl fatty acid esters. However, the current knowledge on acyl chain oxidation products (ACOPs) of phytosteryl/-stanyl fatty acid esters is almost exclusively limited to the formation of unstable, primary hydroperoxides in the fatty acid moieties (cf. 2.3.2.1). Therefore, secondary acyl chain oxidation products formed from phytosteryl/-stanyl fatty acid esters should be identified for the first time. Crucial steps of the investigations were (i) the development of a method suitable to separate the quantitatively dominating non-oxidized esters from the polar oxidation products, (ii) the synthesis of authentic reference compounds of phytosteryl/-stanyl fatty acid esters with oxidized fatty acid moieties, and (iii) the separation and identification of the oxidation products by means of capillary gas chromatography-mass spectrometry.

4.2.1.1. Thermal Treatment and Sample Preparation

Phytosteryl/-stanyl linoleates prepared via esterification of a commercially available mixture of sterols (75% β-sitosterol, 10% campesterol) and stanols (12% sitostanol, 2% campestanol) were used as model substrates for the thermal treatment. Figure 22 shows the ^{1}H NMR spectrum of the phytosteryl/-stanyl linoleate mixture. Such esters account for a large proportion of the phytosteryl/-stanyl fatty acid ester mixtures being added to foods (Scholz *et al.*, 2016). The ester mixture was heated at 180 °C, as heat treatments at this temperature have also been reported to result in oxidation products of fatty acid methyl esters and triacylglycerides (Berdeaux *et al.*, 1999; Marmesat *et al.*, 2012). With regard to the heating time of 40 min, the only study dealing with the quantitation of ACOPs was used for orientation (Julien-David *et al.*, 2014). In this study heat treatment of an enriched margarine, albeit at 170 °C, resulted in a pronounced increase in the formation of sitosteryl 9,10-dihydroxystearate only after heating periods > 30 min.

Non-oxidized phytosteryl/-stanyl linoleates were anticipated to be still quantitatively dominating in the thermally treated sample. Therefore, as a first step, an SPE method was developed to pre-concentrate the acyl chain oxidation products by removal of the non-oxidized esters. The oxidized esters were expected to be more polar than the starting esters. Accordingly, the strategy was based on an elution of the non-oxidized esters from a silica SPE column with a non-polar eluent in a first fraction, followed by a second eluting step for the polar oxidation products.

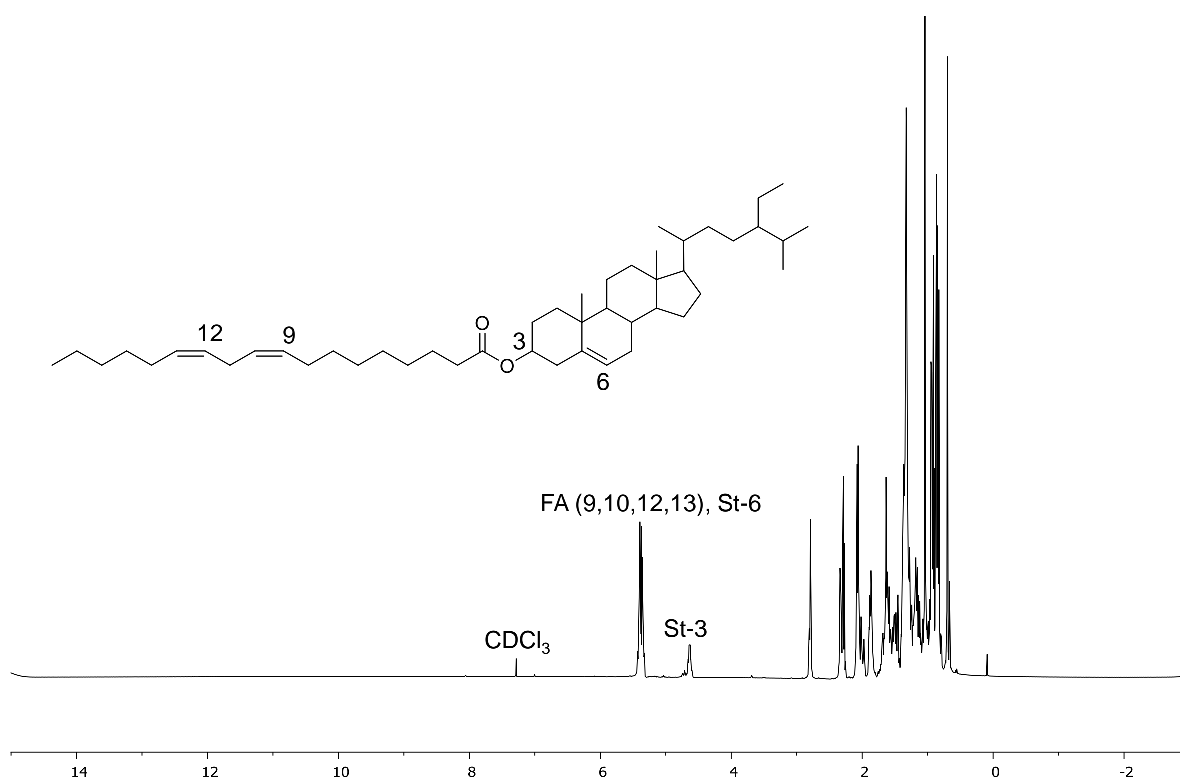

Figure 22. ^{1}H NMR spectrum of the synthesized mixture of phytosteryl/-stanyl linoleates. Signals mainly refer to sitosteryl linoleate, the major ester in the synthesized mixture. Characteristic signals: δ 5.39 (FA-9,10,12,13/ St-6, 5H, m), δ 4.62 (St-3, 1H, m); St, sterol moiety; FA, fatty acid moiety.

In previous studies, SPE methods have also been described for the pre-concentration of cholesteryl ester hydroperoxides formed upon heating of cholesteryl esters and for the separation of oxidized fatty acid methyl esters into non-polar and polar fractions (Dobarganes and Márquez-Ruiz, 2007; Lehtonen *et al.*, 2011b; Lehtonen *et al.*, 2012b). In the course of the method development several non-polar solvents with increasing elution powers were tested to find an eluent just capable of eluting the non-oxidized linoleates from the silica column without causing the elution of polar oxidation products. The compounds retained on the column were subsequently eluted with MTBE, and both fractions were checked for non-oxidized linoleates

by GC-FID/MS. Similar gas chromatographic conditions as previously used for the analysis of intact phytosteryl/-stanyl fatty acid esters were applied (Barnsteiner *et al.*, 2012; Esche *et al.*, 2012). When using heptane or hexane as elution solvents for the first fraction, remainders of non-oxidized esters were still detectable in the MTBE fraction. Using cyclohexane, non-oxidized linoleates could be effectively removed from the silica column in the first elution step (Figure 23A), and no non-oxidized linoleates were detected in the second fraction after eluting with MTBE (Figure 23B). Therefore, this procedure allowed the pre-concentration of polar acyl chain oxidation products.

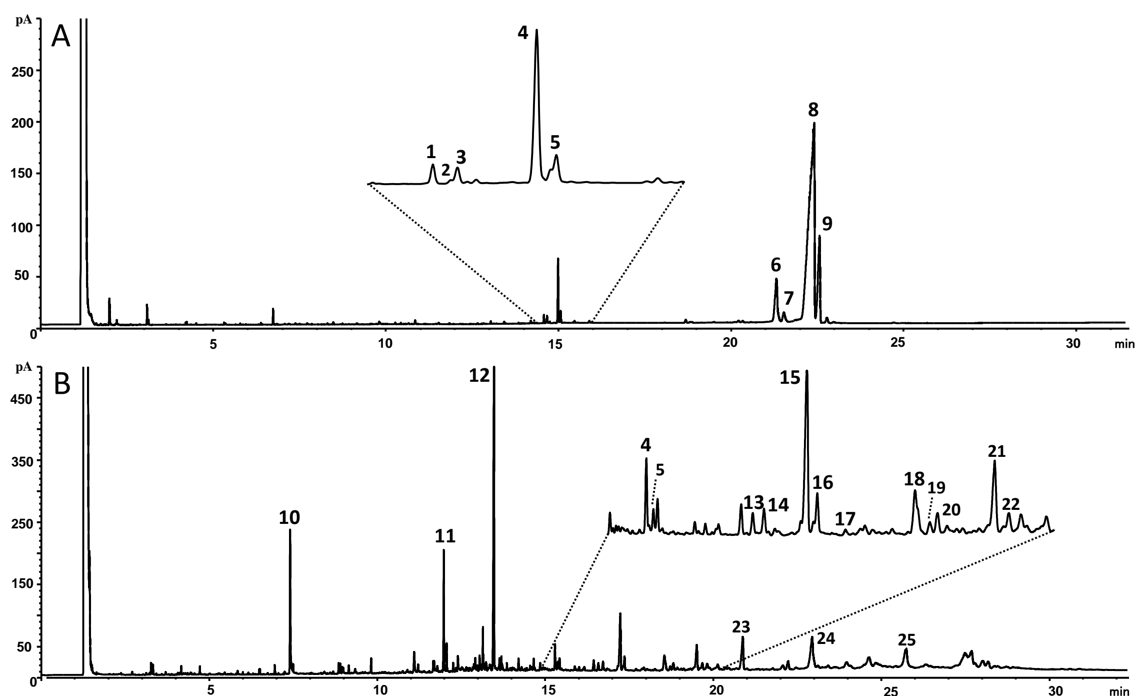

Figure 23. Capillary gas chromatographic separations of (A) the unpolar and (B) the polar fractions obtained via SPE from the heated phytosteryl/-stanyl linoleate mixture. 1, sitosteryl heptanoate; 2, sitostanyl heptanoate; 3, campesteryl octanoate; 4, sitosteryl octanoate; 5, sitostanyl octanoate; 6, campesteryl linoleate; 7, campestanyl linoleate; 8, sitosteryl linoleate; 9, sitostanyl linoleate; 10, linoleic acid; 11, sitosterol; 12, stigmasta-3,5-dien-7-one; 13, sitosteryl 7-hydroxyheptanoate; 14, campesteryl 8-hydroxyoctanoate; 15, sitosteryl 8-hydroxyoctanoate; 16, sitostanyl 8-hydroxyoctanoate; 17, sitosteryl 7-oxoheptanoate; 18, sitosteryl 8-oxooctanoate; 19, sitostanyl 8-oxooctanoate; 20, campesteryl 9-oxononanoate; 21, sitosteryl 9-oxononanoate; 22, sitostanyl 9-oxononanoate; 23/24, *α*/*β*-7-hydroxysitosteryl linoleate (tentative); 25, *α*/*β*-5,6-epoxysitosteryl linoleate (tentative).

4.2.1.2. Identification of Oxidation Products

The capillary gas chromatographic analysis of fraction 1 revealed that in addition to the remaining non-oxidized linoleates several minor peaks were detectable (Figure 23A). Peaks 1-5 were identified via GC/MS as heptanoates and octanoates of sitosterol, campesterol and sitostanol (Table 14).

In fraction 2, three major peaks were detected (Figure 23B). Peaks 10 and 11 were identified as trimethylsilyl derivatives of linoleic acid and *β*-sitosterol, respectively. According to GC and ^{1}H NMR analysis, both were not contained as impurities in the synthesized mixture of phytosteryl/-stanyl linoleates used for the heating experiment. Their occurrence might be explained by a potential formation via hydrolysis during the heating procedure. On the other hand, the presence of linoleic acid may also be related to the formation of compound 12. Based on comparison of the retention time and the mass spectrum to those of a synthesized reference compound, peak 12 was assigned as stigmasta-3,5-dien-7-one. This compound has previously been identified in heartwood and in the smoke of incense (Abramovitch and Micetich, 1962; Dalibalta *et al.*, 2015). In previous investigations involving the cleavage of the ester bond as part of the analytical procedure, 7-ketositosterol has been reported as major oxidation product resulting from the thermal treatment of sitosteryl esters (Soupas *et al.*, 2007; Scholz *et al.*, 2015b). The respective 7-ketositosteryl linoleate was not detected in the present study; stigmasta-3,5-dien-7-one might be explained, for example, as degradation product formed from 7-ketositosteryl linoleate via liberation of linoleic acid. On the other hand, the analogous oxidation product stigmasta-3,5,22-trien-7-one has also been identified upon thermo-oxidation of free stigmasterol (Menéndez-Carreño *et al.*, 2010), indicating that compound 12 might also be the degradation product of intermediately formed *β*-sitosterol.

In addition to these three quantitatively dominating compounds, GC-MS analysis of fraction 2 revealed the presence of a series of minor peaks exhibiting fragmentation patterns similar to those described for non-oxidized phytosteryl/-stanyl fatty acid esters (Barnsteiner *et al.*, 2011; Barnsteiner *et al.*, 2012; Esche *et al.*, 2012). The respective part of the chromatogram is enlarged in Figure 23B, and the MS data are listed in Table 14. The base peaks *m/z* 396 observed for compounds 13, 15, 17, 18 and 21 and *m/z* 382 for compounds 14 and 20 correspond to those described to result from the cleavage of the fatty acid from sitosteryl and campesteryl esters, respectively (Barnsteiner *et al.*, 2011; Esche *et al.*, 2012). The base peak *m/z* 215 observed for compounds 16, 19 and 22 has been described as being generated by cleavage of the fatty acid and further fragmentation involving the sterol side chain of short chain sitostanyl esters (Barnsteiner *et al.*, 2012). This enabled the differentiation of steryl and stanyl esters; exemplary mass spectra are shown in Figure 24. In a previous study in which the same mass spectrometric conditions were used, weak but characteristic molecular ions could be detected for sitostanyl fatty acid esters (Barnsteiner *et al.*, 2012).

Table 14. Characteristic GC-MS Fragment Ions of Phytosteryl/-stanyl Ester Oxidation Products.

acyl chain oxidation products[a]	RT[b] [min]	molecular ion $[M]^+$	characteristic fragments [*m/z*]
1, sitosteryl heptanoate	14.565	526 (-)	397 (33), 396 (100), 381 (27), 275 (15), 255 (22), 213 (16)
2, sitostanyl heptanoate	14.636	528 (6)	513 (0.3), 399 (10), 398 (41), 383 (35), 344 (10), 257 (18), 215 (100)
3, campesteryl octanoate	14.662	526 (-)	383 (36), 382 (100), 367 (33), 261 (22), 255 (26), 213 (20)
4, sitosteryl octanoate	14.972	540 (-)	397 (49), 396 (100), 381 (46), 275 (34), 255 (40), 213 (34)
5, sitostanyl octanoate	15.049	542 (4)	527 (1), 399 (21), 398 (53), 383 (39), 344 (11), 257 (18), 215 (100)
13, sitosteryl 7-hydroxyheptanoate[c]	16.606	614 (-)	397 (47), 396 (100), 381 (24), 275 (14), 255 (17), 213 (14), 524 (0.3)
14, campesteryl 8-hydroxyoctanoate[c]	16.740	614 (-)	383 (39), 382 (100), 367 (23), 261 (13), 255 (15), 213 (26)
15, sitosteryl 8-hydroxyoctanoate[c]	17.277	628 (-)	397 (43), 396 (100), 381 (18), 275 (12), 255 (17), 213 (14), 538 (0.2)
16, sitostanyl 8-hydroxyoctanoate[c]	17.398	630 (0.2)	399 (17), 398 (42), 383 (32), 344 (5), 257 (17), 215 (100), 540 (0.3)
17, sitosteryl 7-oxoheptanoate	17.736	540 (-)	525 (0.1), 397 (35), 396 (100), 381 (28), 275 (14), 255 (20), 213(19)
18, sitosteryl 8-oxooctanoate	18.590	554 (-)	397 (50), 396 (100), 381 (41), 275 (20), 255 (24), 213 (18)
19, sitostanyl 8-oxooctanoate	18.761	556 (0.9)	541 (0.7), 399 (14), 398 (50), 383 (36), 344 (4), 257 (14), 215 (100)
20, campesteryl 9-oxononanoate	18.856	554 (-)	383 (30), 382 (100), 367 (27), 261 (12), 255 (14), 213 (13)
21, sitosteryl 9-oxononanoate	19.559	568 (-)	397 (32), 396 (100), 381 (25), 275 (13), 255 (15), 213 (12)
22, sitostanyl 9-oxononanoate	19.722	570 (1.3)	555 (0.3), 399 (21), 398 (58), 383 (36), 344 (7), 257 (16), 215 (100)

[a] Numbers correspond to those in the chromatogram shown in Figure 23.
[b] Retention times on Rtx-200MS.
[c] As trimethylsilyl ether.

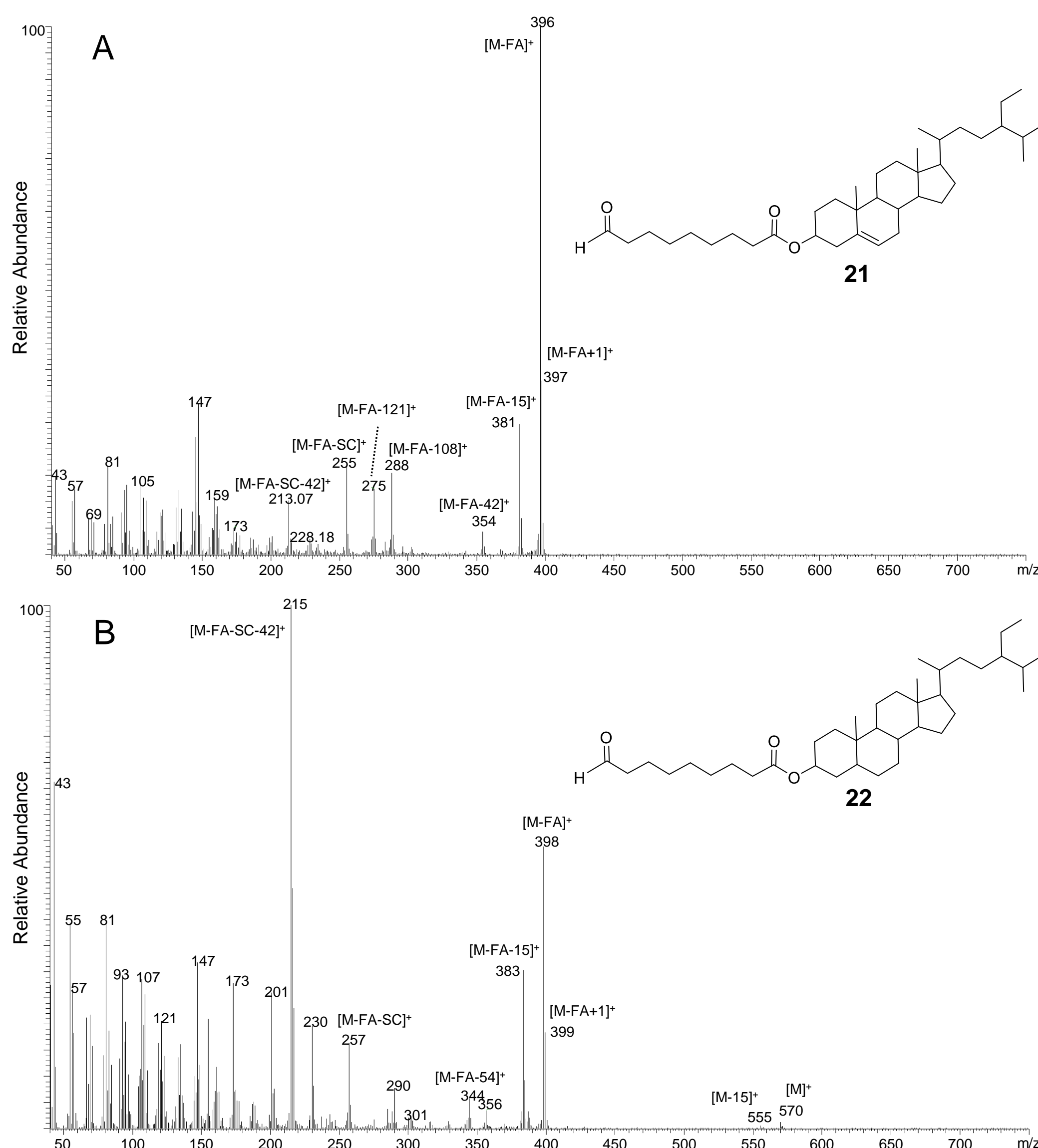

Figure 24. Electron-impact ionization spectra of (A) sitosteryl 9-oxononanoate and (B) sitostanyl 9-oxononanoate; [M]+, molecular ion; [FA], fatty acid; [SC], sterol/stanol side chain.

Therefore, based on the oxidative modifications in the acyl part known from peroxidation of cholesteryl linoleate and high temperature degradation of methyl linoleate and triacylglycerides (Kamido *et al.*, 1992a; Berdeaux *et al.*, 1999; Velasco *et al.*, 2005; Berdeaux *et al.*, 2012), molecular masses of potential acyl chain oxidation products of sitostanyl linoleate, i.e. oxo- and hydroxyalkanoates, were calculated and compared with the mass spectra determined in fraction 2. This screening gave first indications that the peaks 16, 19 and 22 corresponded to sitostanyl 8-hydroxyoctanote, 8-oxooctanoate and 9-oxononanoate, respectively.

These preliminary assignments initiated the next phase of the study, namely the synthesis and characterization of authentic reference compounds. Firstly, potential candidates of oxidized fatty acid moieties were synthesized via established synthetic routes: 9-oxononanoic acid was

obtained by oxidative diol cleavage of aleuritic acid (Reuter and Salomon, 1978); 8-oxooctanoic acid and 7-oxoheptanoic acid were obtained by Baeyer-Villiger reaction of the corresponding cycloalkanones and subsequent oxidation with iodoxybenzoic acid (Rajabi *et al.*, 2014). These synthesized acids as well as heptanoic and octanoic acid were esterified with the same phytosteryl/-stanyl mixture as used for the esterification of linoleic acid. The corresponding hydroxyalkanoates were obtained by reduction of 7-oxoheptanoates and 8-oxooctanoates with sodium borohydride.

The identities of the synthesized reference compounds were confirmed via NMR. With a proportion of 75%, sitosterol was the major phytosterol in the synthesized phytosteryl/-stanyl ester mixtures; therefore, $^{1}H/^{13}C$ NMR signals of sitosteryl esters could be interpreted. By making use of a repertoire of two-dimensional techniques, it was possible to assign almost every carbon and proton signal to their respective atom number (Figure 25).

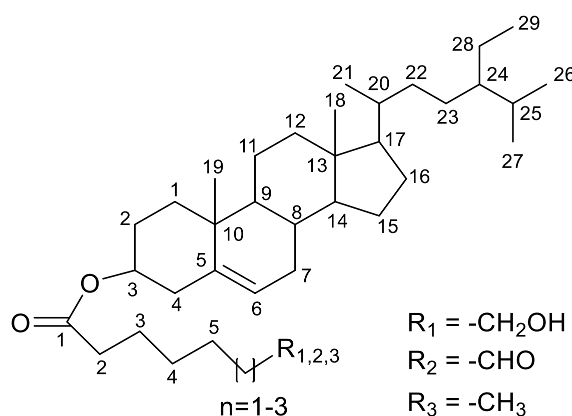

Figure 25. General structures and atom numbering of acyl chain oxidation products of sitosteryl linoleate.

By comparing the ^{13}C spectrum of unesterified sitosterol with ^{13}C spectra of the sitosteryl ester reference compounds, the carbon signals of the acyl chains could be assigned. Each synthesized compound exhibited the same proton and carbon signals in the sterol moiety (Table 15), thus only differing in their signals in the fatty acid part (Table 16).

Equally characteristic for each sitosteryl ester was the carbon atom FA-1 involved in the ester bond showing a chemical shift of 173 ppm and the carbon atom FA-2 with a chemical shift of 34.6 ppm and a well resolved triplet proton signal pattern at 2.3 ppm. Figure 26 shows the heteronuclear coupling (HMBC) between C_1 of the fatty acid moiety and the hydrogen atom at C_3 of the sterol moiety that was characteristic for all synthesized phytosteryl fatty acid esters.

Table 15. $^{1}H/^{13}C$ NMR signals of the sterol moiety of synthesized sitosteryl fatty acid esters.

C-atom[a]	^{13}C (ppm)	^{1}H (ppm)	quantity H	multiplet[b]
St-5	139.7	-	-	-
St-6	122.59	5.39	1	d
St-3	73.67	4.63	1	m
St-14	56.68	1	1	m
St-17	56.02	1.1	1	m
St-9	50.01	0.98	1	m
St-24	45.81	0.93	1	m
St-13	42.31	-	-	-
St-12	39.72	1.17/2.03	2	m
St-4	38.16	2.33	2	d
St-1	37.01	1.13/1.86	2	m
St-10	36.6	-	-	-
St-20	36.17	1.37	2	m
St-22	33.92	1.32	2	m
St-7	31.91	1.96/2.03	2	m
St-8	31.86	1.31	1	m
St-25	29.12	1.32	1	m
St-2	28.27	1.59/1.87	2	m
St-16	27.82	1.57/1.88	2	m
St-23	26.03	1.19	2	m
St-15	24.31	1.04/1.60	2	m
St-28	23.05	0.9	2	m
St-11	21.03	1.01/1.51	2	m
St-26/27[c]	19.85	0.89	3	d
St-19	19.34	1.04	3	s
St-26/27[c]	19.04	0.89	3	d
St-21	18.79	0.94	3	d
St-29	12	0.89	3	t
St-18	11.87	0.7	3	s

[a] Numbering of C-atoms according to Figure 25.
[b] Signal patterns: s, singlet; d, douplet; m, multiplet.
[c] Signals could not be distinguished.

Table 16. $^{1}H/^{13}C$ NMR signals of the fatty acid moieties of synthesized sitosteryl fatty acid esters.

C-atom[a]	^{13}C (ppm)	^{1}H (ppm)	quantity H	multiplet[b]
		heptanoate		
FA-1	173.34	-	0	-
FA-2	34.74	2.29	2	t
FA-5	31.49	1.32	2	m
FA-4	28.81	1.32	2	m
FA-3	25.05	1.64	2	m
FA-6	22.51	1.31	2	m
FA-7	14.07	0.88	3	t

Table 16. Continued.

C-atom	^{13}C (ppm)	^{1}H (ppm)	quantity H	multiplet
		octanoate		
FA-1	173.35	-	-	-
FA-2	34.76	2.29	2	t
FA-6	31.69	1.3	2	m
FA-4/5[c]	29.1	1.32	2	m
FA-4/5[c]	28.96	1.32	2	m
FA-3	25.08	1.63	2	m
FA-7	22.62	1.3	2	m
FA-8	14.1	0.9	3	t
		7-hydroxyheptanoate		
FA-1	173.25	-	-	-
FA-7	62.94	3.67	2	t
FA-2	34.6	2.32	2	t
FA-6	32.55	1.6	2	m
FA-4/5[c]	28.9	1.39	2	m
FA-4/5[c]	28.86	1.31	2	m
FA-3	25.4	1.66	2	m
		8-hydroxyoctanoate		
FA-1	173.29	-	-	-
FA-8	63.06	3.66	2	t
FA-2	34.64	2.29	2	t
FA-7	32.71	1.58	2	m
FA-4/5[c]	29.21	1.37	2	m
FA-4/5[c]	29.02	1.29	2	m
FA-6	25.66	1.37	2	m
FA-3	24.95	1.66	2	m
		7-oxoheptanoate		
FA-7	202.59	9.79	1	t
FA-1	173	-	-	-
FA-6	43.68	2.46	2	d of t
FA-2	34.57	2.31	2	t
FA-4/5[c]	28.57	1.28	2	m
FA-4/5[c]	27.81	1.59	2	m
FA-3	24.73	1.62	2	m
		8-oxooctanoate		
FA-8	203	9.78	1	t
FA-1	173	-	-	-
FA-7	43.82	2.45	2	d of t
FA-2	34.56	2.29	2	t
FA-4/5[c]	28.82	1.36	2	m
FA-4/5[c]	28.8	1.36	2	m
FA-6	27.82	1.66	2	m
FA-3	24.8	1.65	2	m

Table 16. Continued.

C-atom	^{13}C (ppm)	^{1}H (ppm)	quantity H	multiplet
9-oxononanoate				
FA-9	202.87	9.79	1	t
FA-1	173.22	-	-	-
FA-8	43.89	2.44	2	d of t
FA-2	34.63	2.29	2	tr
FA-4/5/6[c]	29.02	1.35	2	m
FA-4/5/6[c]	28.97	1.35	2	m
FA-4/5/6[c]	28.89	1.35	2	m
FA-7	27.82	1.61	2	m
FA-3	24.95	1.63	2	m

[a] Numbering of C-atoms according to Figure 25.
[b] Signal patterns: s, singlet; d, douplet; t, triplet; m, multiplet.
[c] Signals could not be distinguished.

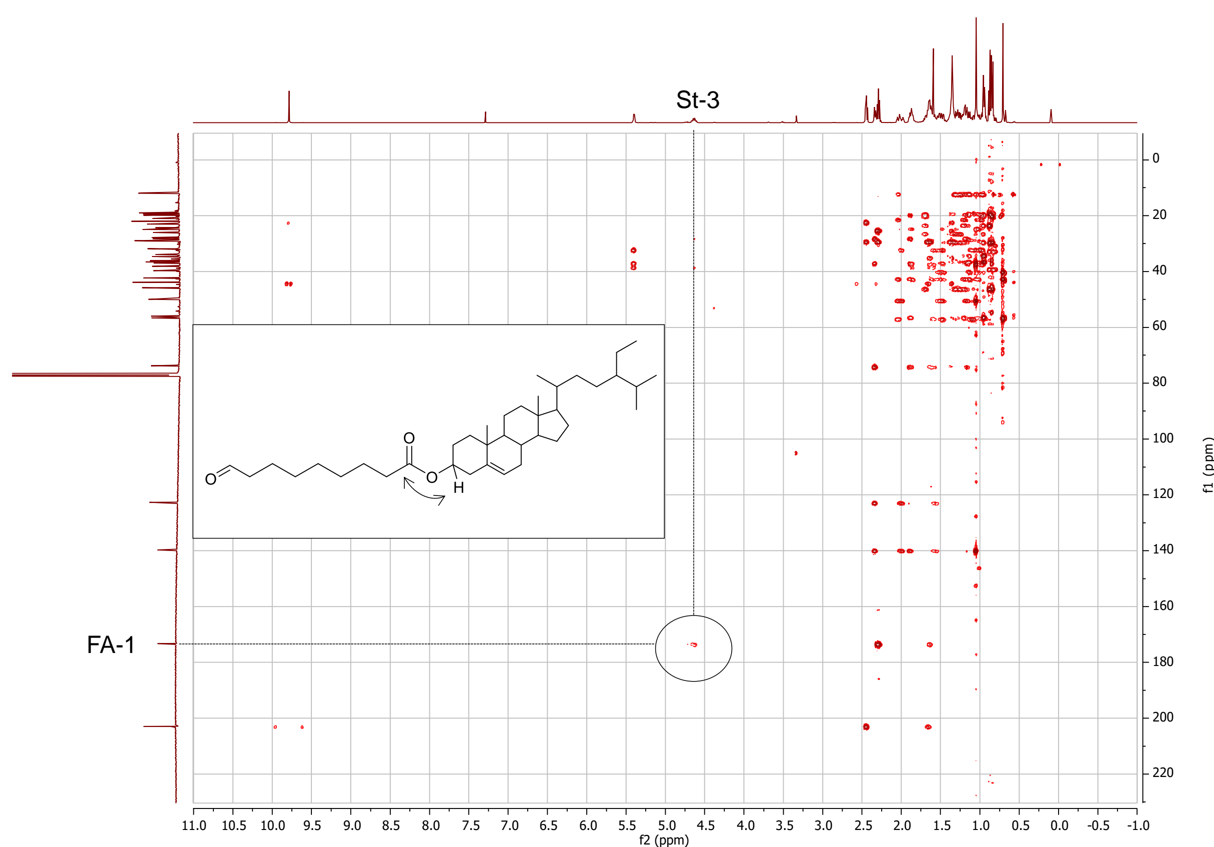

Figure 26. Heteronuclear multiple-bond correlation spectroscopy (HMBC) of synthesized sitosteryl 9-oxononanoate. The horizontal axis represents the ^{1}H spectrum, the vertical axis the ^{13}C spectrum. The coupling between C_1 of the fatty acid moiety and the hydrogen atom at C_3 of the sterol moiety, characteristic for all synthesized phytosteryl fatty acid esters, is highlighted.

The signals of terminal carbon and proton atoms of the fatty acid part enabled the discrimination between the synthesized reference classes. The terminal carbon atom of sitosteryl alkanoates exhibited a signal at 14.1 ppm and the corresponding proton showed a triplet proton signal pattern at 0.9 ppm, whereas the terminal aldehyde function of core aldehyde esters had a

chemical shift at 203 ppm and an intense triplet pattern was observed for the aldehyde proton at 9.8 ppm (Figure 27).

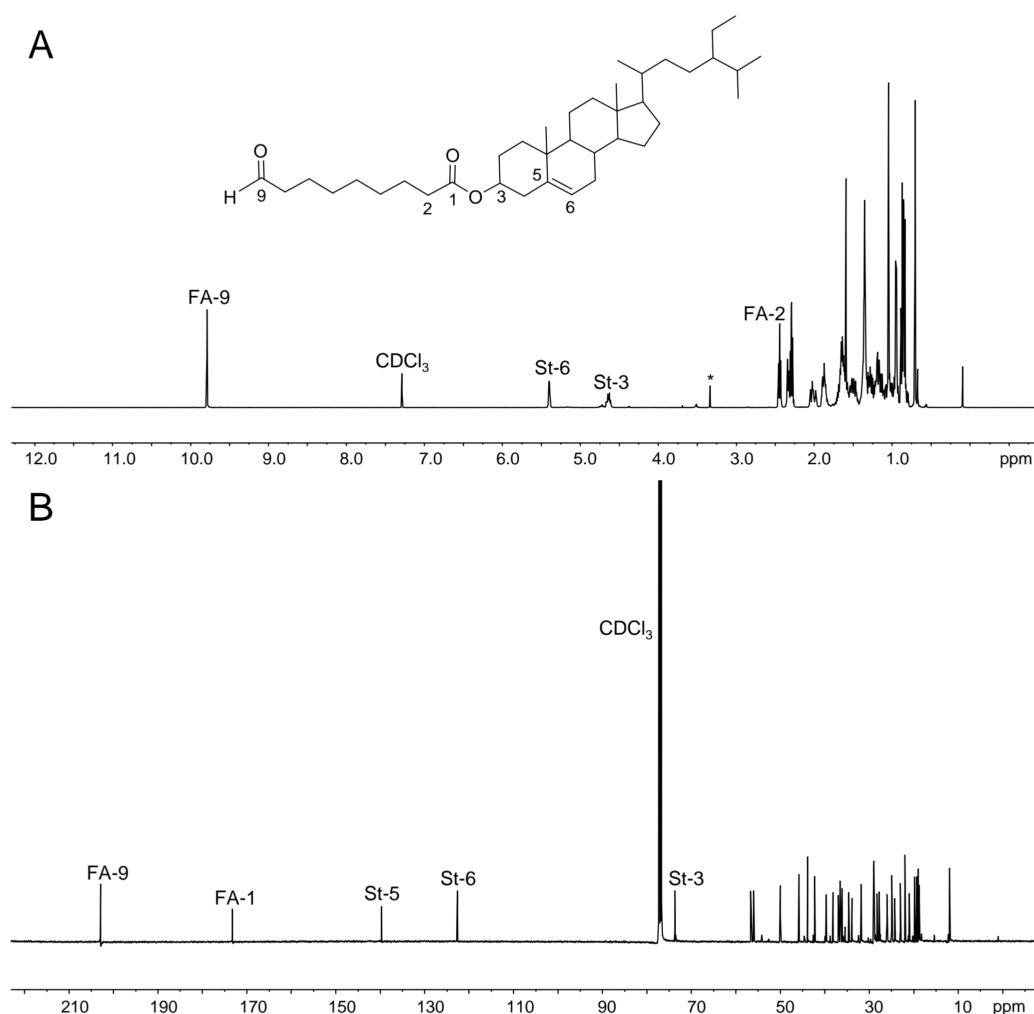

Figure 27. NMR spectra of synthesized sitosteryl 9-oxononanoate. (A) ^{1}H NMR; characteristic proton signals of the sterol moiety and the fatty acid moiety: δ 9.79 (FA-9, 1H, t), 5.39 (St-6, 1H, m), 4.63 (St-3, 1H, m), 2.29 (FA-2, 2H, t). The asterisks represents an impurity. (B) ^{13}C NMR; characteristic carbon signals of the sterol moiety and the fatty acid moiety: δ 202.87 (FA-9), 173.22 (FA-1), 139.7 (St-5), 122.59 (St-6), 73.67 (St-3).

The acquired signal pattern and peak assignments correlated with published ^{1}H and ^{13}C data of cholesteryl 9-oxononanoate (Boechzelt *et al.*, 1998). The terminal carbon atom of sitosteryl hydroxyalkanoates showed a signal at 63 ppm and a well resolved triplet pattern at 3.66 ppm of the terminal protons. The characteristic proton signals of the synthesized reference compounds were also found as minor signals in the ^{1}H NMR spectrum of the heated phytosteryl/-stanyl linoleates (Figure 28). Final peak identifications were carried out by comparing retention times and mass spectra of sample peaks with those of the synthesized reference compounds (Table 14). Due to their slightly higher polarity compared to the

respective linoleates, sitosteryl octanoate (4) and sitostanyl octanoate (5) were also present in fraction 2 (Figure 23B).

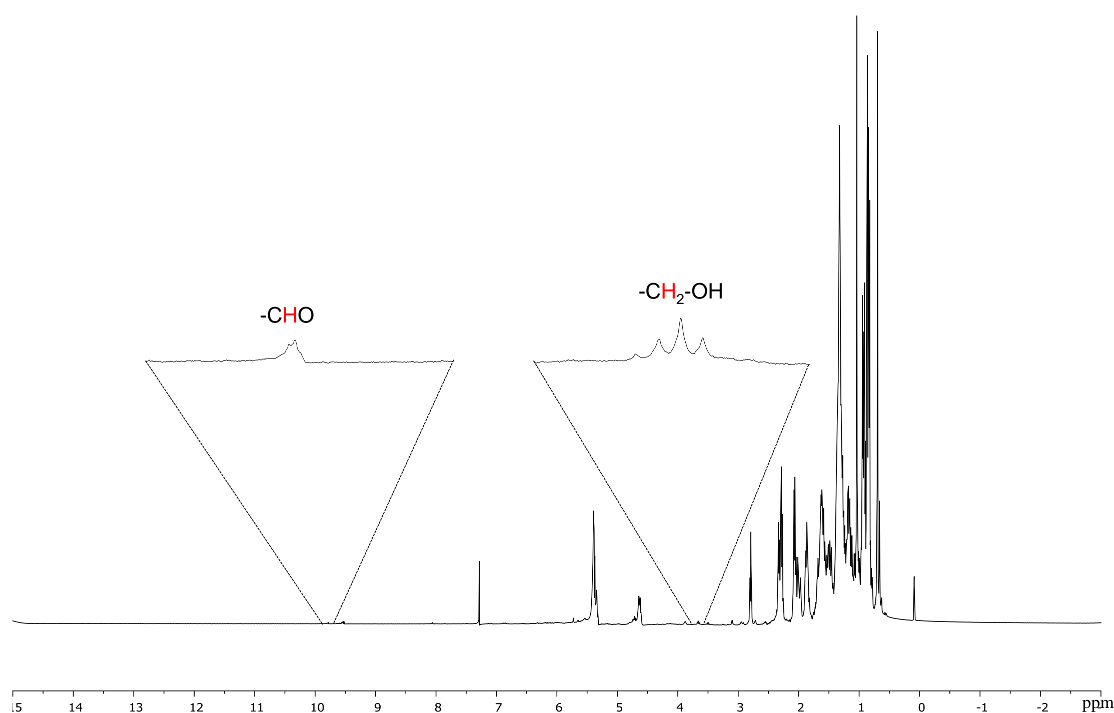

Figure 28. ^{1}H NMR spectrum of the heated mixture of phytosteryl/-stanyl linoleates. Signals corresponding to *n*-alkanals (-CHO) and primary alcohols ($-CH_2-OH$) are enlarged.

In total, 7 types of oxidation products of the fatty acid moiety belonging to three different classes, i.e. alkanoates, hydroxyalkanoates and oxoalkanoates, could be identified upon heating of phytosteryl/phytostanyl linoleates (Figure 29). These classes of acyl chain oxidation products were in line with those previously reported for cholesteryl esters, fatty acid methyl esters and triacylglycerides (Kamido *et al.*, 1993; Berdeaux *et al.*, 1999; Dobarganes and Márquez-Ruiz, 2007).

The formation of these acyl chain oxidation products may be explained analogously to the routes described for the oxidation of fatty acid methyl esters (Frankel *et al.*, 1984; Frankel, 2005; Berdeaux *et al.*, 2012). The primarily formed allylic hydroperoxides on positions 8 and 9, respectively, are degraded by homolytic β-scission according to route I (Figure 30). After interaction of the resulting alkyl radical with a hydrogen radical, heptanoates and octanoates, respectively, are obtained. Alternatively, 7-hydroxyheptanoates and 8-hydroxyoctanoates are formed by reaction of the alkyl radical with a hydroxyl radical. Repeated oxidation of the alkyl radical formed from the degradation of the 8-hydroperoxide with oxygen leads to the formation of 7-hydroperoxides, which can decompose to 7-oxoheptanoates via an alkoxyl radical. On the

other hand, 8-oxooctanoates and 9-oxononanoates emerge directly upon *β*-scission of allylic hydroperoxides on positions 8 and 9 following route II. In addition, 9-oxononanoates may be formed from the allylic hydroperoxide in position 10 following route I and subsequent reaction with a hydroxyl radical.

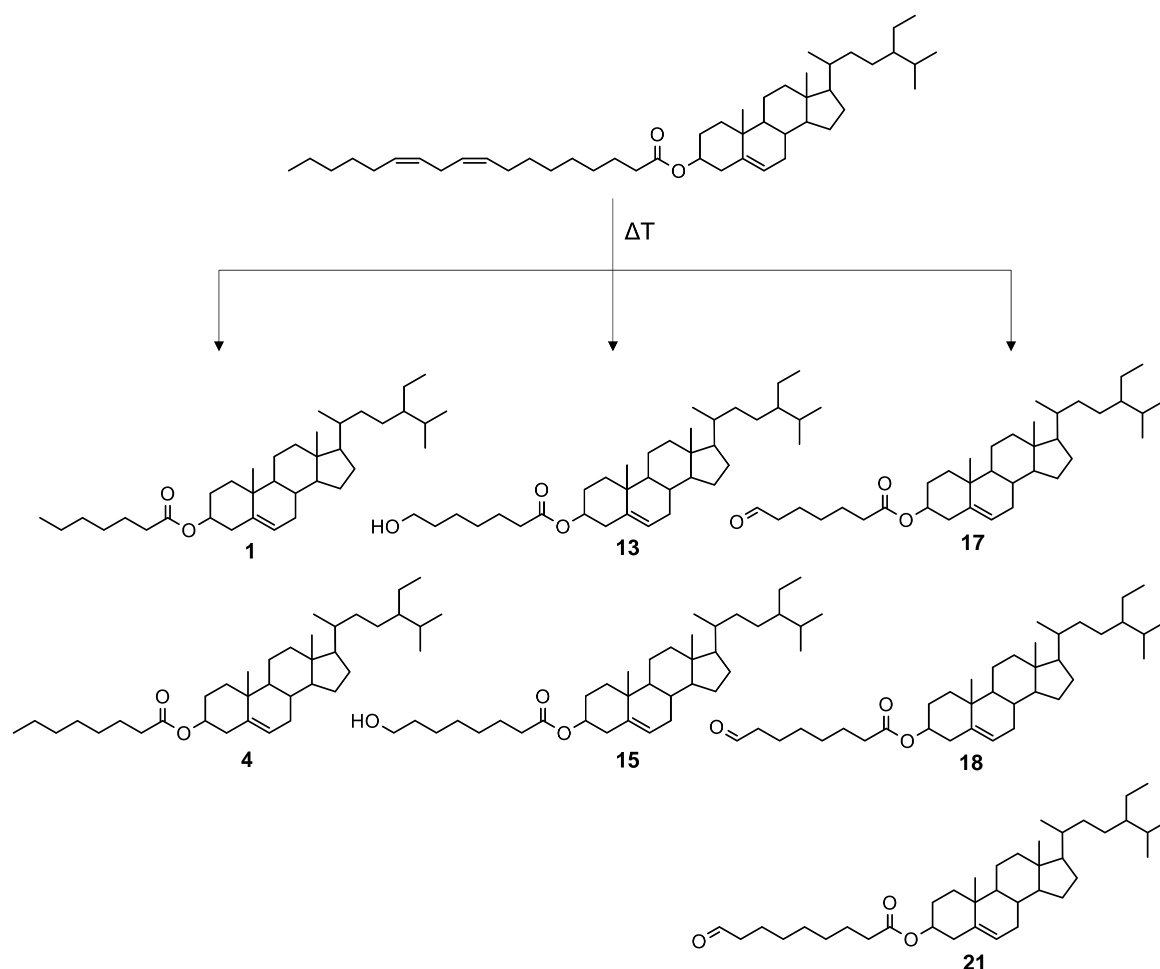

Figure 29. Classes of identified oxidation products in the fatty acid part upon heating of sitosteryl, campesteryl and sitostanyl esters, exemplarily shown for sitosteryl linoleate: Class I (alkanoates): 1, sitosteryl heptanoate; 4, sitosteryl octanoate; class II (hydroxyalkanoates): 13, sitosteryl 7-hydroxyheptanoate; 15, sitosteryl 8-hydroxyoctanoate; class III (oxoalkanoates): 17, sitosteryl 7-oxoheptanoate; 18, sitosteryl 8-oxooctanoate and 21, sitosteryl 9-oxononanoate. The numbering corresponds to that of Figure 23.

In addition to the described acyl chain oxidation products, three representatives of linoleates of oxidized sterols were detected in fraction 2. Peaks 23 and 24 (Figure 23B) were assumed to be silylated *α*/*β*-7-hydroxysitosteryl linoleates, since their mass spectra (Table 17) exhibited a characteristic base peak of *m/z* 484 corresponding to $[M\text{-}FA]^+$, which was also described as the base peak of double silylated 7-hydroxysitosterols in a previous study (Apprich and Ulberth, 2004). Peak 25 was tentatively assigned as *α*- or *β*-5,6-epoxysitosteryl linoleate, exhibiting characteristic fragment ions similar to those reported for silylated *α*/*β*-5,6-epoxysitosterols, with

m/z 395 potentially corresponding to $[M\text{-}FA\text{-}18]^{+}$ and *m/z* 413 resulting from $[M\text{-}FA]^{+}$ (Zhang *et al.*, 2005). As no reference compounds were available for these esters, their identifications were only considered tentative.

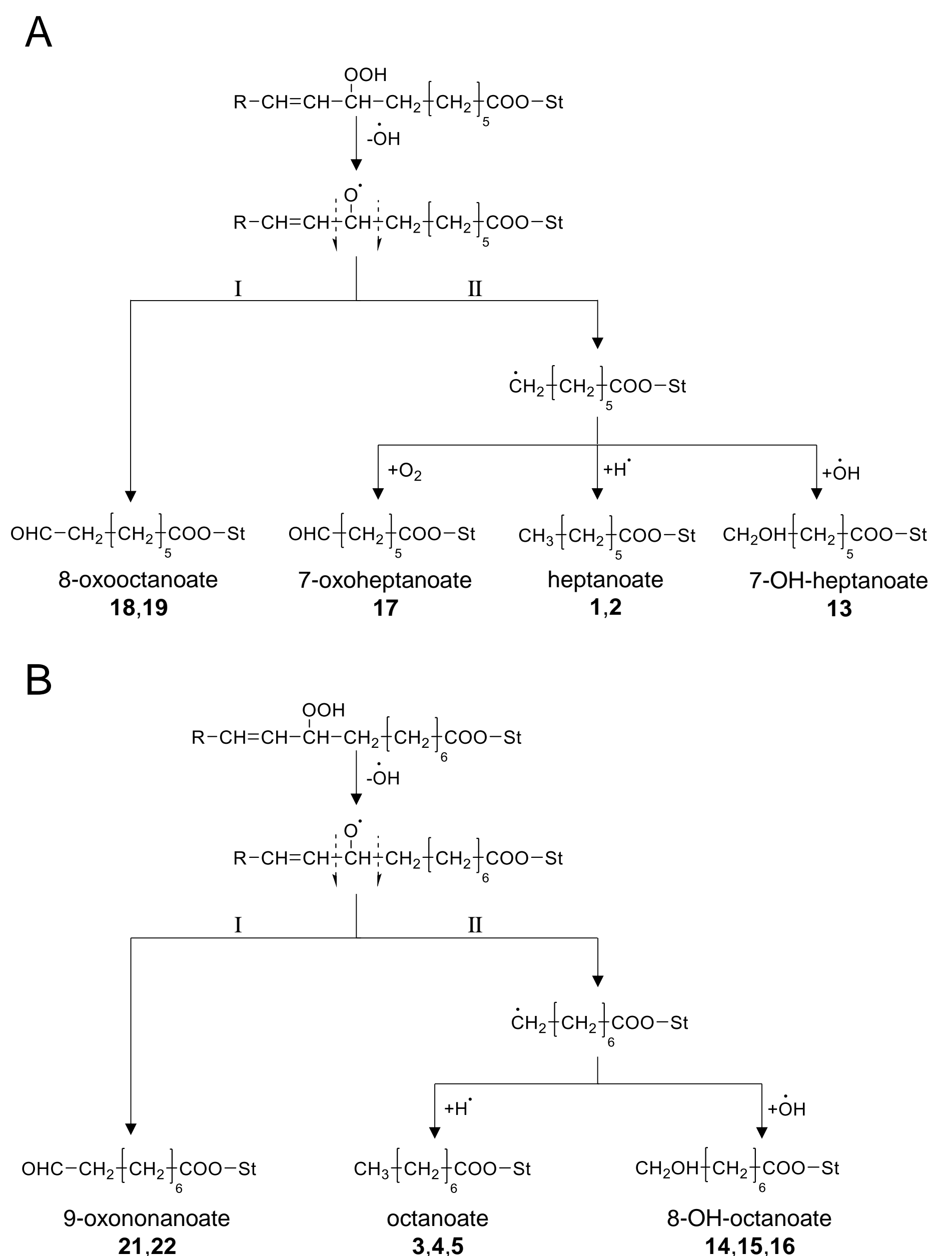

Figure 30. Formation pathways of steryl/stanyl ester oxidation products in the acyl chain by homolytic β-scission of primarily formed allylic hydroperoxides on positions (A) 8 and (B) 9 (modified from Frankel, 2005). Numberings correspond to those in Figure 23 and 29. Only pathways leading to the compounds identified in this study are illustrated.

There are other representatives of potential acyl chain oxidation products that have not been detected in this study, e.g. those deriving from the 13-hydroperoxides. In addition, compounds covered using LC-MS, such as long-chain ACOPs bearing hydroxy or epoxy groups as well as

sitosteryl fatty acid esters oxidized in both the acyl chain and the sterol part (Julien-David *et al.*, 2014), cannot be analyzed using the approach selected in the present study.

Table 17. Characteristic GC-MS Fragment Ions of Tentatively Assigned Sitosteryl Linoleates Oxidized in the Sterol Moiety.

sitosteryl linoleate oxidation products[a]	RT[b] [min]	molecular ion $[M]^+$	characteristic fragments [*m/z*]
23, 7*α*/*β*-hydroxysitosteryl linoleate[c,d]	20.955	765 (-)	486 (12), 485 (55), 484 (100), 483 (33)
24, 7*α*/*β*-hydroxysitosteryl linoleate[c,d]	23.038	765 (-)	486 (16), 485 (46), 484 (100), 483 (56)
25, 5,6*α*/*β*-epoxysitosteryl linoleate[d]	25.845	693 (-)	413 (44), 395 (100)

[a] Peak numbers correspond to those in the chromatogram shown in Figure 23B.
[b] Retention times on Rtx-200MS.
[c] As trimethylsilyl ether.
[d] α/*β*-Stereoisomers could not be distinguished.

4.2.2. Summary

The employed methodology, i.e. a pre-concentration of polar oxidation products via SPE, the establishment of capillary gas chromatographic conditions enabling the separation of various oxidation products, and the synthesis of authentic reference compounds provided data on the formation and the identities of previously unreported acyl chain oxidation products resulting from the thermal treatment of phytosteryl/-stanyl fatty acid esters. In total, 15 sitosteryl, sitostanyl and campesteryl esters, resulting from oxidation of the acyl chain, could be identified by GC-FID/MS. Among the identified ACOPs were (i) nonpolar phytosteryl/-stanyl heptanoates and octanoates, and (ii) polar phytosteryl/-stanyl 7-hydroxyheptanoates, 8-hydroxyoctanoates, 7-oxoheptanoates, 8-oxooctanoates and 9-oxononanoates The observed classes of products correspond to those expected from the thermo-oxidation of unsaturated fatty acids and triglycerides. However, the fact that the approach was not based on the formation of methylesters via transesterification enabled for the first time the analysis of a spectrum of individual phytosteryl/-stanyl fatty acid esters oxidized in the acyl moiety.

4.2.3. Quantitation of Acyl Chain Oxidation Products Formed upon Thermo-Oxidation of Phytosteryl/-stanyl Oleates and Linoleates

Based on the identification of novel acyl chain oxidation products of phytosteryl/-stanyl linoleates, a method for the quantitative determination of ACOPs should be developed. The method should be used to investigate the formation of ACOPs being formed upon thermo-oxidation of four different ester mixtures: (i) phytosteryl/-stanyl oleates, (ii) phytosteryl/-stanyl linoleates, (iii) phytostanyl oleates, and (iv) phytostanyl linoleates. To determine the contributions of ACOPs to the losses of phytosteryl/-stanyl fatty acid esters, non-oxidized fatty acid esters should be determined after thermo-oxidation according to a previously published method used for the analysis of phytosteryl/-stanyl fatty acid esters in enriched foods (Barnsteiner *et al.*, 2012). In addition, oxidation products in the phytosterol moieties (POPs) formed upon thermo-oxidation of the ester mixtures should be analyzed via on-line LC-GC to compare the occurrence of POPs with that of ACOPs (Scholz *et al.*, 2015b).

4.2.3.1. Gravimetric Determination of Nonpolar and Polar Compounds in Heated Phytosteryl/-stanyl Fatty Acid Esters

For the synthesis of phytosteryl/-stanyl fatty acid esters as model substrates, both a commercially available mixture of predominantly sterols (75 % sitosterol, 10 % campesterol, 12 % sitostanol and 2 % campestanol) and a mixture of stanols (98 % sitostanol, 2 % campestanol) were esterified with oleic and linoleic acid. The four obtained mixtures of phytosteryl/-stanyl oleates and linoleates represented the quantitatively dominating proportion of phytosteryl/-stanyl fatty acid esters being added to margarines enriched with phytosteryl/-stanyl fatty acid esters. Analysing the phytosteryl/-stanyl fatty acid ester profiles in two enriched margarines, a recent study determined a proportion of 86 % for sitosteryl/campesteryl/sitostanyl oleates and linoleates of sitosterol, campesterol and sitostanol in a margarine enriched with phytosteryl esters, and a proportion of 83 % sitostanyl/campestanyl oleates and linoleats in a margarine enriched with phytostanyl esters (Scholz *et al.*, 2016).

As part of the quality control procedure of heated oils or the FAME derived thereof, nonpolar and polar compounds are commonly separated via silica column chromatography and the fractions obtained are determined gravimetrically (IUPAC, 1992). This procedure allows a first unspecific estimation of the extent of primary, secondary and tertiary oxidation products. Therefore, the four ester mixtures under study were heated and subjected to the SPE procedure

that was used for the identification of nonpolar and polar acyl chain oxidation products (cf. 4.2.1.1). The results of the gravimetric determinations of the nonpolar und polar fractions for each of the four ester mixtures are given in Table 18.

Increased unsaturation both in the sterol moieties and in the fatty acid moieties led to higher amounts of polar compounds. Thus, 76 wt.% of originally present phytosteryl/-stanyl linoleates and only 45 wt.% of phytostanyl oleates were present in a polar, oxidized form. Interestingly, phytosteryl/-stanyl oleates and phytostanyl linoleates showed similar proportions, 60 and 65 wt. %, respectively, of polar compounds upon thermo-oxidation. These findings corresponded to a recent study that reported higher amounts of total polar compounds obtained for stigmasteryl linoleate heated at 180 °C compared to stigmasteryl oleate (Raczyk *et al.*, 2017a).

Table 18. Gravimetrically determined proportions [%] of the nonpolar and polar SPE fractions of heated (180 °C/ 40 min) phytosteryl/-stanyl fatty acid esters.

	proportions[a] [%]	
heated ester mixture	nonpolar fraction[b]	polar fraction[b]
phytosteryl/-stanyl oleates	33 ± 3	60 ± 3
phytosteryl/-stanyl linoleates	27 ± 1	76 ± 2
phytostanyl oleates	53 ± 3	45 ± 3
phytostanyl linoleates	38 ± 5	65 ± 5

[a] Values represent the mean ± standard deviation. Heating and gravimetric determinations were performed in triplicate (n=3); proportions were calculated on the basis of 11 mg of esters subjected to the heating procedure.
[b] The nonpolar fraction was obtained after eluting with cyclohexane, the polar fraction after eluting with MTBE.

4.2.3.2. Quantitation of ACOPs

To quantitate the phytosteryl/-stanyl alkanoates, oxoalkanoates and hydroxyalkanoates previously identified upon thermal treatment of a mixture of phytosteryl/-stanyl linoleates at the chosen time/temperature combination of 40 min/180 °C, structurally related cholesteryl esters representative for these classes (cholesteryl octanoate, cholesteryl 9-oxononanoate and cholesteryl 9-hydroxynonanoate) were synthesized and employed as internal standards. The NMR spectra obtained for synthesized cholesteryl octanoate and cholesteryl 9-oxononanoate corresponded to previously published spectra (Cameron *et al.*, 1972; Boechzelt *et al.*, 1998). The identity of cholesteryl 9-hydroxynonanoate was confirmed by two-dimensional NMR of the fatty acid part (Table 19).

The nonpolar ACOPs (heptanoates, octanoates) were quantitated directly in aliquots of samples obtained after thermal treatment, while the non-oxidized phytosteryl/-stanyl fatty acid esters

after an additional dilution step. The dilution was required to obtain concentrations of non-oxidized esters that were in the range reported to be suitable for quantitative analysis (Barnsteiner *et al.*, 2012).

Table 19. $^{1}H/^{13}C$ NMR signals of the fatty acid moiety of synthesized cholesteryl 9-hydroxynonanoate.

C-atom[a]	^{13}C (ppm)	^{1}H (ppm)	quantity H	multiplet[b]
FA-1	173.29	-	-	-
FA-9	63.06	3.66	2	t
FA-2	34.7	2.29	2	t
FA-8	32.71	1.58	2	m
FA-4/5/6[c]	29.22	1.35	2	m
FA-4/5/6[c]	29.22	1.35	2	m
FA-4/5/6[c]	29.03	1.35	2	m
FA-7	25.66	1.64	2	m
FA-3	25.02	1.65	2	m

[a] Numbering of C-atoms according to Figure 25.
[b] Signal patterns: s, singlet; d, douplet; t, triplet; m, multiplet.
[c] Signals could not be distinguished.

To quantitate the polar oxidation products (oxo- and hydroxyalkanoates), the described SPE-based methodology resulting in a separation of a fraction containing the polar oxidation products from the non-oxidized fatty acid esters was applied. A schematic flow of the quantitation procedure is depicted in Figure 31.

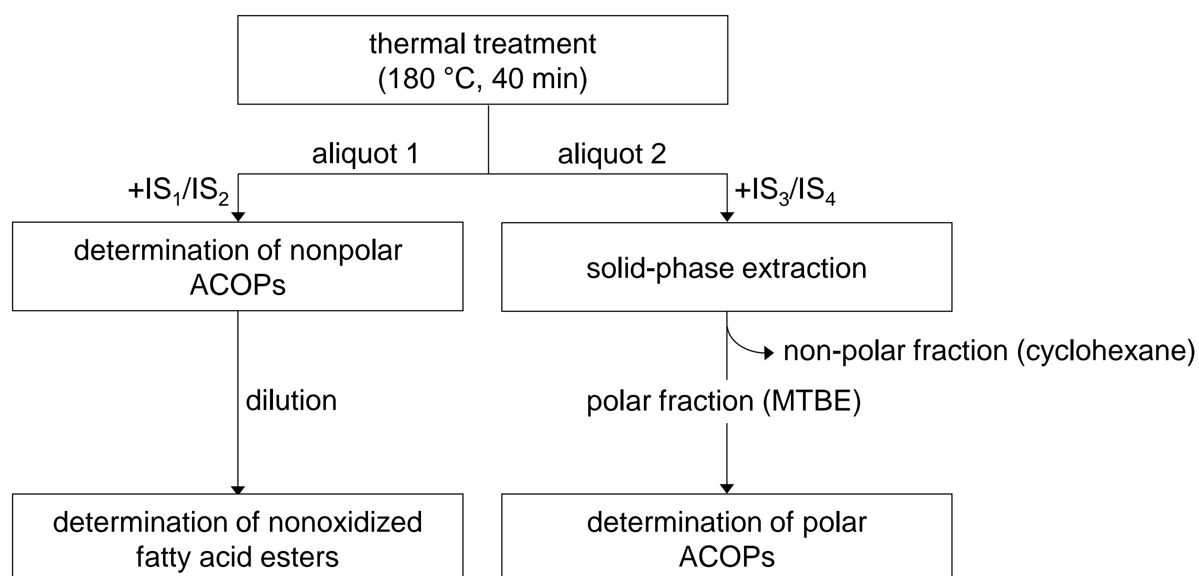

Figure 31. Sample preparation scheme for the quantitation of nonpolar and polar ACOPs and non-oxidized fatty acid esters; internal standards: IS_1, cholesteryl octanoate; IS_2, cholesteryl palmitate; IS_3, cholesteryl 9-hydroxynonanoate; IS_4, cholesteryl 9-oxononanoate.

The chromatograms resulting from the analysis of heat-treated mixtures of phytosteryl/-stanyl and phytostanyl linoleates are exemplarily shown in Figure 32.

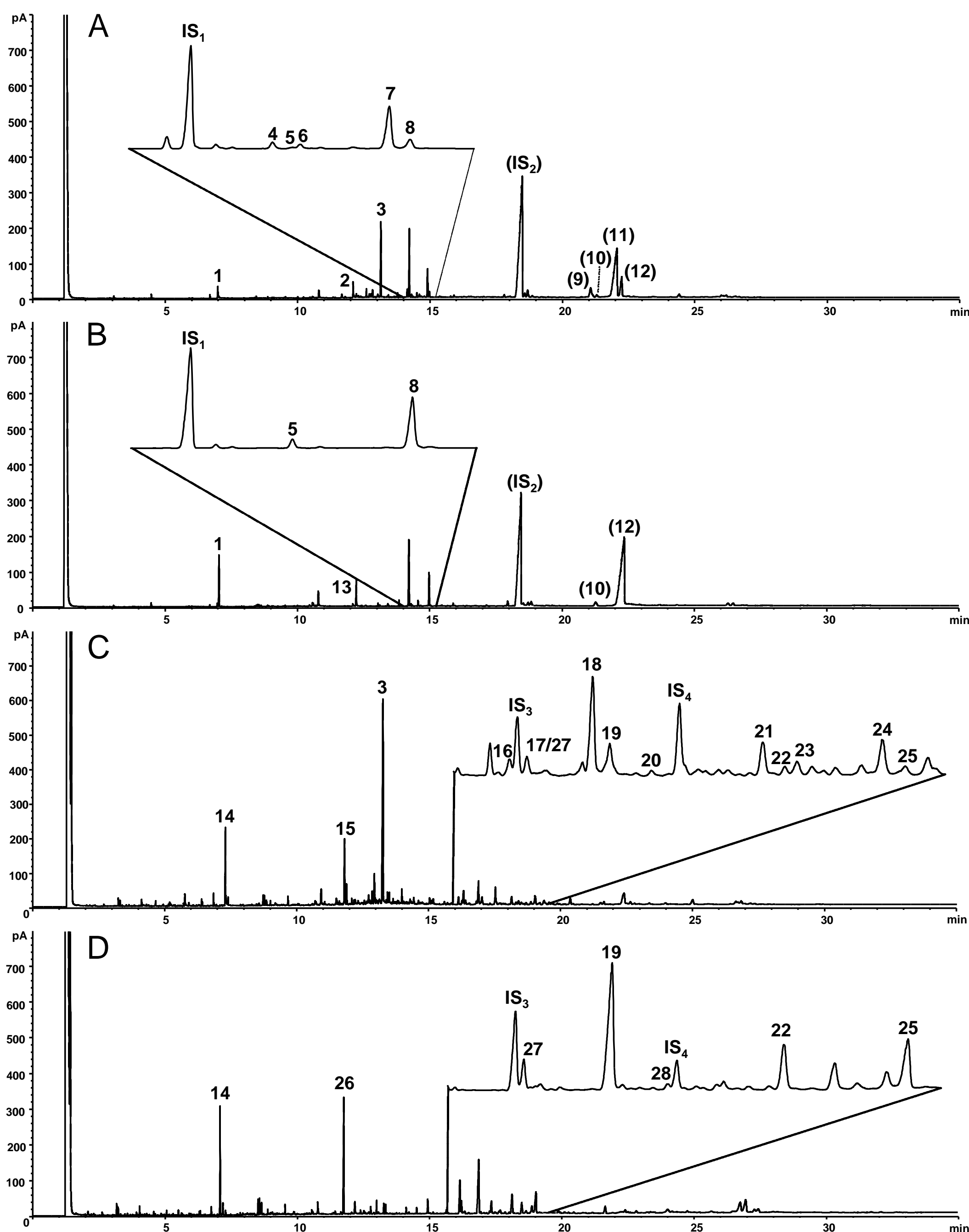

Figure 32. Capillary gas chromatographic analyses of thermo-oxidized phytosteryl/-stanyl linoleates and phytostanyl linoleates: (A) phytosteryl/-stanyl linoleates (nonpolar ACOPs), (B) phytostanyl linoleates (nonpolar ACOPs), (C) phytosteryl/-stanyl linoleates (polar ACOPs), (D) phytostanyl linoleates (polar ACOPs). Peak assignments: 1, linoleic acid; 2, sitosterol; 3, stigmasta-3,5-dien-7-one; 4, sitosteryl heptanoate; 5, sitostanyl heptanoate; 6, campesteryl octanoate; 7, sitosteryl octanoate; 8, sitostanyl octanoate; 9, campesteryl linoleate; 10, campestanyl linoleate; 11, sitosteryl linoleate; 12, sitostanyl linoleate; 13, sitostanol; 14, linoleic acid (TMS); 15, sitosterol (TMS); 16, sitosteryl 7-hydroxyheptanoate; 17, campesteryl 8-hydroxyoctanoate; 18, sitosteryl 8-hydroxyoctanoate; 19, sitostanyl 8-hydroxyoctanoate; 20, sitosteryl 7-oxoheptanoate; 21, sitosteryl 8-oxooctanoate; 22, sitostanyl 8-oxooctanoate; 23, campesteryl 9-oxononanoate; 24, sitosteryl 9-oxononanoate; 25, sitostanyl 9-oxononanoate; 26, sitostanol (TMS); 27, sitostanyl 7-hydroxyheptanoate; 28, sitostanyl 7-oxoheptanoate; internal standards: IS_1, cholesteryl octanoate; IS_2, cholesteryl palmitate; IS_3, cholesteryl 9-hydroxynonanoate; IS_4, cholesteryl 9-oxononanoate. Numbering of internal standards corresponds to that of Figure 31. The peaks assigned by numbers in parentheses have not been considered for quantitation in these chromatograms.

LODs and LOQs were determined for representatives of each class of oxidation products. (Table 20). The linearity in the working range was determined for these oxidized sitosteryl esters as well as for the corresponding oxidized cholesteryl esters used as internal standards. Coefficients of correlation (r2) higher than 0.999 for all compounds indicated good linearity in the working range.

To determine the potential influence of the preponderance of non-oxidized esters compared to the formed ACOPs on the recoveries, sitosteryl 9-oxononanoate and sitosteryl 9-hydroxynonanoate were spiked to non-heated sitosteryl linoleate before being subjected to the SPE-step. Good recovery rates for these representative ACOPs from 96.1 ± 2.5 to 102.4 ± 4.8 % were obtained over the whole concentration range.

Table 20. Limits of Detection (LOD), Limits of Quantitation (LOQ), and Characteristics of Calibration Curves of Representative ACOPs.

ACOPs	LOD [µg/mL][a]	LOQ [µg/mL][a]	slope	intercept	r^2
sitosteryl octanoate	0.09	0.25	2.4848	0.3123	0.9994
sitosteryl 9-hydroxynonanoate[b]	0.49	1.55	0.3374	0.1689	0.9994
sitosteryl 9-oxononanoate	0.10	0.28	1.8268	0.1605	0.9992

[a] LODs and LOQs expressed as µg/mL of injected test solutions (GC-FID); determined on the basis of 1 µL injection volume.
[b] Determined as TMS ether.

Thermo-oxidation and analysis of phytostanyl linoleates were repeated by a second operator in triplicate on another day; relative standard deviations <5 % for nonpolar and polar oxidation products indicated good reproducibility. Taking these validation data into account, analyses of three samples of each of the four ester mixtures resulting from independently performed heating experiments were considered to be appropriate.

4.2.3.3. Profiles of ACOPs in Heat-treated Phytosteryl-/stanyl Oleates and Linoleates

The concentrations of individual ACOPs determined upon heating of the four phytosteryl/-stanyl fatty acid ester mixtures are compiled in Tables 21 and 22. Analyses before the heating steps revealed that the concentrations of polar ACOPs were below the LODs, whereas nonpolar ACOPs could already be quantitated in the starting materials. This can be explained by the heat-catalyzed synthesis of the phytosteryl/-stanyl fatty acid ester mixtures and their subsequent SPE-purification using cyclohexane as eluent. Despite this initial presence of nonpolar oxidation products, the analyses demonstrated pronounced increases of the concentrations of both polar as well as nonpolar ACOPs upon heat treatment.

Table 21. ACOPs Formed upon Heating of Phytostanyl Esters at 180 °C [µg/mg Esters][a]

oxidation products[b] [µg/mg]	RRT[c]	phytostanyl oleates[d]		phytostanyl linoleates[d]	
		0 min	40 min	0 min	40 min
non-polar oxidation products					
5 sitostanyl heptanoate***	1.024	0.60 ± 0.02	3.69 ± 0.14	0.15 ± 0.00	1.02 ± 0.01
8 sitostanyl octanoate***	1.053	0.73 ± 0.04	5.17 ± 0.20	1.31 ± 0.01	7.07 ± 0.08
total non-polar oxidation products*		1.33 ± 0.02	8.86 ± 0.34	1.46 ± 0.01	8.08 ± 0.07
polar oxidation products					
27 sitostanyl 7-hydroxyheptanoate**	1.006	nd[e]	1.97 ± 0.09	nd	1.43 ± 0.04
19 sitostanyl 8-hydroxyoctanoate***	1.043	nd	2.39 ± 0.10	nd	8.90 ± 0.21
28 sitostanyl 7-oxoheptanoate***	0.996	nd	2.72 ± 0.12	nd	0.84 ± 0.01
22 sitostanyl 8-oxooctanoate	1.049	nd	5.21 ± 0.18	nd	5.81 ± 0.30
25 sitostanyl 9-oxononanoate***	1.092	nd	6.37 ± 0.26	nd	11.18 ± 0.26
total polar oxidation products***		nd	18.65 ± 0.72	nd	28.15 ± 0.72
total oxidation products***		1.33 ± 0.02	27.51 ± 0.99	1.46 ± 0.01	36.24 ± 0.76
molar oxidation rate [%][f]		-	3.2 ± 0.07	-	4.3 ± 0.04

[a] Within the same row, statistically significant differences between ACOPs formed upon heating of oleates and linoleates were identified using unpaired Student's *t*-test. Values represent the mean ± standard deviation (n = 3). Levels of significance: $p < 0.001$, highly significant (***); $p < 0.01$, very significant (**); $p < 0.05$, significant (*).
[b] Numbering corresponds to Figure 32.
[c] Retention times on Rtx-200MS relative to the internal standards cholesteryl octanoate, cholesteryl 9-hydroxynonanoate and cholesteryl 9-oxononanoate.
[d] Phytostanols esterified to oleic/linoleic acid: 98% sitostanol and 2% campestanol.
[e] <LOD (0.09 µg alkanoates, 0.10 µg oxoalkanoates and 0.49 µg hydroxyalkanoates per mL injection solution); determined on the basis of 1 µL injection volume (GC-FID).
[f] Molar oxidation rate [%] = ((total oxidation products (40 min) – total oxidation products (0 min) [mol])/ fatty acid esters subjected to thermo-oxidation [mol]) × 100.

Table 22. ACOPs Formed upon Heating of Phytosteryl/-stanyl Esters at 180 °C [µg/mg Esters] [a].

oxidation products[b] [µg/mg]		RRT[c]	phytosteryl/-stanyl oleates[d]		phytosteryl/-stanyl linoleates[d]	
			0 min	40 min	0 min	40 min
non-polar oxidation products						
4	sitosteryl heptanoate***	1.020	0.42 ± 0.02	1.84 ± 0.08	0.27 ± 0.02	0.89 ± 0.05
5	sitostanyl heptanoate***	1.024	0.06 ± 0.00	0.46 ± 0.02	nd	0.21 ± 0.02
6	campesteryl octanoate*	1.027	nd[e]	0.20 ± 0.01	0.23 ± 0.01	0.54 ± 0.05
7	sitosteryl octanoate**	1.048	0.51 ± 0.02	2.45 ± 0.07	2.39 ± 0.16	6.27 ± 0.35
8	sitostanyl octanoate***	1.053	0.08 ± 0.01	0.59 ± 0.03	0.33 ± 0.01	1.36 ± 0.05
total non-polar oxidation products**			1.13 ± 0.04	5.54 ± 0.13	3.25 ± 0.20	9.27 ± 0.34
polar oxidation products						
16	sitosteryl 7-hydroxyheptanoate***	1.001	nd	0.85 ± 0.06	nd	0.44 ± 0.00
17 27	campesteryl 8-hydroxyoctanoate/ sitostanyl 7-hydroxyheptanoate**	1.006	nd	0.42 ± 0.04	nd	0.61 ± 0.02
18	sitosteryl 8-hydroxyoctanoate***	1.035	nd	0.95 ± 0.08	nd	3.97 ± 0.17
19	sitostanyl 8-hydroxyoctanoate**	1.043	nd	0.46 ± 0.02	nd	1.33 ± 0.11
20	sitosteryl 7-oxoheptanoate***	0.989	nd	1.06 ± 0.04	nd	0.65 ± 0.04
28	sitostanyl 7-oxoheptanoate	0.996	nd	nd	nd	nd
21	sitosteryl 8-oxooctanoate***	1.034	nd	1.32 ± 0.04	nd	3.54 ± 0.31
22	sitostanyl 8-oxooctanoate	1.049	nd	nd	nd	0.99 ± 0.05
23	campesteryl 9-oxononanoate	1.054	nd	nd	nd	1.09 ± 0.08
24	sitosteryl 9-oxononanoate***	1.083	nd	1.53 ± 0.04	nd	5.05 ± 0.49
25	sitostanyl 9-oxononanoate***	1.092	nd	0.80 ± 0.01	nd	1.15 ± 0.03
total polar oxidation products**			nd	7.39 ± 0.19	nd	18.80 ± 0.81
total oxidation products***			1.13 ± 0.04	12.92 ± 0.24	3.25 ± 0.20	28.07 ± 1.11
molar oxidation rate [%][f]			-	1.4 ± 0.02	-	3.0 ± 0.08

[a] Within the same row, statistically significant differences between ACOPs formed upon heating of oleates and linoleates were identified using unpaired Student's *t*-test. Values represent the mean ± standard deviation (n = 3). Levels of significance: $p < 0.001$, highly significant (***); $p < 0.01$, very significant (**); $p < 0.05$, significant (*).

[b] Numbering corresponds to Table 21 and Figure 32.
[c] Retention times on Rtx-200MS relative to the internal standards cholesteryl octanoate, cholesteryl 9-hydroxynonanoate and cholesteryl 9-oxononanoate.
[d] Phytosterols/-stanols esterified to linoleic/oleic acid: 75% sitosterol, 12% sitostanol, 10% campesterol, 2% campestanol and 1% others.
[e] <LOD (0.09 µg alkanoates, 0.10 µg oxoalkanoates and 0.49 µg hydroxyalkanoates per mL injection solution); determined on the basis of 1 µL injection volume (GC-FID).
[f] Molar oxidation rate [%] = ((total oxidation products (40 min) – total oxidation products (0 min) [mol])/ fatty acid esters subjected to thermo-oxidation [mol]) × 100.

As shown in Figure 33, the distributions of ACOPs were strongly impacted by the fatty acid moieties. The profiles are in agreement with the knowledge on the formation and degradation of hydroperoxides upon oxidation of unsaturated fatty acids. The autoxidation of oleates is known to result in four hydroperoxides in positions 8, 9, 10 and 11 (Frankel, 2005). Amongst the possible breakdown products of these hydroperoxides, ACOPs resulting from the degradation of 8- and 9-hydroperoxides were detected in the heat-treated phytostanyl oleates (Figure 33A). The concentrations of ACOPs resulting from the cleavage of the 9-hydroperoxide, i.e. sitostanyl octanoate, 8-hydroxyoctanoate, 8-oxooctanoate and sitostanyl 9-oxononanoate, were higher than those resulting from the cleavage of the respective 8-hydroperoxide, i.e. sitostanyl heptanoate, 7-hydroxyheptanoate and 7-oxoheptanoate. These differences are in agreement with the higher concentration of the 9-hydroperoxide compared to the 8-hydroperoxide detected upon thermal treatment of cholesteryl oleate (Lehtonen *et al.*, 2011b). The quantitatively dominating sitostanyl 9-oxononanoate may be formed by the degradation of both the 9- and the 10-hydroperoxide. The other longer-chain and unsaturated ACOPs potentially resulting from the cleavage of the 10- or the 11-hydroperoxide are not covered by the applied analytical methodology; they may not be amenable to GC-analysis.

Due to the presence of the two neighbouring double bonds in linoleates, the formation of a different spectrum of hydroperoxides and thus another profile of ACOPs in the course of autoxidation was to be expected. The pentadienyl radical initially generated in position 11 can be stabilized resulting in the preferred formation of two hydroperoxides in positions 9 and 13 in nearly equal amounts, each retaining a conjugated diene system. Accordingly, in heat-treated phytostanyl linoleates the concentrations of sitostanyl octanoate, 9-oxononanoate, 8-hydroxyoctanoate and 8-oxooctanoate resulting from the cleavage of the 9-hydroperoxide were higher than in the oleates (Figure 33A and B), and constitute the quantitatively predominating ACOPs. These findings are in line with a previous study reporting high amounts of the 9-hydroperoxide upon heating of cholesteryl linoleate (Lehtonen *et al.*, 2011b). Again, the longer-chain and unsaturated ACOPs expected from the degradation of the 13-hydroperoxide, also previously reported to be formed in high amounts from heated cholesteryl linoleate (Lehtonen *et al.*, 2011b), are not covered by the employed GC-based methodology. The other possible linoleate hydroperoxides in positions 8, 10, 12 and 14 are known to occur only in small amounts (Frankel, 2005). Amongst the possible breakdown products of these hydroperoxides, the short-chain ACOPs resulting from the 8-hydroperoxide, i.e. sitostanyl heptanoate, 7-hydroxyheptanoate and 7-oxoheptanoate, were detected. However, the concentrations of these breakdown products were lower than in the heat-treated oleates (Figure 29A and B). Differences

in the distributions of ACOPs similar to those between heat-treated phytostanyl oleates and linoleates were also observed between phytosteryl/-stanyl oleates and linoleates (Figure 33C and D).

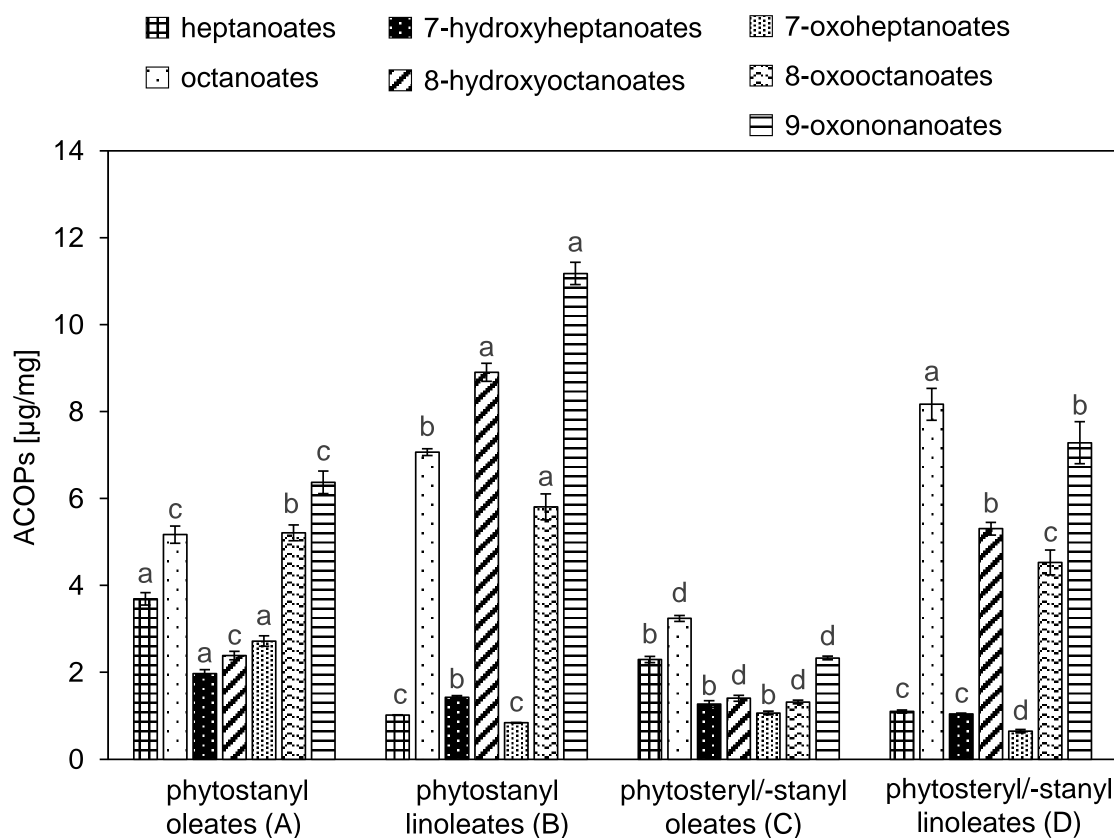

Figure 33. Distributions of acyl chain oxidation products in the phytosteryl/-stanyl fatty acid mixtures [µg/mg esters] upon thermo-oxidation (180 °C/ 40 min). The values represent the means ± standard deviations obtained from three heating experiments. For each type of ACOPs their formation was compared between the four ester mixtures (A-D). The data were statistically assessed via ANOVA; different letters above bars of the same types of ACOPs (identical fillings) indicate significant differences (Tukey-Kramer test) among the four ester mixtures ($p < 0.05$).

4.2.3.4. Quantitation of the Contributions of Oxidation Products to the Decreases of Phytosteryl/-stanyl Fatty Acid Esters

The analysis of non-oxidized fatty acid esters demonstrated that approximately 61% of the phytostanyl oleates, 54% of the phytostanyl linoleates, 40% of the phytosteryl/-stanyl oleates and 32% of the phytosteryl/-stanyl linoleates remained intact after the heating procedure (Table 23 and 24). The fact that the heat-induced losses of the linoleates were more pronounced than those of the respective oleates is in accordance with the existing knowledge on the susceptibility of unsaturated phytosteryl/-stanyl fatty acid esters to oxidations (Scholz *et al.*, 2016; Raczyk *et al.*, 2017a). These differences in ester losses were reflected in significantly higher amounts of

total ACOPs formed upon heating of phytostanyl or phytosteryl/-stanyl linoleates compared to the respective oleates (Tables 21 and 22). The findings are also in line with the previously reported higher formation rate of primary hydroperoxides in the fatty acid moiety upon heating of cholesteryl linoleate compared to cholesteryl oleate (Lehtonen *et al.*, 2011b).

Table 23. Amounts of Non-oxidized Fatty Acid Esters [µg/mg Esters] Remaining in the Phytostanyl Fatty Acid Ester Mixtures after Thermal Treatment (180 °C/ 40 min) and Percentage Proportions of Ester Losses.

phytostanyl fatty acid esters [µg/mg]	phytostanyl oleates[b]	phytostanyl linoleates[b]
campestanyl fatty acid ester**	15.2 ± 0.6[a]	12.1 ± 0.7
sitostanyl fatty acid ester***	598.4 ± 6.7	528.0 ± 11.6
total fatty acid esters***	613.6 ± 7.0	540.1 ± 11.0
losses of fatty acid esters [%]***	38.6 ± 0.7	46.0 ± 1.1

[a] Statistically significant differences between the means were identified using unpaired Student´s *t*-test. Values represent the mean ± standard deviation (n = 3). Levels of significance: $p < 0.001$, highly significant (***); $p < 0.01$, very significant (**); $p < 0.05$, significant (*).
[b] Phytostanols esterified to linoleic/oleic acid: 98% sitostanol and 2% campestanol.

Table 24. Amounts of Non-oxidized Fatty Acid Esters [µg/mg Esters] Remaining in the Phytosteryl/-stanyl Fatty Acid Ester Mixtures after Thermal Treatment (180 °C/ 40 min) and Percentage Proportions of Ester Losses.

phytosteryl/-stanyl fatty acid esters [µg/mg]	phytosteryl/-stanyl oleates[b]	phytosteryl/-stanyl linoleates[b]
campesteryl fatty acid ester***	36.0 ± 1.8[a]	22.7 ± 1.1
campestanyl fatty acid ester***	13.9 ± 0.3	9.0 ± 0.6
sitosteryl fatty acid ester*	282.2 ± 10.6	259.0 ± 8.4
sitostanyl fatty acid ester***	68.8 ± 3.4	43.8 ± 2.2
total fatty acid esters**	400.9 ± 13.4	319.0 ± 17.0
losses of fatty acid esters [%]**	60.0 ± 1.3	68.1 ± 1.7

[a] Statistically significant differences between the means were identified using unpaired Student´s *t*-test. Values represent the mean ± standard deviation (n = 3). Levels of significance: $p < 0.001$, highly significant (***); $p < 0.01$, very significant (**); $p < 0.05$, significant (*).
[b] Phytosterols/-stanols esterified to linoleic/oleic acid: 75% sitosterol, 12% sitostanol, 10% campesterol, 2% campestanol and 1% others.

In contrast, the presence of a double bond in the sterol moiety resulted in more pronounced heat-induced losses of both oleates and linoleates compared to the respective stanyl esters. However, the total amounts of ACOPs formed upon heating of steryl/stanyl oleates and linoleates were significantly lower than those formed from the stanyl esters (Tables 21 and 22). This indicated that oxidative reactions of the sterol moieties additionally contribute to the losses of these esters. Therefore, 7-hydroxy, 5,6-epoxy and 7-keto derivatives of the sitosterol and campesterol moieties were quantitated via on-line LC-GC after cleavage of the ester bonds

(Scholz *et al.*, 2015b). Figure 34 shows the amounts of these three classes of POPs formed upon thermo-oxidation of the phytosteryl/-stanyl fatty acid mixtures and the amounts of individual POPs are given in Table 25.

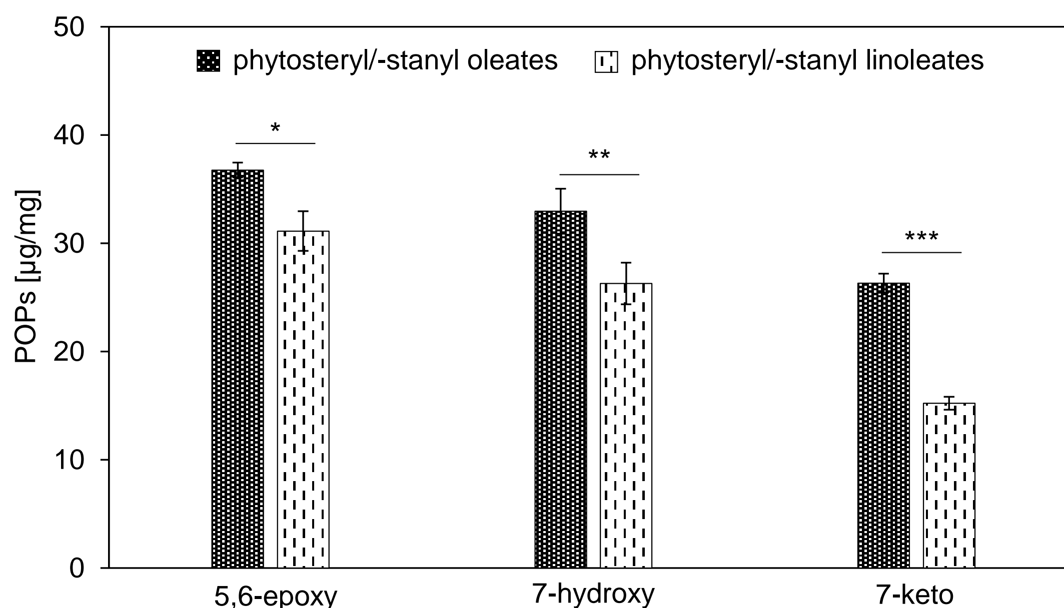

Figure 34. Distributions of phytosterol oxidation products (POPs) in the phytosteryl/-stanyl fatty acid mixtures [µg/mg esters] upon thermo-oxidation (180 °C/ 40 min). Values represent the means ± standard deviations obtained from three heating experiments. For each class of POPs their formation was compared between phytosteryl/-stanyl oleates and phytosteryl/-stanyl linoleates. Statistically significant differences were identified using unpaired Student's *t*-test. The number of asterisks indicates the levels of significance: $p < 0.001$, highly significant (***); $p < 0.01$, very significant (**); $p < 0.05$, significant (*).

Comparisons on a molar basis (Figure 35) revealed that the formation of ACOPs accounted for 8.7% and 9.6% of the thermally induced losses of phytostanyl oleates and linoleates, respectively. The observed contributions (2.7% and 5.2%) of ACOPs to the losses of phytosteryl/-stanyl oleates and phytosteryl linoleates, respectively, were significantly lower. However, much higher contributions of oxidation products in the sterol moieties (26.2% and 17.6%) were determined. These results correspond to a previous study in which the formation of POPs accounted for on average 20% of degraded phytosteryl esters upon various heating treatments of a phytosteryl ester-enriched margarine (Scholz *et al.*, 2016).

The observation that oxidation products in the sterol moieties were more abundant than oxidation products in the acyl chains in heat-treated steryl/stanyl esters is in agreement with previously reported data on hydroperoxides upon heating of cholesteryl oleate and linoleate. Higher concentrations of hydroperoxides in the sterol moieties, i.e. the direct precursors of

POPs, were reported than hydroperoxides in the acyl moieties, i.e. the direct precursors of ACOPs (Lehtonen *et al.*, 2011b).

Table 25. Phytosterol Oxidation Products (POPs) [µg/mg Esters] Formed upon Heating (180 °C/ 40 min) of Phytosteryl/-stanyl Fatty Acid Esters.

phytosterol oxidation products [µg/mg]	phytosteryl/-stanyl oleates[b]	phytosteryl/-stanyl linoleates[b]
5,6*β*-epoxycampesterol (*)	2.21 ± 0.08[a]	1.92 ± 0.14
5,6*β*-epoxysitosterol (*)	22.81 ± 1.55	19.09 ± 1.03
5,6*α*-epoxycampesterol	1.11 ± 0.04	1.01 ± 0.06
7*α*-hydroxycampesterol (**)	1.35 ± 0.10	1.00 ± 0.06
5,6*α*-epoxysitosterol	10.63 ± 0.95	9.10 ± 0.64
7*α*-hydroxysitosterol (*)	15.81 ± 1.35	11.62 ± 0.94
7*β*-hydroxycampesterol	1.35 ± 0.06	1.19 ± 0.08
7*β*-hydroxysitosterol	14.45 ± 0.88	12.48 ± 0.92
7-ketocampesterol (***)	2.25 ± 0.04	1.23 ± 0.03
7-ketositosterol (***)	24.07 ± 0.91	13.99 ± 0.57
total POPs (***)	96.04 ± 2.80	72.63 ± 3.16
molar oxidation rate[c] [%]	15.05 ± 0.51	11.36 ± 0.44

[a] Statistically significant differences between the means were identified using unpaired Student´s *t*-test. Values represent the mean ± standard deviation (n = 3). Levels of significance: $p < 0.001$, highly significant (***); $p < 0.01$, very significant (**); $p < 0.05$, significant (*).
[b] Phytosterols/-stanols esterified to linoleic/oleic acid: 75% sitosterol, 12% sitostanol, 10% campesterol, 2% campestanol and 1% others.
[c] Molar oxidation rate [%] = (total POPs [mol]/ fatty acid esters subjected to thermo-oxidation [mol]) × 100.

The amounts of ACOPs and POPs quantitated in the present study accounted in total for approximately 29% and 23% of degraded phytosteryl/-stanyl oleates and linoleates, respectively. It remains unclear to which extent oxidation products oxidized both in the sterol and in the fatty acid moieties were formed in the course of the thermo-oxidation. A direct analysis of this type of oxidation products was not achieved. The oxidized sterol moieties are comprised by the POPs determined after the transesterification step; however, oxidations in the fatty acid moieties of steryl/stanyl esters might be underestimated. So far, only one study reported an oxidation product of sitosteryl oleate, oxidized in both the sterol and in the fatty acid moiety upon heating of a spread enriched with phytosteryl esters (Julien-David *et al.*, 2014). Only minor concentrations were determined for 7-ketositosteryl 9,10-dihydroxysteate compared to 7-ketositosteryl oleate. The contributions of a potential heat-catalyzed hydrolysis to the losses of linoleates and oleates were considered to be little (<1.5 %), as determined via semi-quantitation of sitosterol found in the polar SPE fractions. However, further ester losses might be caused by polymerization reactions as indicated previously (Lehtonen *et al.*, 2012a). Under the heating conditions in this study, high polymerization rates might be expected, as the

polymerization of lipids was generally reported to occur at frying temperatures (150-190 °C) (Blekas and Boskou, 1999).

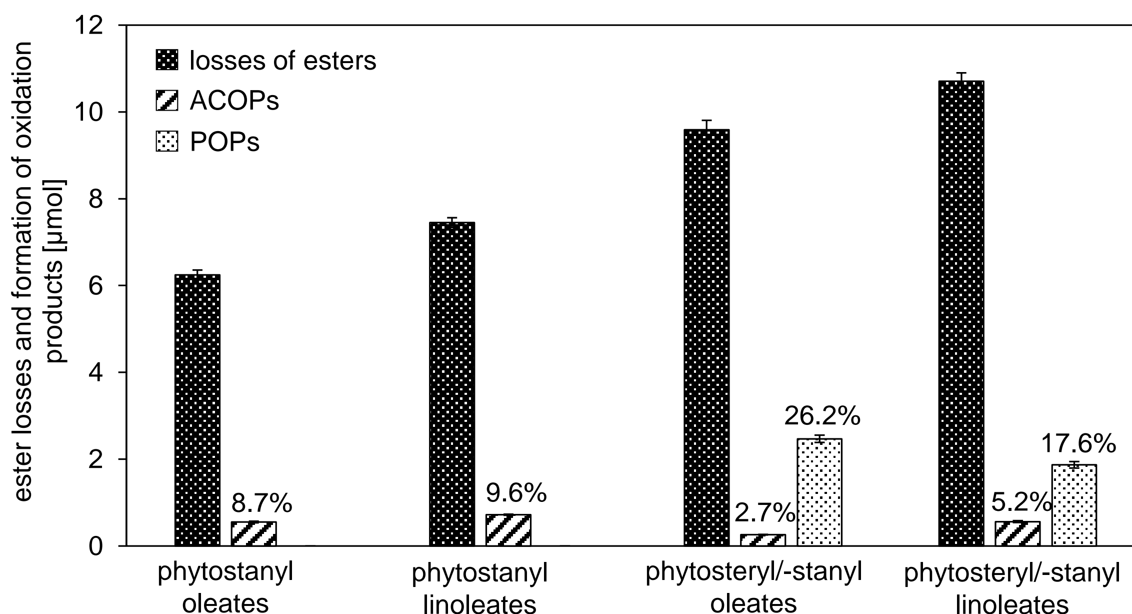

Figure 35. Comparisons of ester losses and formations of oxidation products in the sterol (POPs) and in the fatty acid moieties (ACOPs) upon thermal treatment (180 °C/ 40 min) of 11 mg (≙ 16 µmol) of each ester mixture, on a molar basis. POPs were only determined for phytosteryl/-stanyl esters; the values represent the means ± standard deviations from three heating experiments. Percentages above the POP and ACOP bars represent the contributions of these oxidation products to the respective ester losses; they were calculated as follows: (i) POPs [µmol] × 100 / losses of esters [µmol] and (ii) ACOPs [µmol] × 100 / losses of esters [µmol].

Compared with the previously reported very low contribution (< 1%) of oxidation products in the phytostanol moieties to the decreases of phytostanyl fatty acid esters in heated margarine (Scholz *et al.*, 2016), the present quantitative data demonstrate for the first time a substantial contribution of ACOPs to the decreases of these esters upon thermal treatment. The lower amounts of ACOPs observed upon heating of unsaturated esters of phytosterols and the higher proportions of POPs indicate preferred contributions of oxidation reactions of the phytosterol moieties to the decreases of these esters under the applied heating conditions.

The employed analytical approach does not cover the whole range of possible ACOPs. Owing to the GC-based approach, several types of potentially formed compounds are not amenable to the analysis. This might include unsaturated and longer-chain scission products, e.g. from the 11-hydroperoxides of oleates or the 13-hydroperoxides of linoleates. In addition, compounds covered using LC-MS, such as long-chain ACOPs bearing hydroxy or epoxy groups as well as sitosteryl fatty acid esters oxidized in both the acyl chain and the sterol part (Julien-David *et al.*, 2014), cannot be analyzed using the approach selected in the present study. Furthermore,

the selected time/temperature combination of 40 min/180 °C, of course, can only result in a snapshot, both qualitatively and quantitatively. Therefore, further studies under various conditions will be required to provide a more complete picture of the oxidation products. Nevertheless, the elaborated data enable first quantitative insights into different oxidation pathways of unsaturated phytosteryl/-stanyl esters and particularly into the formation of so far neglected classes of oxidation products.

4.2.4. Summary

Based on the methodologies used for the identification of ACOPs, a GC-based approach that involved the preseparation of polar ACOPs via solid phase extraction, was established for the quantitation of acyl chain oxidation products (ACOPs) formed upon thermo-oxidation of phytosteryl/-stanyl fatty acid esters. For the quantitation of ACOPs, structurally related cholesteryl fatty acid esters, showing oxidized acyl moieties, were synthesized and used as internal standards. The novel method was used for the quantitation of ACOPs formed upon thermo-oxidation in four phytosteryl/-stanyl fatty acid mixtures that account for large proportions in ester mixtures commonly used for the enrichment of foods: (i) phytosteryl/-stanyl oleates, (ii) phytosteryl/-stanyl linoleates, (iii) phytostanyl oleates and (iv) phytostanyl linoleates. The remaining non-oxidized phytosteryl/-stanyl fatty acid esters were analyzed via GC-FID to determine the contributions of ACOPs to the decreases of phytosteryl/-stanyl fatty acid esters. In addition, phytosterol oxidation products were analyzed in the heated phytosteryl fatty acid esters via on-line LC-GC to compare their occurrence with that of ACOPs.

The concentrations of ACOPs resulting from initially formed 9-hydroperoxides (octanoates, 8-hydroxyoctanoates, 9-oxononanoates) were higher than those from 8-hydroperoxides (heptanoates, 7-hydroxy- and 7-oxoheptanoates, 8-oxooctanoates) in both oleates and linoleates. Significantly higher amounts of ACOPs were found in heat-treated linoleates compared to oleates. However, despite lower thermally induced losses of phytostanyl oleates and linoleates compared to the respective phytosteryl/-stanyl esters, higher concentrations of ACOPs (approx. 9% and 10% of the ester losses, respectively) were observed in the heat-treated phytostanyl esters. In contrast, in the heated phytosteryl/-stanyl oleates and linoleates the contribution of ACOPs to the ester losses was lower (approx. 3% and 5%, respectively), and there was a more pronounced formation of oxidation products of the sterol moieties (approx. 26% and 18% of the ester losses, respectively).

5. SUMMARY

Cooking and baking applications with foods that contain phytosterols/-stanols and the respective fatty acid esters may lead to the formation of potentially harmful phytosterol oxidation products (POPs), particularly in foods that were enriched with phytosteryl/-stanyl fatty acid esters. Therefore, data on POP contents in phytosterol/-stanol containing foods upon cooking and baking applications and the identification of parameters that influence the formation of POPs are required.

A commercially available liquid margarine was enriched with a mixture of phytosteryl/-stanyl fatty acid esters, and both the non-enriched and enriched liquid margarine were subjected to various heat treatments that may represent typical applications in the home. The contents of phytosterol oxidation products were determined after cleavage of the ester bonds via a previously published method based on on-line LC-GC. POP contents determined in the non-enriched and enriched liquid margarine, respectively, depending on the heat treatment were in the following ascending order: pan-frying < vial (oven) < casserole (oven). Higher POP contents were found in the enriched liquid margarine compared to the non-enriched liquid margarine upon the same heat treatment owing to the higher availability of phytosterols/-stanols for oxidation reactions. However, the proportion of initially present phytosterols/-stanols that were oxidized in the course of pan-frying and vial oven-heating was higher in the non-enriched compared to the enriched liquid margarine. Only upon casserole oven-heating comparable oxidation rates were determined in the non-enriched and the enriched liquid margarine. This indicated that the access of phytosterols to air oxygen, reflected in a low mass-to-surface ratio, was less restricted in the course of casserole oven-heating compared to the other heat treatments. Moreover, a reduced amount of enriched liquid margarine subjected to pan-frying and vial oven-heating was shown to result in a lower mass-to-surface ratio and thus, higher POP contents.

To investigate the formation of POPs in the course of a baking application, the non-enriched and enriched liquid margarine, respectively, was used as an ingredient for the preparation of a dough that was used for muffin baking. Heating of the liquid margarines in a muffin matrix was demonstrated to be a low-oxidizing process, as reflected in negligible formations of POPs and unaltered phytosterol/-stanol contents after baking. In addition, neither the increase of the dough surface nor the reduction of the amount of dough, as assessed via cake and cookie baking, respectively, had a significant influence on the formation of POPs and degradation of phytosterols/-stanols. By doubling the baking time, a burnt cookie was produced that served as

a model to study the influence of the heating time on the formation of POPs. Very high POP levels and a significant reduction of phytosterols/-stanols in the burnt cookie suggested the temperature of the dough to be the crucial factor for the oxidation of phytosterols/-stanols in the course of baking.

The formation of phytosterol oxidation products did not account for all degraded phytosteryl fatty acid esters upon thermo-oxidations in previous studies. Therefore, the identification and quantitation of novel phytosteryl/-stanyl fatty acid esters that are oxidized in the fatty moieties, was targeted upon thermo-oxidation of phytosteryl/-stanyl fatty acid ester mixtures in the second part of this thesis.

The employed methodology, i.e. a pre-concentration of polar oxidation products via SPE, the establishment of capillary gas chromatographic conditions enabling the separation of various oxidation products, and the synthesis of authentic reference compounds provided data on the formation and the identities of previously unreported acyl chain oxidation products resulting from the thermal treatment of phytosteryl/-stanyl linoleates. In total, 15 sitosteryl, sitostanyl and campesteryl esters, resulting from oxidation of the acyl chain, could be identified by GC-FID/MS. Among the identified ACOPs were (i) nonpolar phytosteryl/-stanyl heptanoates and octanoates, and (ii) polar phytosteryl/-stanyl 7-hydroxyheptanoates, 8-hydroxyoctanoates, 7-oxoheptanoates, 8-oxooctanoates and 9-oxononanoates. The fact that the approach was not based on the formation of methylesters via transesterification enabled for the first time the analysis of a spectrum of individual phytosteryl/-stanyl fatty acid esters oxidized in the acyl moiety.

Based on the methodologies used for the identification of ACOPs, a quantitative approach was established for the quantitation of acyl chain oxidation products formed upon thermo-oxidation in four phytosteryl/-stanyl fatty acid ester mixtures: (i) phytosteryl/-stanyl oleates, (ii) phytosteryl/-stanyl linoleates, (iii) phytostanyl oleates and (iv) phytostanyl linoleates. For the quantitation of ACOPs, structurally related cholesteryl fatty acid esters were synthesized and used as internal standards. The remaining non-oxidized phytosteryl/-stanyl fatty acid esters were analyzed via GC-FID to determine the contributions of ACOPs to the decreases of phytosteryl/-stanyl fatty acid esters. In addition, phytosterol oxidation products were analyzed in the heated phytosteryl fatty acid esters via on-line LC-GC to compare their occurrence with that of ACOPs. The concentrations of ACOPs resulting from initially formed 9-hydroperoxides (octanoates, 8-hydroxyoctanoates, 9-oxononanoates) were higher than those from 8-hydroperoxides (heptanoates, 7-hydroxy- and 7-oxoheptanoates, 8-oxooctanoates) in both

oleates and linoleates. Significantly higher amounts of ACOPs were found in heat-treated linoleates compared to oleates. However, despite lower thermally induced losses of phytostanyl oleates and linoleates compared to the respective phytosteryl/-stanyl esters, higher concentrations of ACOPs (approx. 9% and 10% of the ester losses, respectively) were observed in the heat-treated phytostanyl esters. In contrast, in the heated phytosteryl/-stanyl oleates and linoleates the contribution of ACOPs to the ester losses was lower (approx. 3% and 5%, respectively), and there was a more pronounced formation of oxidation products of the sterol moieties (approx. 26% and 18% of the ester losses, respectively).

6. ZUSAMMENFASSUNG

Koch- und Backanwendungen mit Lebensmitteln, die Phytosterole/-stanole und die entsprechenden Fettsäureester enthalten, können zur Bildung von möglicherweise gesundheitsschädlichen Phytosterol-Oxidationsprodukten führen. Dies gilt insbesondere für Lebensmittel, die mit Phytosteryl-/Phytostanylfettsäureestern aufgrund ihrer Cholesterol senkenden Eigenschaften angereichert wurden. Daher besteht die Notwendigkeit der Erhebung von POP-Gehalten in phytosterol/-stanolhaltigen Lebensmitteln nach Koch- und Backanwendungen, sowie der Identifizierung von Parametern, welche die Bildung von POPs während dieser Prozesse beeinflussen.

Eine kommerziell erhältliche Pflanzenölcreme wurde mit einer Mischung aus Phytosteryl-/Phytostanylfettsäureestern angereichert, und sowohl die nicht angereicherte als auch die angereicherte Pflanzenölcreme wurden Hitzebehandlungen unterzogen, die typischen Koch- und Backanwendungen im Haushalt entsprachen. Die resultierenden Gehalte an Phytosterol-Oxidationsprodukten wurden mittels einer etablierten Methode basierend auf on-line LC-GC nach vorheriger Spaltung der Esterbindungen bestimmt. Die Gehalte an Phytosterol-Oxidationsprodukten in der nicht angereicherten bzw. in der angereicherten Pflanzenölcreme in Abhängigkeit der Hitzebehandlung waren in folgender aufsteigender Reihenfolge: Pfanne (Herd) < Vial (Ofen) < Auflaufform (Ofen). Nach derselben Hitzebehandlung wurden in der nicht angereicherten Pflanzenölcreme niedrigere Gehalte an Phytosterol-Oxidationsprodukten als in der angereicherten Pflanzenölcreme bestimmt, aufgrund der erhöhten Verfügbarkeit an Phytosterolen/-stanolen in der angereicherten Creme. Jedoch war der Anteil an ursprünglich vorhandenden Phytosterolen/-stanolen, die während der Erhitzung in der Pfanne (Herd) und im Vial (Ofen) oxidiert wurden, in der nicht angereicherten Creme größer als in der angereicherten Creme. Nur nach Erhitzung in der Auflaufform (Ofen) wurden vergleichbare Oxidationsraten in der nicht angereicherten und angereicherten Creme bestimmt. Dies deutete darauf hin, dass im Verlauf der Erhitzung in der Auflaufform (Ofen) der Zugang der Phytosterole zu Luftsauerstoff im Vergleich zu den anderen Hitzebehandlungen weniger stark eingeschränkt war, was sich anhand eines geringen Masse-zu-Oberflächen Verhältnisses zeigen ließ. Ferner wurde gezeigt, dass eine verringerte Menge an angereicherter Creme, die zur Erhitzung in der Pfanne (Herd) oder im Vial (Ofen) eingesetzt wurde, zu einem geringeren Masse-zu-Oberflächen Verhältnis und folglich zu höheren POP-Gehalten führte.

Um die Bildung von Phytosterol-Oxidationsprodukten im Verlauf einer Backanwendung zu untersuchen, wurde die nicht angereicherte bzw. angereicherte Creme als Bestandteil zur

Herstellung eines Teiges verwendet, welcher zum Backen eines Muffins eingesetzt wurde. Die Erhitzung der Pflanzenölcremes in einer Teig-Matrix hatte nur einen geringfügig oxidationsfördernden Effekt, was sich anhand einer nur geringen Bildung von POPs und eines unveränderten Gehaltes an Phytosterolen/-stanolen nach dem Backen zeigte. Ferner hatten weder die Vergrößerung der Teigoberfläche noch die Reduzierung der Teigmenge einen bedeutenden Einfluss auf die Bildung von POPs bzw. den Abbau von Phytosterolen/-stanolen, was anhand des Backens eines Kuchens und eines Kekses untersucht wurde. Nach Verdoppelung der Backzeit wurde ein verbrannter Keks hergestellt, der als Modell diente, um den Einfluss der Backzeit auf die Bildung von POPs zu untersuchen. Sehr hohe Mengen an POPs und eine signifikante Reduktion von Phytosterolen/-stanolen im verbrannten Keks, wiesen darauf hin, dass die Teigtemperatur den entscheidenden Faktor darstellt, welcher die Oxidation von Phytosterolen/-stanolen im Verlauf von Backprozessen bestimmt.

In bisherigen Studien konnte der thermisch induzierte Abbau von Phytosteryl-/Phytostanylfettsäureestern nur teilweise mit der Bildung von Phytosterol-Oxidationsprodukten erklärt werden. Daher sollten im zweiten Teil dieser Arbeit bisher unbekannte Phytosteryl-/Phytostanylfettsäureester mit oxidiertem Fettsäurerest in thermisch behandelten Mischungen von Phytosteryl-/Phytostanylfettsäureestern identifiert und quantifiziert werden.

Die angewandte Methodik, d.h. die Aufkonzentrierung von polaren Oxidationsprodukten mittels SPE, die Etablierung geeigneter gaschromatographischer Bedingungen und die Synthese von Referenzsubstanzen, ermöglichte die eindeutige Identifizierung und Charakterisierung bisher unbekannter Oxidationsprodukte am Fettsäurerest, welche durch thermische Oxidation von Phytosteryl-/Phytostanyllinoleaten gebildet wurden. Insgesamt konnten 15 Sitosteryl-, Sitostanyl- und Campesterylester mit oxidiertem Fettsäurerest mittels GC-FID/MS identifiziert werden. Darunter waren (i) unpolare Phytosteryl-/Phytostanylheptanoate und –oktanoate, (ii) polare Phytosteryl-/Phytostanyl-7-hydroxyheptanoate, -8-hydroxyoktanoate, -7-oxoheptanoate, -8-oxooktanoate und -9-oxononanoate. Das analytische Vorgehen ohne vorherige Umesterung der Fettsäuren zu Methylestern ermöglichte erstmalig die Analytik eines Spektrums an einzelnen Phytosteryl-/Phytostanylfettsäureestern mit oxidiertem Fettsäurerest.

Basierend auf dem analytischen Vorgehen zur Identifizierung von ACOPs, wurde eine Methode zur quantitativen Erfassung von ACOPs in vier thermisch behandelten Phytosteryl-/Phytostanylfettsäureestern Mischungen etabliert:

(i) Phytosteryl-/Phytostanyloleate, (ii) Phytosteryl-/Phytostanyllinoleate, (iii) Phytostanyloleate und (iv) Phytostanyllinoleate. Für die Quantifizierung von ACOPs wurden strukturell verwandte Cholesterylfettsäureester zur Verwendung als interne Standards synthetisiert. Die nach Erhitzung noch intakten Phytosteryl-/Phytostanylfettsäureester wurden mittels GC-FID bestimmt, um auf den Beitrag der ACOPs zum Abbau der Phytosteryl-/Phytostanylfettsäureester schließen zu können. Außerdem wurden die POPs nach Spaltung der Esterbindungen mittels on-line LC-GC analysiert, um einen quantitativen Vergleich zwischen ACOPs und POPs zu ermöglichen. Die Gehalte an ACOPs, die über das intermediär gebildete 9-Hydroperoxid (Oktanoate, 8-Hydroxyoktanoate, 9-Oxononanoate) entstanden sind, waren sowohl in den Oleaten als auch in den Linoleaten höher als jene, die über das 8-Hydroperoxid (Heptanoate, 7-Hydroxyheptanoate, 7-Oxoheptanoate, 8-Oxooktanoate) gebildet wurden. In den erhitzten Linoleaten wurden signifikant höhere Mengen an ACOPs gefunden als in den Oleaten. Jedoch wurden trotz eines geringeren thermischen Abbaus der Phytostanyloleate und –linoleate verglichen mit den jeweiligen Phytosteryl-/Phytostanylfettsäureestern, höhere Konzentrationen an ACOPs in den erhitzen Phytostanylfettsäurestern (ca. 9% bzw. 10% der abgebauten Ester) gefunden. Im Gegensatz dazu war der Beitrag an ACOPs in Phytosteryl-/Phytostanyloleaten und –linoleaten zum thermischen Abbau der Ester geringer (ca. 3% bzw. 5%), jedoch war ein deutlich höherer Beitrag zum Abbau der Ester auf die Bildung von POPs (ca. 26% bzw. 18%) zurückzuführen.

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8. APPENDIX

Table 26. Individual phytosterol oxidation products determined in the non-enriched and enriched liquid margarine before and after pan-frying treatments.

phytosterol oxidation product [mg/kg]	non-enriched (1 g)		
	no treatment	day 1	day 2
5,6*β*-epoxycampesterol	nd[a]	2.7 ± 0.1[b]	2.7 ± 0.1
5,6*β*-epoxysitosterol	nd	4.7 ± 0.3	5.3 ± 0.1
5,6*α*-epoxycampesterol	nd	1.8 ± 0.2	1.5 ± 0.1
7*α*-hydroxycampesterol	nd	2.9 ± 0.3	2.8 ± 0.2
5,6*α*-epoxysitosterol	nd	2.6 ± 0.2	3.5 ± 0.2
7*α*-hydroxysitosterol	nd	3.2 ± 0.2	4.6 ± 0.2
7*β*-hydroxycampesterol	nd	3.1 ± 0.2	2.6 ± 0.1
7*β*-hydroxysitosterol	nd	2.1 ± 0.1	2.9 ± 0.3
7-ketocampesterol	3.0 ± 0.1	3.3 ± 0.3	3.3 ± 0.1
7-ketositosterol	8.2 ± 0.6	8.1 ± 0.7	7.8 ± 0.8
total	11.1 ± 0.6	34.6 ± 1.6	37.1 ± 1.0

phytosterol oxidation product [mg/kg]	enriched (1 g)		
	no treatment	day 1	day 2
5,6*β*-epoxycampesterol	4.4 ± 0.3	9.2 ± 0.6	10.6 ± 0.3
5,6*β*-epoxysitosterol	14.1 ± 1.0	37.5 ± 1.1	46.5 ± 0.7
5,6*α*-epoxycampesterol	5.9 ± 0.5	7.7 ± 0.1	8.9 ± 1.2
7*α*-hydroxycampesterol	5.1 ± 0.4	10.4 ± 0.5	9.9 ± 0.2
5,6*α*-epoxysitosterol	22.0 ± 1.7	28.8 ± 2.2	30.0 ± 1.1
7*α*-hydroxysitosterol	7.2 ± 0.7	21.4 ± 2.0	17.0 ± 1.1
7*β*-hydroxycampesterol	nd	8.4 ± 0.8	7.3 ± 0.2
7*β*-hydroxysitosterol	nd	14.8 ± 1.0	13.9 ± 0.6
7-ketocampesterol	14.1 ± 0.4	24.3 ± 1.2	23.8 ±1.3
7-ketositosterol	27.0 ± 1.6	82.6 ± 4.0	86.8 ± 5.2
total	99.9 ± 3.0	244.9 ± 3.8	254.7 ± 6.7

phytosterol oxidation product [mg/kg]	enriched (3 g)		
	no treatment	day 1	day 2
5,6*β*-epoxycampesterol	1.3 ± 0.0	5.7 ± 0.3	5.5 ± 0.3
5,6*β*-epoxysitosterol	5.3 ± 0.5	25.5 ± 1.8	19.4 ± 1.0
5,6*α*-epoxycampesterol	1.8 ± 0.2	3.8 ± 0.2	4.1 ± 0.2
7*α*-hydroxycampesterol	2.1 ± 0.2	6.9 ± 0.4	8.0 ± 0.2
5,6*α*-epoxysitosterol	9.7 ± 0.9	18.8 ± 0.7	15.4 ± 0.9
7*α*-hydroxysitosterol	4.3 ± 0.3	13.8 ± 1.3	14.7 ± 0.5
7*β*-hydroxycampesterol	1.9 ± 0.2	5.4 ± 0.3	7.7 ± 0.2
7*β*-hydroxysitosterol	1.4 ± 0.1	9.1 ± 0.5	10.1 ± 0.4
7-ketocampesterol	11.0 ± 0.9	15.6 ± 1.2	17.2 ± 7.1
7-ketositosterol	11.8 ± 2.4	55.9 ± 1.7	48.5 ± 8.2
total	50.7 ± 4.2	160.6 ± 4.5	150.5 ± 8.6

[a] <LOD (0.16 µg 5,6-epoxysterol/g liquid margarine; 0.22 µg 7-hydroxysterol/g liquid margarine; 0.22 µg 7-ketosterol/g liquid margarine).
[b] Values represent the mean ± standard deviation (n=3).

Table 27. Individual phytosterol oxidation products determined in the non-enriched and enriched liquid margarine before and after vial oven-heating.

phytosterol oxidation product [mg/kg]	non-enriched (1 g)			
	day 1		day 2	
	no treatment	after heating	no treatment	after heating
5,6*β*-epoxycampesterol	nd[a]	4.4 ± 0.2[b]	nd	5.5 ± 0.2
5,6*β*-epoxysitosterol	nd	8.6 ± 0.3	nd	9.5 ± 0.0
5,6*α*-epoxycampesterol	nd	2.7 ± 0.2	nd	2.5 ± 0.1
7*α*-hydroxycampesterol	nd	3.7 ± 0.2	nd	4.0 ± 0.2
5,6*α*-epoxysitosterol	nd	6.2 ± 0.4	nd	6.2 ± 0.4
7*α*-hydroxysitosterol	nd	6.1 ± 0.6	nd	6.9 ± 0.4
7*β*-hydroxycampesterol	nd	4.3 ± 0.5	nd	5.3 ± 0.3
7*β*-hydroxysitosterol	nd	4.6 ± 0.3	nd	5.7 ± 0.3
7-ketocampesterol	4.3 ± 0.2	9.3 ± 0.5	3.0 ± 0.1	5.0 ± 0.2
7-ketositosterol	9.7 ± 0.6	11.7 ± 0.9	8.2 ± 0.6	11.9 ± 1.0
total	14.0 ± 0.6	61.7 ± 1.0	11.1 ± 0.6	62.5 ± 1.2

phytosterol oxidation product [mg/kg]	enriched (0.5 g)			
	day 1		day 2	
	no treatment	after heating	no treatment	after heating
5,6*β*-epoxycampesterol	4.4 ± 0.3	64.9 ± 1.8	1.3 ± 0.0	62.4 ± 2.7
5,6*β*-epoxysitosterol	14.1 ± 1.0	252.4 ± 10.3	5.8 ± 0.5	238.2 ± 5.2
5,6*α*-epoxycampesterol	5.9 ± 0.5	32.2 ± 1.4	1.8 ± 0.2	30.2 ± 1.1
7*α*-hydroxycampesterol	5.1 ± 0.4	48.5 ± 1.4	2.1 ± 0.2	55.3 ± 5.0
5,6*α*-epoxysitosterol	22.0 ± 1.7	137.6 ± 9.8	9.7 ± 0.9	121.1 ± 2.9
7*α*-hydroxysitosterol	7.2 ± 0.7	137.2 ± 5.2	4.3 ± 0.3	157.5 ± 9.0
7*β*-hydroxycampesterol	nd	56.5 ± 0.7	1.9 ± 0.2	63.1 ± 1.6
7*β*-hydroxysitosterol	nd	135.1 ± 9.5	1.4 ± 0.1	152.4 ± 3.2
7-ketocampesterol	14.1 ± 0.4	64.2 ± 1.0	11.0 ± 0.9	64.1 ± 2.5
7-ketositosterol	27.0 ± 1.6	252.7 ± 5.7	11.8 ± 2.4	222.9 ± 1.7
total	99.9 ± 3.0	1181.3 ± 38.6	50.7 ± 4.2	1167.3 ± 24.8

phytosterol oxidation product [mg/kg]	enriched (1 g)			
	day 1		day 2	
	no treatment	after heating	no treatment	after heating
5,6*β*-epoxycampesterol	4.4 ± 0.3	40.1 ± 1.3	1.3 ± 0.0	36.4 ± 1.2
5,6*β*-epoxysitosterol	14.1 ± 1.0	154.0 ± 2.5	5.8 ± 0.5	129.9 ± 2.6
5,6*α*-epoxycampesterol	5.9 ± 0.5	21.9 ± 1.1	1.8 ± 0.2	17.8 ± 0.7
7*α*-hydroxycampesterol	5.1 ± 0.4	28.6 ± 2.3	2.1 ± 0.2	30.9 ± 0.5
5,6*α*-epoxysitosterol	22.0 ± 1.7	83.3 ± 1.8	9.7 ± 0.9	67.7 ± 0.7
7*α*-hydroxysitosterol	7.2 ± 0.7	77.5 ± 2.3	4.3 ± 0.3	85.1 ± 2.6
7*β*-hydroxycampesterol	nd	24.0 ± 1.2	1.9 ± 0.2	36.0 ± 0.8
7*β*-hydroxysitosterol	nd	71.9 ± 2.3	1.4 ± 0.1	79.3 ± 1.9
7-ketocampesterol	14.1 ± 0.4	40.5 ± 2.7	11.0 ± 0.9	50.0 ± 4.8
7-ketositosterol	27.0 ± 1.6	158.0 ± 4.6	11.8 ± 2.4	159.7 ± 5.1
total	99.9 ± 3.0	699.9 ± 3.5	50.7 ± 4.2	692.9 ± 0.9

[a] <LOD (0.16 µg 5,6-epoxysterol/g liquid margarine; 0.22 µg 7-hydroxysterol/g liquid margarine; 0.22 µg 7-ketosterol/g liquid margarine).
[b] Values represent the mean ± standard deviation (n=3).

Table 28. Individual phytosterol oxidation products determined in the non-enriched and enriched liquid margarine before and after casserole oven-heating.

phytosterol oxidation product [mg/kg]	non-enriched			
	day 1		day 2	
	no treatment	after heating	no treatment	after heating
5,6*β*-epoxycampesterol	nd[a]	29.5 ± 1.6[b]	nd	33.2 ± 1.3
5,6*β*-epoxysitosterol	nd	54.4 ± 2.1	nd	53.8 ± 1.0
5,6*α*-epoxycampesterol	nd	18.8 ± 1.7	nd	16.8 ± 0.6
7*α*-hydroxycampesterol	nd	32.5 ± 6.0	nd	26.3 ± 2.2
5,6*α*-epoxysitosterol	nd	29.8 ± 1.5	nd	29.2 ± 2.1
7*α*-hydroxysitosterol	nd	36.9 ± 3.0	nd	34.2 ± 2.2
7*β*-hydroxycampesterol	nd	13.7 ± 1.5	nd	22.2 ± 1.6
7*β*-hydroxysitosterol	nd	30.9 ± 2.7	nd	25.9 ± 0.8
7-ketocampesterol	4.3 ± 0.2	29.3 ± 0.5	3.0 ± 0.1	30.8 ± 0.6
7-ketositosterol	9.7 ± 0.6	73.0 ± 3.9	8.2 ± 0.6	60.5 ± 0.6
total	14.0 ± 0.6	348.9 ± 9.9	11.1 ± 0.6	332.9 ± 3.4

phytosterol oxidation product [mg/kg]	enriched			
	day 1		day 2	
	no treatment	after heating	no treatment	after heating
5,6*β*-epoxycampesterol	4.4 ± 0.3	336.9 ± 20.8	4.4 ± 0.3	361.6 ± 13.4
5,6*β*-epoxysitosterol	14.1 ± 1.0	1263.3 ± 36.6	14.1 ± 1.0	1342.5 ± 47.4
5,6*α*-epoxycampesterol	5.9 ± 0.5	152.5 ± 3.1	5.9 ± 0.5	166.7 ± 17.3
7*α*-hydroxycampesterol	5.1 ± 0.4	203.9 ± 19.9	5.1 ± 0.4	256.4 ± 3.3
5,6*α*-epoxysitosterol	22.0 ± 1.7	575.4 ± 23.9	22.0 ± 1.7	600.0 ± 5.4
7*α*-hydroxysitosterol	7.2 ± 0.7	563.7 ± 34.3	7.2 ± 0.7	625.0 ± 121.9
7*β*-hydroxycampesterol	nd	173.7 ± 9.3	nd	142.5 ± 12.6
7*β*-hydroxysitosterol	nd	543.0 ± 39.7	nd	621.9 ± 37.5
7-ketocampesterol	14.1 ± 0.4	332.7 ± 3.3	14.1 ± 0.4	333.8 ± 25.3
7-ketositosterol	27.0 ± 1.6	1270.1 ± 5.4	27.0 ± 1.6	1326.8 ± 69.6
total	99.9 ± 3.0	5415.2 ± 165.0	99.9 ± 3.0	5777.2 ± 158.5

[a] <LOD (0.16 µg 5,6-epoxysterol/g liquid margarine; 0.22 µg 7-hydroxysterol/g liquid margarine; 0.22 µg 7-ketosterol/g liquid margarine).
[b] Values represent the mean ± standard deviation (n=3).

Table 29. Individual phytosterol oxidation products determined in the non-enriched and enriched liquid margarine before and after muffin baking.

phytosterol oxidation product [mg/kg]	non-enriched					
	day 1		day 2		day 3	
	before heating	after heating	before heating	after heating	before heating	after heating
5,6*β*-epoxycampesterol	nd[a]	1.6 ± 0.1[b]	nd	1.4 ± 0.1	nd	1.5 ± 0.1
5,6*β*-epoxysitosterol	nd	6.6 ± 0.6	nd	6.0 ± 0.2	nd	6.2 ± 0.2
5,6*α*-epoxycampesterol	nd	1.3 ± 0.1	nd	0.9 ± 0.1	nd	1.3 ± 0.1
7*α*-hydroxycampesterol	nd	1.6 ± 0.1	nd	1.9 ± 0.0	nd	1.7 ± 0.0
5,6*α*-epoxysitosterol	nd	2.7 ± 0.0	nd	2.4 ± 0.2	nd	3.4 ± 0.1
7*α*-hydroxysitosterol	nd	3.6 ± 0.3	nd	3.8 ± 0.2	nd	3.1 ± 0.0
7*β*-hydroxycampesterol	nd	1.5 ± 0.1	nd	1.4 ± 0.1	nd	1.7 ± 0.1
7*β*-hydroxysitosterol	nd	3.9 ± 0.3	nd	3.2 ± 0.3	nd	3.1 ± 0.2
7-ketocampesterol	2.3 ± 0.0	3.2 ± 0.2	2.8 ± 0.0	3.2 ± 0.3	3.0 ± 0.1	2.9 ± 0.1
7-ketositosterol	7.1 ± 0.5	12.1 ± 0.1	6.5 ± 0.4	10.3 ± 1.1	9.0 ± 0.6	8.5 ± 0.2
total	9.4 ± 0.5	38.1 ± 0.3	9.2 ± 0.6	34.4 ± 0.6	12.0 ± 0.7	33.3 ± 0.1

phytosterol oxidation product [mg/kg]	enriched					
	day 1		day 2		day 3	
	before heating	after heating	before heating	after heating	before heating	after heating
5,6*β*-epoxycampesterol	1.3 ± 0.0	1.6 ± 0.1	1.7 ± 0.2	2.3 ± 0.1	1.7 ± 0.2	1.5 ± 0.1
5,6*β*-epoxysitosterol	7.8 ± 0.2	8.5 ± 0.2	5.8 ± 0.2	8.0 ± 0.6	5.8 ± 0.2	7.8 ± 0.5
5,6*α*-epoxycampesterol	2.1 ± 0.1	1.7 ± 0.1	2.3 ± 0.2	2.3 ± 0.1	2.3 ± 0.2	2.6 ± 0.2
7*α*-hydroxycampesterol	3.9 ± 0.1	3.4 ± 0.1	3.4 ± 0.2	4.1 ± 0.2	3.4 ± 0.2	4.1 ± 0.4
5,6*α*-epoxysitosterol	8.6 ± 0.3	8.8 ± 0.5	7.5 ± 0.6	8.9 ± 0.1	7.5 ± 0.6	9.2 ± 1.1
7*α*-hydroxysitosterol	4.9 ± 0.4	6.3 ± 0.3	6.4 ± 0.7	6.4 ± 0.4	6.4 ± 0.7	5.0 ± 0.2
7*β*-hydroxycampesterol	1.9 ± 0.0	1.8 ± 0.1	3.3 ± 0.3	3.8 ± 0.1	3.3 ± 0.3	2.3 ± 0.1
7*β*-hydroxysitosterol	0.9 ± 0.0	1.8 ± 0.0	2.0 ± 0.1	2.9 ± 0.2	2.0 ± 0.1	2.1 ± 0.1
7-ketocampesterol	9.6 ± 0.4	7.9 ± 0.1	9.6 ± 0.9	11.6 ± 0.9	9.6 ± 0.9	10.4 ± 0.1
7-ketositosterol	20.2 ± 1.1	27.7 ± 1.8	16.2 ± 5.8	20.6 ± 3.4	16.2 ± 5.8	20.7 ± 1.7
total	61.3 ± 1.9	69.5 ± 2.2	58.2 ± 5.2	70.9 ± 4.5	58.2 ± 5.2	65.6 ± 2.2

[a] <LOD (0.16 µg 5,6-epoxysterol/g liquid margarine; 0.22 µg 7-hydroxysterol/g liquid margarine; 0.22 µg 7-ketosterol/g liquid margarine).
[b] Values represent the mean ± standard deviation (n=3).

Table 30. Individual phytosterol oxidation products determined in the enriched liquid margarine before and after cake baking.

phytosterol oxidation product [mg/kg]	enriched			
	no treatment	day 1	day 2	day 3
5,6*β*-epoxycampesterol	1.4 ± 0.1[a]	2.3 ± 0.1	2.0 ± 0.1	2.3 ± 0.1
5,6*β*-epoxysitosterol	5.2 ± 0.3	7.4 ± 0.3	9.2 ± 0.6	8.0 ± 0.8
5,6*α*-epoxycampesterol	1.5 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.7 ± 0.4
7*α*-hydroxycampesterol	2.1 ± 0.1	2.5 ± 0.2	3.1 ± 0.4	3.1 ± 0.2
5,6*α*-epoxysitosterol	7.0 ± 0.8	8.3 ± 1.1	8.3 ± 0.4	8.8 ± 0.6
7*α*-hydroxysitosterol	3.8 ± 0.3	7.0 ± 0.2	9.0 ± 0.8	7.5 ± 0.8
7*β*-hydroxycampesterol	1.9 ± 0.1	1.7 ± 0.1	2.7 ± 0.2	2.0 ± 0.6
7*β*-hydroxysitosterol	1.3 ± 0.1	3.2 ± 0.3	3.5 ± 0.1	2.7 ± 0.1
7-ketocampesterol	12.3 ± 0.9	13.3 ± 1.1	12.8 ± 0.8	11.7 ± 1.1
7-ketositosterol	19.5 ± 0.6	27.9 ± 2.6	34.0 ± 1.8	29.8 ± 1.6
total	55.9 ± 0.1	75.0 ± 2.3	86.1 ± 2.7	77.6 ± 4.9

[a] Values represent the mean ± standard deviation (n=3).

Table 31. Individual phytosterol oxidation products determined in the enriched liquid margarine before and after cookie baking for 20 and 40 min.

phytosterol oxidation product [mg/kg]	enriched (20 min)			
	no treatment	day 1	day 2	day 3
5,6*β*-epoxycampesterol	1.3 ± 0.0[a]	2.2 ± 0.7	1.9 ± 0.7	2.2 ± 0.6
5,6*β*-epoxysitosterol	5.6 ± 0.4	10.9 ± 1.3	8.6 ± 0.8	8.9 ± 0.8
5,6*α*-epoxycampesterol	1.2 ± 0.2	1.7 ± 0.5	1.4 ± 0.6	1.3 ± 0.3
7*α*-hydroxycampesterol	0.7 ± 0.3	3.3 ± 1.6	2.2 ± 0.8	2.4 ± 0.6
5,6*α*-epoxysitosterol	7.7 ± 0.6	9.2 ± 1.3	8.1 ± 1.4	7.2 ± 0.8
7*α*-hydroxysitosterol	3.5 ± 0.7	10.0 ± 0.6	7.1 ± 0.6	7.0 ± 1.3
7*β*-hydroxycampesterol	0.8 ± 0.3	2.1 ± 1.1	1.3 ± 0.6	1.4 ± 0.4
7*β*-hydroxysitosterol	0.8 ± 0.5	4.6 ± 1.1	3.4 ± 0.9	2.3 ± 0.6
7-ketocampesterol	9.7 ± 1.0	16.6 ± 4.6	17.7 ± 4.0	19.0 ± 1.4
7-ketositosterol	24.4 ± 1.8	37.0 ± 3.1	37.2 ± 5.4	44.4 ± 7.2
total	55.7 ± 0.3	97.5 ± 0.2	88.8 ± 1.4	96.1 ± 3.5

phytosterol oxidation product [mg/kg]	enriched (40 min)			
	no treatment	day 1	day 2	day 3
5,6*β*-epoxycampesterol	1.3 ± 0.0	410.4 ± 0.8	358.5 ± 4.2	481.4 ± 22.1
5,6*β*-epoxysitosterol	5.6 ± 0.4	1700.7 ± 15.5	1509.7 ± 3.8	1893.8 ± 32.4
5,6*α*-epoxycampesterol	1.2 ± 0.2	190.7 ± 3.9	158.3 ± 7.6	218.3 ± 7.2
7*α*-hydroxycampesterol	0.7 ± 0.3	466.5 ± 12.6	388.0 ± 9.8	525.1 ± 27.2
5,6*α*-epoxysitosterol	7.7 ± 0.6	818.2 ± 8.1	722.2 ± 22.2	937.0 ± 18.1
7*α*-hydroxysitosterol	3.5 ± 0.7	1526.3 ± 14.4	1312.9 ± 40.5	1693.8 ± 31.2
7*β*-hydroxycampesterol	0.8 ± 0.3	332.0 ± 0.9	312.7 ± 9.2	367.9 ± 3.1
7*β*-hydroxysitosterol	0.8 ± 0.5	1401.0 ± 3.3	1237.5 ± 27.9	1511.0 ± 11.2
7-ketocampesterol	9.7 ± 1.0	311.3 ± 7.6	246.1 ± 6.3	317.2 ± 3.4
7-ketositosterol	24.4 ± 1.8	1279.1 ± 15.4	997.8 ± 16.4	1307.6 ± 20.0
total	55.7 ± 0.3	8436.2 ± 20.3	7243.6 ± 12.6	9253.0 ± 46.4

[a] Values represent the mean ± standard deviation (n=3).

Table 32. Amounts of internal standards added during the sample preparation for the analysis of POPs in liquid margarines (cf. 3.2.1.5).

Heat treatment	IS_1/IS_2 [µg]
Non-enriched/enriched liquid margarine: no treatment, muffin baking, cake baking, cookie baking (20 min)	5-10
Non-enriched/enriched liquid margarine: vial oven-heating (0.5 g/1 g), pan-frying (1 g/ 3 g) Non-enriched liquid margarine: casserole oven-heating	20-50
Enriched liquid margarine: casserole oven-heating, cookie baking (40 min)	200-340

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Wocheslander, S.; Eisenreich, W.; Scholz, B.; Lander, V.; Engel, K.-H., Identification of acyl chain oxidation products upon thermal treatment of a mixture of phytosteryl/-stanyl linoleates. *J. Agric. Food Chem.* **2016**, *64*, 9214-9223.
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Wocheslander, S.; Gross, F.; Scholz, B.; Engel, K.-H., Quantitation of acyl Chain oxidation products formed upon thermo-oxidation of phytosteryl/-stanyl oleates and linoleates. *J. Agric. Food Chem.* **2017**, *65*, 2435-2442.
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9. PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS (peer-reviewed)

Wocheslander, S.; Eisenreich, W.; Scholz, B.; Lander, V.; Engel, K.-H., Identification of acyl chain oxidation products upon thermal treatment of a mixture of phytosteryl/-stanyl linoleates. *J. Agric. Food Chem.* **2016**, *64*, 9214-9223.

Wocheslander, S.; Gross, F.; Scholz, B.; Engel, K.-H., Quantitation of acyl Chain oxidation products formed upon thermo-oxidation of phytosteryl/-stanyl oleates and linoleates. *J. Agric. Food Chem.* **2017**, *65*, 2435-2442.

Scholz, B.; Wocheslander, S.; Lander, V.; Engel, K.-H., On-line liquid chromatography–gas chromatography: A novel approach for the analysis of phytosterol oxidation products in enriched foods. *J. Chromatogr. A* **2015**, *1396*, 98-108.

ORAL PRESENTATIONS

Wocheslander, S.; Eisenreich, W.; Engel, K.-H., Thermal oxidation of phytosteryl/-stanyl fatty acid esters: Identification of novel acyl chain oxidation products. *1st Food Chemistry Conference*, 30 Oct.-1 Nov. **2016**, Amsterdam, The Netherlands.

Scholz, B.; Wocheslander, S.; Lander, V.; Engel, K.H., Novel foods enriched with phytostery/ stanyl fatty acid esters – new analytical approaches for a comprehensive analysis. *7th Internati onal Symposium on Recent Advances in Food Analysis*, 36 November **2015**, Prague,
Czech Republic.

Mestdagh, F.; Wocheslander, S.; Rodriguez, A.; Egli, A.; Davidek, T.; Blank, I., Conversion of green coffee precursors into flavor during roasting of Arabica and Robusta coffees. *14th Weurman Flavour Research Symposium*, 15-19 Sept. **2014**, Cambridge, United Kingdom.

POSTER PRESENTATIONS

Wocheslander, S.; Eisenreich, W.; Engel, K.-H., Thermal Oxidation of Phytosteryl/-stanyl Linoleates. *45th Annual Meeting of German Food Chemists*, 12-14 Sept. **2016**, Freising, Germany.

Scholz, B.; Wocheslander, S.; Lander, V.; Engel, K.-H., Phytosterol oxidation products in enriched foods – the other side of the coin. *EFSA's second scientific conference Shaping the future of food safety, together*, 14-16 October **2015**, Milan, Italy.

10. CURRICULUM VITAE

WORK EXPERIENCE

Feb. 2014 – July 2017	Research assistant/PhD candidate Chair of General Food Technology (Prof. Dr. Karl-Heinz Engel) Technical University of Munich Freising, Germany
Nov. 2012 – Feb. 2013	Research assistant Mass Spectrometry Research Facility University of Oxford (Prof. Dr. James McCullagh) Oxford, United Kingdom

ACADEMIC STUDIES AND EDUCATION

Oct. 2010 – Sept. 2013	State examination in Food Chemistry (“Staatsexamen”) Technical University of Munich Final Thesis: “Impact of Profile Roasting on Coffee Aroma Formation”, Nestlé Product Technology Center, Orbe-VD, Switzerland
Oct. 2008 – Sept. 2010	Intermediate degree in Food Chemistry Technical University of Munich (“Vorexamen”)
Sept. 2000 – July 2008	A-levels (“Abitur”) Donau-Gymnasium, Kelheim, Germany

AWARDS AND GRANTS

Nov. 2011	Scholarship for academic studies granted by the German National Foundation („Studienstiftung des deutschen Volkes“)
Oct. 2011 – Oct. 2012	Financial grants (“Deutschlandstipendium”)
Apr. 2010 - Sept. 2011	Financial grants by the Technical University of Munich
Oct. 2010	Book award for the best intermediate degree in Food Chemistry