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Novel Approaches for the Analysis of Phytosteryl/-stanyl Fatty Acid Esters and Phytosterol Oxidation Products in Enriched Foods

Birgit Anette Scholz

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ABBREVIATIONS

ABC	ATP-binding cassette transporter
ACh	acetylcholine
ADI	acceptable daily intake
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
apoE	apolipoprotein E
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
bw	body weight
CoA	coenzyme A
CV	coefficient of variation
EI	electron impact
ESI	electrospray ionization
Et_2O	diethyl ether
EtOAc	ethyl acetate
eV	electron volt
FA	fatty acid
FID	flame ionization detector
FL	fluorescence
GC	gas chromatography
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography
i.d.	internal diameter
IL	interleukin
IPP	isopentenyl pyrophosphate
IS	internal standard
IUPAC-IUB	International Union of Pure and Applied Chemistry and International Union of Biochemistry
LC	liquid chromatography
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LPL	lipoprotein lipase
MeOH	methanol
MMI	multimode inlet

MS	mass spectrometry
MTBE	methyl *tert*-butyl ether
NMR	nuclear magnetic resonance spectroscopy
NOAEL	no observed adverse effect level
NP	normal phase
NPC1L1	Niemann-Pick C1 like 1 protein
PCM	pneumatics control module
PLM	post launch monitoring
PTV	programmable temperature vaporizer
Rf	response factor
ROS	reactive oxygen species
RP	reversed phase
RRT	relative retention time
SC	side chain
SCF	Scientific Committee on Food
SIM	selected ion monitoring
SMT	sterol methyltransferase
SPE	solid phase extraction
TEAC	trolox equivalent antioxidant capacity
TLC	thin layer chromatography
TMS	trimethylsilyl
TNFα	tumor necrosis factor α
UHPLC	ultra high performance liquid chromatography
VLDL	very low density lipoprotein

1 INTRODUCTION AND OBJECTIVES

An elevated plasma low-density lipoprotein (LDL)-cholesterol level is a scientifically established risk factor for the development of cardiovascular diseases, the leading causes of death worldwide (WHO, 2015). A daily dietary intake of approximately 2 g phytosterols/-stanols has been demonstrated to result in a reduction of LDL and total plasma cholesterol levels of approximately 10 %, thus showing anti-atherogenic effects (Katan *et al.*, 2003; AbuMweis *et al.*, 2008). Therefore, nutrition-based concepts as therapy measures have attained particular attention: Owing to their beneficial qualities, phytosterols/-stanols are commercially being added to a broad spectrum of foods. In order to improve their solubility particularly in fat-based matrices, the plant sterols/stanols are mostly esterified to fatty acids prior to their incorporation into the foods (Moreau *et al.*, 2002). The use of phytosterols/-stanols and their fatty acid esters in foods falls within 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), now repealed by Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 (EU, 2015). The ester preparations are specified and may either consist of pure phytostanyl fatty acid esters, or of mixtures of both, phytostanyl and phytosteryl fatty acid esters (SCF, 2003b; EFSA, 2008).

Owing to their structural resemblance, phytosterols, either in their free forms or esterified to fatty acids, are susceptible to oxidation reactions similar to those known for cholesterol due to their unsaturation in the ring system. Comparable to cholesterol oxidation products, the formation of phytosterol oxidation products in foods and their subsequent dietary intake might be detrimental to human health: Several *in vitro* studies showed cytotoxic and pro-inflammatory effects of phytosterol oxidation products (García-Llatas and Rodríguez-Estrada, 2011; Vanmierlo *et al.*, 2013). There are indications for a pro-atherogenic potential of plant sterol oxides deriving from both, *in vitro* and *in vivo* experiments; however, the data are still inconclusive (Scholz *et al.*, 2015). Further, a reduced anti-atherogenic potency of phytosterol oxidation products is being discussed (Alemany *et al.*, 2014). In addition to the phytosteryl moieties, also the fatty acid moieties are prone to oxidation reactions (Julien-David *et al.*, 2008; Lehtonen *et al.*, 2012). The oxidation of both, unsaturated fatty acids and phytosterols is known to be catalyzed by light, transition metals, water, heat, and oxygen (Nawar, 1984; García-Llatas and Rodríguez-Estrada, 2011).

As these factors are present during several household ways of handling and preparing foods, an intensive investigation of their effects on particularly phytosteryl/-stanyl ester-enriched foods

seems to deserve closer attention. Such procedures might favor changes of the two crucial compound classes determining efficacy and safety of enriched foods: A decrease of the originally added phytosteryl and/or phytostanyl fatty acid esters, in their intact forms providing the functionality of the product, and a concurrent formation of potentially health-relevant phytosterol and/or phytostanol oxidation products. Thus, for a comprehensive understanding of oxidation processes in enriched foods, the investigation of both, the phytosterol oxidation products and the intact esters, should be in the focus of analytical efforts. However, previous approaches were mainly limited to the determination of only oxidized sterol or stanol moieties (Dutta, 2004), with some of the existing studies additionally investigating the decreasing contents of the intact sterol/stanol moieties after cleavage of the ester bonds (Soupas *et al.*, 2007; Menéndez-Carreño *et al.*, 2008a; Rudzińska *et al.*, 2014; Menéndez-Carreño *et al.*, 2016).

Though, the chromatographic analysis of individual intact phytosteryl/-stanyl fatty acid esters is not trivial. The ester preparations used for food enrichment vary regarding sterols and stanols as well as fatty acid moieties; on the other hand, they exhibit a high degree of structural similarity. Quantitative approaches that have been described, either based on gas chromatography (GC) (Barnsteiner *et al.*, 2011; 2012) or liquid chromatography (LC) (Billheimer *et al.*, 1983; Mezine *et al.*, 2003; Caboni *et al.*, 2005) suffer, most notably, from insufficient chromatographic resolution or do not allow the analysis of complex mixtures of phytosteryl and –stanyl fatty acid esters (Wewer *et al.*, 2011; Ishida, 2014; Hammann and Vetter, 2015). Methodologies allowing the determination of individual intact phytostanyl fatty acid esters or individual intact esters in mixtures of phytostanyl and phytosteryl fatty acid esters as occurring in enriched foods are lacking.

The oxidation products originating from the phytosterol moieties of the fatty acid esters are usually determined after saponification or transesterfication of the lipid extracts. They are subsequently subjected to off-line procedures such as column chromatography, thin layer chromatography (TLC) or solid phase extraction (SPE) for clean-up and pre-concentration prior to their chromatographic analysis via LC or, more frequently, via GC (Dutta, 2004; Guardiola *et al.*, 2004). However, the off-line procedures are time-consuming and carry the risk of sample losses, degradations of the labile oxides or artifact formation. Due to these difficulties, the analytical portfolio and the quantitative data derived thereof show several gaps.

Thus, the aim of the present study was the establishment and validation of appropriate analytical methodologies allowing (i) the detailed quantitative analysis of individual intact phytosteryl and/or phytostanyl fatty acid esters in enriched foods and (ii) the sensitive and reliable determination of oxidation products that may be generated thereof. Due to the high boiling

points of the intact steryl/stanyl fatty acid esters, LC was considered to be more appropriate compared to GC; as the use of mass selective detectors has proven to be effective in order to improve the differentiation of individual intact esters, the analysis of the phytosteryl/-stanyl fatty acid esters should be based on LC-MS. For the analysis of the phytosterol oxidation products, the on-line coupling of liquid chromatography and gas chromatography (on-line LC-GC) was regarded to be a suitable novel analytical platform. This two-dimensional chromatographic technique offers an elegant alternative to laborious off-line approaches as analyses can be performed in a closed and fully automated system, circumventing the drawbacks of conventional sample preparation techniques. Finally, a complementary use of the developed approaches should allow to extend the knowledge on oxidation processes in enriched foods: Via the monitoring of qualitative and quantitative changes of both, individual intact phytosteryl and/or phytostanyl fatty acid esters and the concurrently formed phytosterol/phytostanol oxidation products, a more complete picture of the impact of oxidation processes on phytosteryl and/or phytostanyl ester-enriched foods should be generated.

2 BACKGROUND

2.1 Phytosterols/-stanols

Since the discovery of cholesterol, sterols and their conjugates have been in the focus of research activities of several scientific disciplines. Whereas mammalian cells contain one single sterol, cholesterol, plants have a characteristically complex sterol mixture, the ubiquitous phytosterols, making up the greatest proportion of the unsaponifiable fraction (Hartmann, 1998).

2.1.1 Structure, Biosynthesis, and Biological Function in Plants

Phytosterols are steroid alcohols, being biosynthetically derived from squalene and belonging to the group of triterpenes (Piironen *et al.*, 2000a). Figure 1 shows the basic structure of a sterol with standard carbon numbering according to the 1989 IUPAC-IUB recommendations (IUPAC-IUB, 1989). Phytosterols are made up of a tetracyclic nucleus, two methyl groups at C10 and C13, a side chain consisting of 9-10 carbon atoms at C17, and an additional hydroxyl group at C3 (Piironen *et al.*, 2000a).

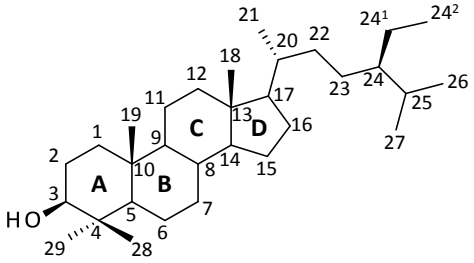

Figure 1. Basic structure of sterols according to IUPAC-IUB recommendations (IUPAC-IUB, 1989).

More than 100 different phytosterols/-stanols have been described in literature (Moreau *et al.*, 2002). Dependent on structure and biochemistry, they can be grouped into 4-desmethyl sterols, 4α-monomethyl sterols, and 4,4-dimethyl sterols. Thereby, 4-desmethyl sterols are the most abundant phytosterols and may be further categorized into Δ^5-sterols, Δ^7-sterols, and $\Delta^{5,7}$-sterols, according to the position and number of double bonds in the B-ring (Hartmann and Benveniste, 1987). The most common phytosterols, e.g. sitosterol and campesterol, have a Δ^5-double bond and, in contrast to cholesterol, an additional methyl or ethyl group at C24 (Figure 2). This alkylation at C24 is a reaction exclusively occurring in plants (Piironen *et al.*, 2000a). The presence of a *trans*-Δ^{22}-double bond, as e.g. in brassicasterol and stigmasterol, is another attribute of phytosterols (Figure 2) (Piironen *et al.*, 2000a).

Phytostanols, the fully saturated forms of phytosterols, are usually found at lower concentrations, as well as Δ^7-sterols (Figure 2). Cholesterol also occurs naturally in several plant cells (Moreau *et al.*, 2002).

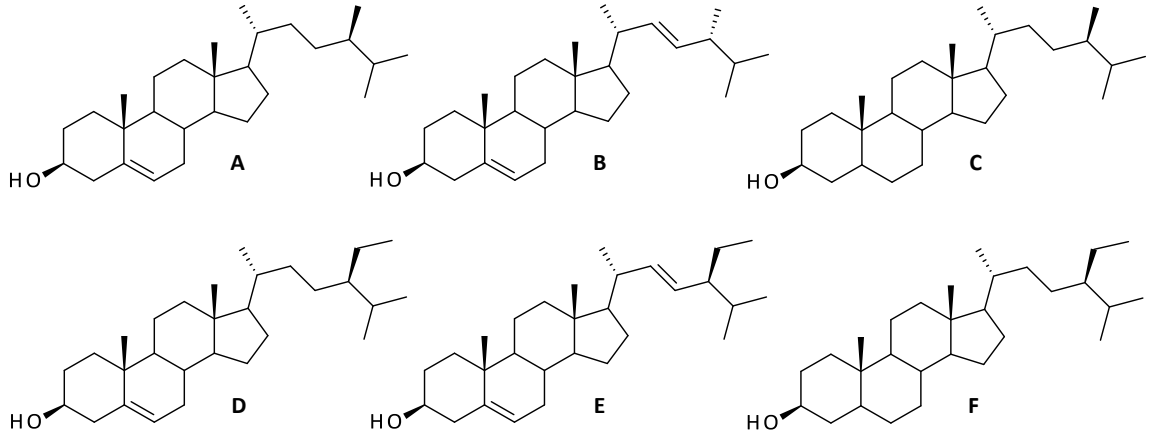

Figure 2. Structures of important 4-desmethyl sterols: campesterol (**A)**, brassicasterol (**B**), campestanol (**C**), sitosterol (**D**), stigmasterol (**E**), and sitostanol (**F**).

The sterol biosynthetic pathway in plants includes a sequence of more than 30 enzyme-catalyzed reactions. The enzymes are mostly localized in the membrane of the plant endoplasmic reticulum, catalyzing the sterol biosynthesis in the cytoplasm (Hartmann, 1998; Piironen *et al.*, 2000a; Benveniste, 2004; Nes, 2011). The phytosterols are products of a branch of the isoprenoid biosynthetic pathway, leading to squalene as intermediate product via the mevalonate pathway (Holmberg *et al*., 2002; Schaller, 2004). The mevalonate pathway starts with acetyl-CoA as initial substrate and finally leads to the production of isopentenyl pyrophosphate (IPP). IPP, the universal precursor of isoprenoids, is then further converted to farnesyl pyrophosphate; direction into the sterol pathway is controlled by the activity of squalene synthase (Piironen *et al.*, 2000a; Liao *et al.*, 2014). The biosynthetic scheme leading from squalene to cholesterol and ergosterol in mammals and fungi, and the one leading to phytosterols in plants differs significantly (Schaller, 2004). Figure 3 shows the principal steps in the post-squalene pathway in plants with a series of methylation, desmethylation, and desaturation reactions, resulting in a plant sterol profile characterized by a mixture consisting of predominantly sitosterol, campesterol, and stigmasterol (Piironen *et al.*, 2000a; Holmberg *et al.*, 2002). Regulation of the pathways occurs by critical rate-limiting steps such as the methylation of the dimethylsterol cycloartenol into 4-methylene cycloartenol via the enzyme sterol C24-methyltransferase (SMT) (Piironen *et al.*, 2000a).

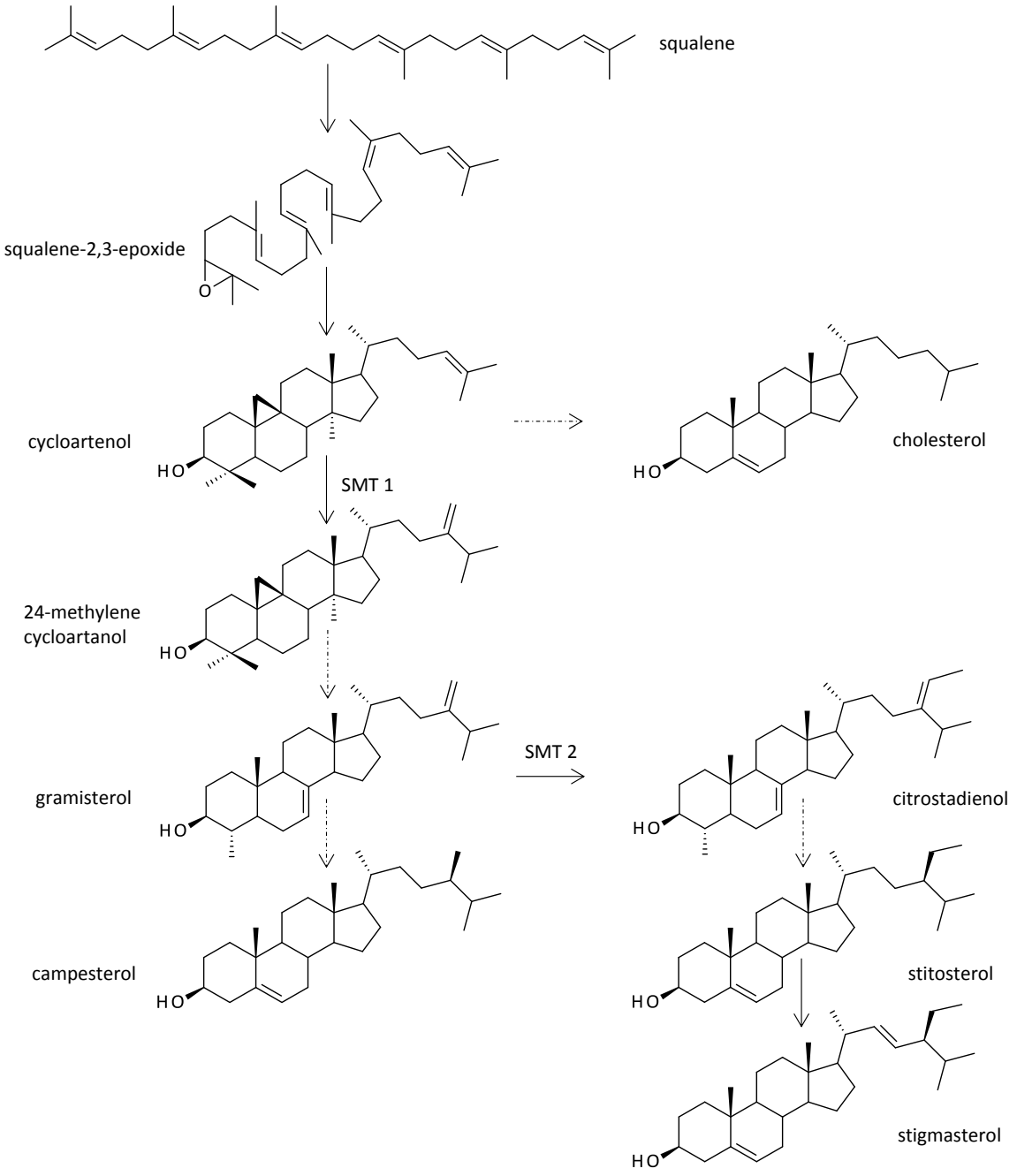

Figure 3. Schematic representation of the sterol pathway post-squalene. Solid lines indicate that one enzymatic step is involved in the conversion and dashed lines indicate the involvement of more than one enzyme.

After their synthesis, the sterols are thought to be transported via the Golgi to the plasma membrane where they accumulate (Hartmann and Benveniste, 1987). The side chain extends into the hydrophobic core of the phospholipid bilayer and interacts with the fatty acyl chains of the phospholipids and proteins (Piironen *et al.*, 2000a). Thereby, the sterols regulate the fluidity of membranes and probably play a role in the adaptation to temperature changes, sitosterol and campesterol being the most efficient regulators. Moreover, sterols participate in the control of

membrane-associated metabolic processes, such as the modulation of membrane-bound H^+-ATPase activity. Studies using sterol biosynthesis inhibitors indicated that phytosterols might play a role in cellular differentiation and proliferation, acting as precursors for the biosynthesis of plant steroid hormones, the brassinosteroids. Finally, they also serve as substrates for a wide variety of secondary metabolites, such as glycoalkaloids, cardenolides, and saponins (Hennessey, 1992; Hartmann, 1998; Piironen *et al.*, 2000a; Moreau *et al.*, 2002; Boutté and Grebe, 2009).

Along with free sterols, plants also contain sterol conjugates in which the 3-hydroxyl group is either esterified to a fatty acid or a phenolic acid or is *ß*-linked to the 1' position of a carbohydrate to form steryl glycosides or acylated steryl glycosides (Figure 4).

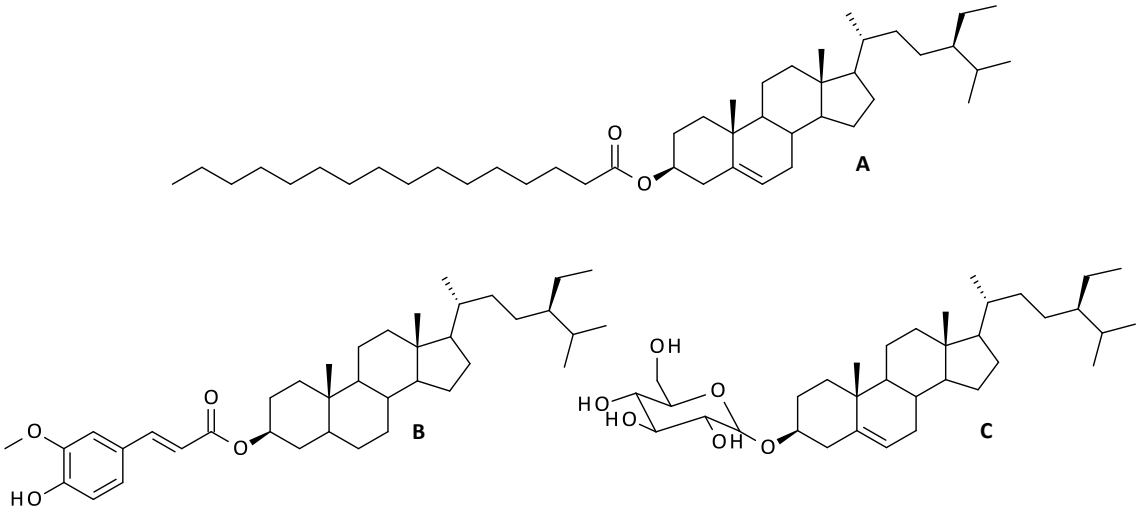

Figure 4. Representative structures of phytosteryl/-stanyl conjugates: sitosteryl palmitate (**A**), *trans*-sitostanyl ferulate (**B**), and sitosteryl-*ß*-D-glucopyranoside (**C**).

Unlike the free sterols, steryl fatty acid esters are presumed to be of storage and transport function, as they are localized in lipid bodies in the cytosol and in soluble forms in droplets or in vesicles, but their precise role remains unclear (Dyas, 1993; Gondet *et al.*, 1994; Piironen *et al.*, 2000a). In many plants, the composition of the sterol moieties of the fatty acid esters consists of the same major 4-desmethylsterols as found in the free sterol fraction. The fatty acid moieties in turn typically range from C12 to C22, with palmitic, stearic, oleic, linoleic, and linolenic acid being the dominant species, and it is suggested that several acyl donors are involved including phospholipids, diacylglycerols or triacylglycerols (Chen *et al.*, 2007; Dyas, 1993). Like free sterols, steryl glycosides and acylated glycosides are membrane structural components (Grille *et al.*, 2010). Steryl and stanyl phenolic acid esters, however, seem to be unique compounds in cereals (Moreau *et al.*, 2002; Esche *et al.*, 2012; Mandak and Nyström, 2012).

Particularly rich sources of phytosterols are vegetable oils, whole grains and cereal-based products, and nuts (Piironen *et al.*, 2000a; Piironen *et al.*, 2000b; Esche *et al.*, 2013d). Based on the dietary intake of natural foods, between 228 and 338 mg/d are estimated to be consumed as part of a typical Western diet (Normen *et al.*, 2001; Valsta *et al.*, 2004; Escurriol *et al.*, 2009; Hearty *et al.*, 2009; Sioen *et al.*, 2011). In vegetarians, the intakes may be two- to threefold increased (Piironen *et al.*, 2000a).

2.1.2 Metabolism of Cholesterol and Phytosterols

Similar to phytosterols in plants, cholesterol serves several essential physiological purposes in mammals. Thus, various tightly coordinated processes with complex feedback mechanisms are involved in maintaining cholesterol homeostasis within a narrow range, consisting of cholesterol biosynthesis, absorption of cholesterol from the diet, and cholesterol excretion into the bile or directly via the feces. Cholesterol can originate from dietary intake as well as from *de novo* synthesis; a dietary intake of cholesterol of approximately 300 mg/d is generally recognized as important in the regulation of serum cholesterol (Ostlund *et al.*, 2002b). When intracellular free cholesterol concentrations decrease, the sterol regulatory element-binding protein 2 (SREBP-2) transcription factor is activated, regulating several target genes being responsible for cholesterol biosynthesis via the mevalonate pathway (McLean *et al.*, 2012). The liver is the major site of cholesterol biosynthesis in humans. Dietary cholesterol in turn can be present in its free form or esterified to fatty acids; after intestinal hydrolysis of esterified cholesterol by the pancreatic cholesterol esterase, free cholesterol is emulsified into mixed micelles to become available for absorption, the micelles interacting with the apical brush-border membrane (de Smet *et al.*, 2012). The principal metabolic pathways of dietary cholesterol in the intestine and in the liver are summarized in Figure 5.

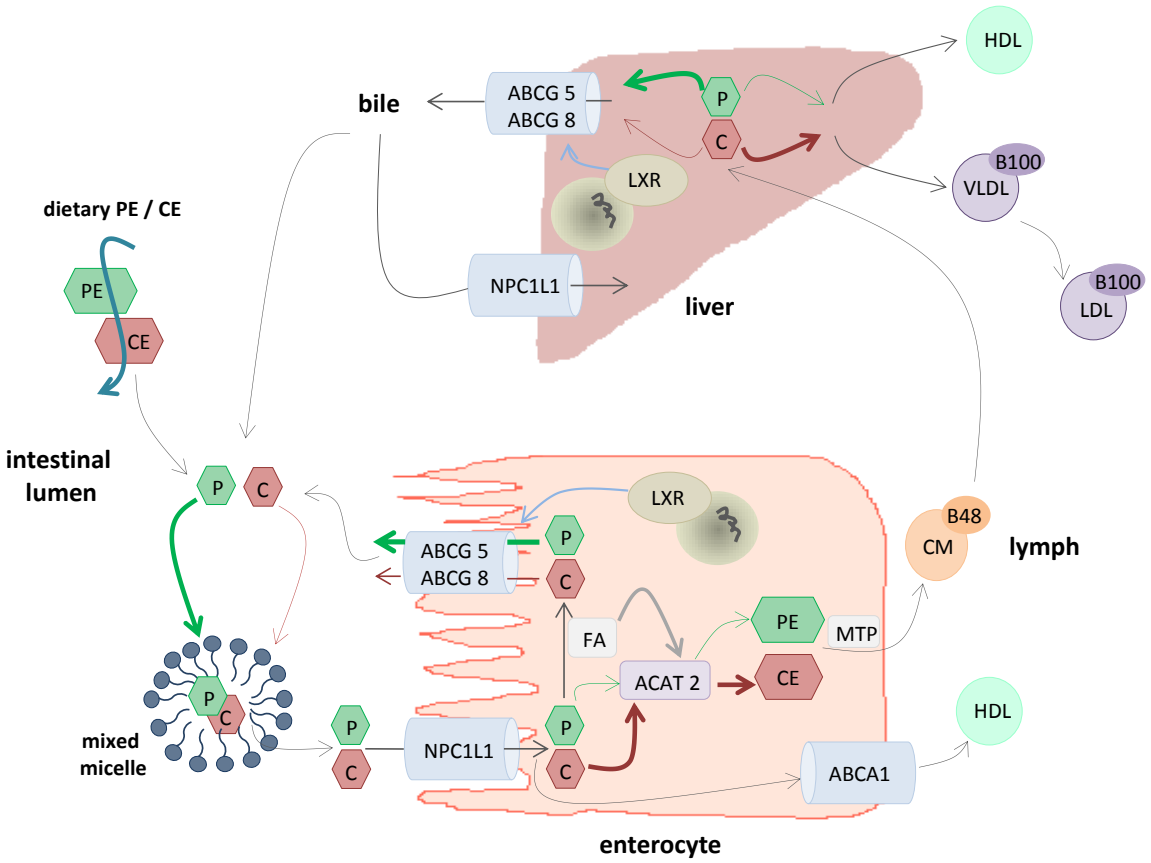

Figure 5. Major metabolic pathways in the absorption and intracellular traffic of cholesterol and plant sterols in the intestine and in the liver (modified according to von Bergmann *et al.*, 2005; Calpe-Berdiel *et al.*, 2009).
ABCG5/G8/A1, adenosine triphosphate-binding cassette G5/G8/A1; ACAT, acyl-CoA:cholesterol acyltransferase; B48/100, apolipoprotein B48/100; C, cholesterol; CE, cholesteryl fatty acid ester; CM, chylomicron; FA, fatty acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LXR, liver X receptor; MTP, microsomal triglyceride transport protein; NPC1L1, Niemann-Pick C1 like 1 protein; P, phytosterol/-stanol; PE, phytosteryl/-stanyl fatty acid ester; VLDL, very low-density lipoprotein.

The micellar cholesterol is mostly absorbed by the influx transporter protein Niemann-Pick C1 like 1 (NPC1L1) located in the brush border membrane of the enterocytes. In a cholesterol-deficient state, the absorbed cholesterol is esterified by acyl-CoA:cholesterol acyltransferase 2 (ACAT 2) to fatty acids and exported to the intestinal lymph in the form of two different lipoprotein particles. Via the microsomal triglyceride transport protein (MTP), cholesteryl esters are, along with triglycerides or phospholipids, and together with apolipoprotein B48 secreted as chylomicrons to the lymph. After hydrolysis of the triglycerides via endothelial lipoprotein lipase (LPL), the chylomicron remnants are taken up the liver. In the liver, the absorbed cholesterol, together with *de novo* synthesized cholesterol, is either stored or secreted into the peripheral circulation via very low density lipoproteins (VLDL). Via LPL, low density lipoprotein (LDL) particles are formed, finally accomplishing the transport of cholesterol to peripheral tissues, the uptake of LDL-cholesterol being mediated by LDL receptors (Elshourbagy *et al.*, 2014). The reverse transport of

cholesterol from the periphery to the liver, in turn, is mediated via high-density lipoprotein (HDL) particles (Calpe-Berdiel *et al.*, 2009; Elshourbagy *et al.*, 2014). In the intestine, free cholesterol can additionally be secreted via nascent HDL-cholesterol, its biogenesis mediated via ATP-binding cassette transporter (ABC) A1 (Calpe-Berdiel *et al.*, 2009). Cellular cholesterol levels in enterocytes can also be regulated by cholesterol secretion back into the intestinal lumen via the ABCG5/ABCG8 heterodimer, regulated by liver X receptor (LXR) (Calpe-Berdiel *et al.*, 2009). Cholesterol that has been secreted into the intestinal lumen is either included in the enterohepatic circulation or may enter the colon and be excreted via the feces as free cholesterol or modified by colon microorganisms (Macdonald *et al.*, 1983).

The intestinal absorption of dietary phytosterols and –stanols differs from that of cholesterol, particularly in quantitative regards (Figure 5). Similarly to cholesteryl esters, fatty acid esters of phytosterols/-stanols are being hydrolyzed in the intestine. It was demonstrated that phytosteryl/-stanyl esters are generally accepted as substrates by pancreatic cholesterol esterase (Moreau and Hicks, 2004). However, *in vitro* and *in vivo* observations on intestinal hydrolysis rates showed dependencies on both, the phytosterol/-stanol structures and the fatty acid moieties of distinct esters (Brown *et al.*, 2010b; Ash *et al.*, 2011; Lubinus *et al.*, 2013). After incorporation into the mixed micelles, whereby phytosterols are preferred over cholesterol, phytosterols/-stanols are also absorbed via the NPC1L1 protein (Brufau *et al.*, 2008). The absorbed phytosterols may also be esterified via ACAT, they are, however, a poor substrate; the majority of the phytosterols remains free and is transported back into the lumen by ABCG5 and ABCG8, whereas sitosterol was shown to be secreted at a higher rate than campesterol (Sudhop, 2002; von Bergmann *et al.*, 2005; Brufau *et al.*, 2008). Esterified phytosterols that enter the circulation after their incorporation into chylomicrons are preferentially excreted from the liver into the bile by the hepatic ABCG5/ABCG8 efflux transporter (MacKay and Jones, 2011). These mechanisms result in an overall low intestinal absorption of phytosterols, ranging from 0.04 % for sitostanol up to 16 % for campesterol (Salen *et al.*, 1970; Heinemann *et al.*, 1993; Lütjohann *et al.*, 1995; Ostlund *et al.*, 2002a; Sudhop, 2002). The differential absorption of phytosterols and phytostanols is reflected in their circulating levels, as plant sterols (7-24 μmol/L) are higher concentrated in plasma than plant stanols (0.05-0.3 μmol/L) (MacKay and Jones, 2011; Vanmierlo *et al*., 2015). Levels of circulating cholesterol, in turn, are considerably higher (~5 mmol/L) than those of the phytosterols/-stanols, even when the dietary intakes of cholesterol and plant sterols are similar (Vanmierlo *et al*., 2015).

2.1.3 Cholesterol-Lowering Properties of Phytosterols/-stanols

The complex regulatory mechanisms responsible for the maintenance of intracellular cholesterol levels within a healthy limit may, however, be disturbed, leading to an imbalanced homeostasis, which is one of the major risk factors associated with cardiovascular diseases, the principal cause of death in developed countries (McLean *et al.*, 2012). Known factors contributing to the development of atherosclerosis include high LDL-cholesterol and low HDL-cholesterol (Elshourbagy *et al.*, 2014). Elevated LDL-cholesterol levels are mostly of genetic origin such as mutations in the LDL receptor gene (Ramasamy, 2016). Further, secondary hypercholesterolemia may be caused by obesity, metabolic syndrome, diabetes, or pregnancy (Ramasamy, 2016). As early as in the 1950s, a negative correlation between sitosterol intake and LDL-cholesterol levels has been observed in humans, due to a reduction of intestinal cholesterol absorption (Pollak, 1953). Since then, several meta-analyses have shown an LDL-cholesterol-lowering effect of dietary consumed phytosterols/-stanols (Katan *et al.*, 2003; Berger *et al.*, 2004; AbuMweis *et al.*, 2008; Demonty *et al.*, 2009; Musa-Veloso *et al.*, 2011). Different hypotheses have been suggested regarding the molecular mechanisms underlying these LDL-cholesterol lowering properties.

A restricted micellar solubilization of intestinal cholesterol in the presence of phytosterols/-stanols due to a higher hydrophobicity of the plant sterols/stanols is unequivocally acknowledged to be a major mechanism (Trautwein *et al.*, 2003; Plat *et al*., 2015). Esterified sterols/stanols are probably not able to affect micellar cholesterol solubilization (Brown *et al.*, 2010a). Differences between phytosterols and phytostanols regarding the efficiency of replacing cholesterol from the mixed micelles are discussed, with phytostanols being considered to be more efficient (de Smet *et al.*, 2012). As studies showed the LDL-cholesterol-lowering efficacy of consuming the same amount of phytosterols within one meal or as part of several meals divided over the day to be equal, it is suggested that additional mechanisms exist (Plat *et al.*, 2000; Matvienko *et al.*, 2002). A co-crystallization of cholesterol and phytosterols in the intestinal lumen has been proposed; however, under physiological conditions, cholesterol and phytosterols were shown to be highly soluble in hydrolysis products of dietary lipids (Mel'nikov *et al.*, 2004). Further, a reduced uptake of cholesterol via the NPC1L1 transporter due to competition with phytosterols, as well as an increased secretion into the intestinal lumen due to increased ABCAG5/G8 activity, possibly upon activation of LXR target genes by plant sterols, are taken into account (Trautwein *et al.*, 2003; de Smet *et al.*, 2012). All of these potential mechanisms would require phytosterols/-stanols in their free forms, revealing previous hydrolysis of esterified phytosterols/-stanols as an essential precondition for the exertion of cholesterol-lowering properties (de Smet *et al.*, 2012; Carden *et al.*, 2015).

The reduced availability of dietary cholesterol in the presence of phytosterols is being compensated by an increased endogenous cholesterol biosynthesis, but also by an increased expression and synthesis of LDL-receptors, leading to a total reduction of levels of circulating LDL-cholesterol (Plat and Mensink, 2005; Brufau *et al.*, 2008).

Besides the LDL-cholesterol-lowering impact, several other beneficial health effects are attributed to dietary phytosterols/-stanols and their conjugates. In the context of reducing risk factors for developing cardiovascular diseases, the effects of phytosterols are probably pleiotropic and tissue specific (de Smet *et al*., 2015), as, for example, it has also been shown that they are lowering serum triglyceride levels (Demonty *et al.*, 2013), probably being related to a decreased hepatic VLDL secretion (Plat *et al.*, 2009; Schonewille *et al.*, 2014). Enhancing the immune function and an anti-inflammatory activity (Othman and Moghadasian, 2011; Plat *et al.*, 2014a; Plat *et al*., 2015) have also been proposed as well as preventive effects again certain types of cancer (Awad and Fink, 2000). However, a recent meta-analysis evaluating the effects of regular phytosterol supplementation via enriched foods on markers of inflammation could not establish an anti-inflammatory effect of dietary phytosterol consumption (Rocha *et al.*, 2016).

2.2 Enrichment of Foods with Phytosteryl/-stanyl Fatty Acid Esters

Based on the observed cholesterol-lowering properties, free phytosterols suspended in oil or methylcellulose were commercially available for the treatment of hypercholesterolemia between 1957 and 1982 (MacKay and Jones, 2011). However, high doses of up to 30 g/d were required as the phytosterols were delivered in their crystalline form (Trautwein *et al.*, 2003). The transfer of the phytosterols from this poorly soluble crystalline form to the micellar phase was considered to be too slow for being effective (Trautwein *et al.*, 2003). Esterification of the phytosterols to fatty acids, in turn, was shown to significantly improve the physical properties regarding both, intestinal solubility and the solubility in particularly fat-based matrices, which would be a preferred carrier for oral phytosterol administration (Mattson *et al.*, 1977; Mattson *et al.*, 1982; Trautwein *et al.*, 2003; MacKay and Jones, 2011). As the esters have been described to be hydrolyzed by intestinal enzymes, the way for an industrial production of phytosteryl/-stanyl fatty acid esters and commercial incorporation of the esters into a broad spectrum of foods was paved. Meta-analyses regarding the effects of the consumption of phytosteryl or phytostanyl fatty acid ester-enriched foods showed significant reductions of plasma LDL-cholesterol levels (Law, 2000; Katan *et al.*, 2003; Chen *et al.*, 2005; AbuMweis *et al.*, 2008; Demonty *et al.*, 2009; Talati *et al.*, 2010; Ras *et al.*, 2014; Rocha *et al.*, 2016). These studies established a significant reduction of total and LDL-cholesterol levels of 9-12 % upon a daily dietary intake of approximately 2-3 g phytosterols/-stanols (Katan *et al.*, 2003; Demonty *et al.*, 2009; Ras *et al.*, 2014). The dose-response effects established in the most recent meta-analysis (Ras *et al.*, 2014) are shown in Figure 6.

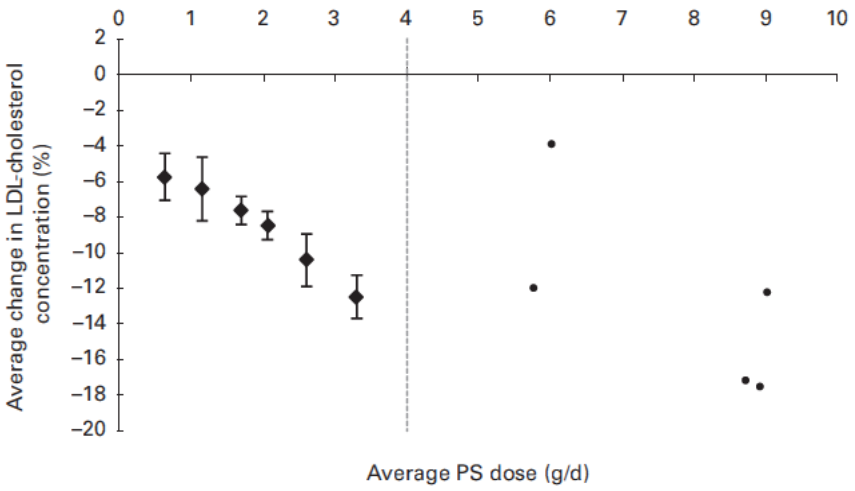

Figure 6. Average effects on LDL-cholesterol concentration for different dose ranges of phytosterols up to 4 g/d; the • represent outcomes of single high-dose studies that were not pooled (Ras *et al.*, 2014).

Doses higher than 3 g/d were shown to offer no additional beneficial effect and might even yield undesired consequences (Miettinen and Gylling, 2004; Demonty *et al.*, 2009), while an intake below 1 g/d is probably not sufficient for a substantial reduction of LDL-cholesterol levels (Vanhanen *et al.*, 1994; Hallikainen *et al.*, 2000). The available epidemiological studies are generally considering phytosteryl and phytostanyl fatty acid esters to be equal regarding short-term reduction of LDL-cholesterol (Moreau *et al.*, 2002), as it was once again recently shown in a meta-analysis by Ras *et al.* (2014). However, phytostanyl fatty acid esters are being discussed to be more effective at high doses (Musa-Veloso *et al.*, 2011) and upon long-term consumption (Miettinen and Gylling, 2004; O'Neill *et al.*, 2005) .

2.2.1 Regulations and Food Products

In the European Union, foods enriched with phytosteryl and/or phytostanyl fatty acid esters fall within 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), now repealed by Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 (EU, 2015). The phytosterols and their fatty acid esters are being considered as novel food ingredients as their consumed amounts via enriched foods exceed eight- to twelvefold those amounts consumed via traditional sources within a normal diet. For approval, a safety assessment prior to final authorization pursuant to Article 4 of the Novel Food Regulation is required. The authorization covers conditions of use, specifications, and labelling requirements. A simplified notification procedure pursuant to Article 5 may take place for foods with proven substantial equivalence to an already authorized novel food.

However, the first such product, a margarine enriched with phytostanyl fatty acid esters ("Benecol", produced by Raisio), was introduced on the Finnish market already before the adoption of the Novel Food Regulation. The first product that has been authorized in the European Union in 1997 as a novel food on the basis of a safety assessment by the Scientific Committee on Food (SCF) (SCF, 2000) was a yellow fat spread "Becel® pro.activ" produced by Unilever, enriched with a mixture of phytosteryl and phytostanyl fatty acid esters (EU, 1997; EC, 2000). In the meantime, a broad spectrum of other foods with added phytosteryl/phytostanyl fatty acid esters has been placed on the market in the European Union. They comprise milk-type products, yoghurt-type products, milk-based fruit drinks, soy-based drinks, cheese-type products, salad dressings, spice sauces, rye bread, rice drinks, and oils (EFSA, 2008). The concentrations of the phytosterols range from 0.3 % in milk-type products to 8 % in margarines and spreads (Barnsteiner *et al.*, 2012). Currently, there is a pending application aiming at extending the use of

phytosteryl esters to spreads and liquid vegetable fat-based emulsions specifically intended for cooking and baking (Unilever, 2013). Phytosterols/phytostanols and their fatty acid esters are among those food ingredients for which health claims referring to the reduction of disease risk – i.e. the reduction of cardiovascular risks via the scientifically established reduction of LDL-cholesterol levels - have been permitted (EC, 2009; 2010).

Based on the lack of evidence for additional benefits at intakes of phytosterols higher than 3 g/d, in its general view on the long-term effects of the intake of elevated levels of phytosterols from multiple sources, the SCF considered it prudent to avoid intakes exceeding a range of 1-3 g/d (SCF, 2002). This precautionary limit is in line with the group acceptable daily intake (ADI) of 0-40 mg/kg body weight (bw) for the group of phytosterols, phytostanols and their esters, expressed as the sum of phytosterols and phytostanols in their free form, later derived by the Joint FAO/WHO Expert Committee on Food Additives (WHO, 2009).

The production of the phytosterols is based on their isolation from edible vegetable oil distillates, such as soybean, sunflower, or rapeseed oil, after hydrolysis and/or interesterification. Alternatively, they can be derived from tall oil from coniferous woods, a by-product of the wood pulp industry (Weber and Mukherjee, 2006). The sterol profiles differ between the sources, particularly regarding the higher proportion of stanols in phytosterol isolates from tall oil; therefore, the following phytosterol/phytostanol profile has been specified by the SCF (2003b): *ß*-sitosterol ≤80 %, campesterol ≤40 %, stigmasterol ≤30 %, sitostanol ≤15 %, campestanol ≤5 %, brassicasterol ≤3 %, and others ≤3 %. Pure saturated phytostanols as used for the foods produced by Raisio can be obtained by hydrogenation. Phytostanols produced from tall oil typically contain ~90 % sitostanol and ~10 % campestanol (Cantrill and Kawamura, 2008).

The fatty acids employed for production of phytosteryl and phytostanyl fatty acid esters are originating from vegetable oils; thus the fatty acid composition of the esters reflects that of the oil used as source (Cantrill and Kawamura, 2008). The esters can be prepared by chemical esterification with fatty acids or by interesterification with fatty acid methyl esters or vegetable oils (Weber and Mukherjee, 2006).

The Senate Commission on Food Safety (SKLM) of the German Research Council previously published two scientific opinions regarding the use of phytosteryl/phytostanyl fatty acid esters in foods (SKLM, 2005; 2007). They focused on the need for assessment of individual phytosteryl/phytostanyl ester preparations and the importance of the corresponding specifications. In addition, they drew particular attention to challenges arising from the broad spectrum of enriched food categories and the uncertainties in ensuring that an intake of 1-3 g/d is

not exceeded. The need for current and reliable consumption data and for measures that the products are only consumed by the target groups was emphasized.

Specific provisions regarding the labelling of foods with added phytosterols/phytostanols have been implemented; for example, a label is required indicating that a consumption of more than 3 g phytosterols per day should be avoided (EC, 2004). However, a consumer awareness study performed in Germany revealed that 45 % of the consumers did not belong to the target group, 3.5 % were children and only 1 % were aware that an intake of 3 g phytosterols/d should not be exceeded (Niemann *et al.*, 2007). Data on the actual exposure of consumers to phytosterols via the multiple sources of enriched foods are also inconsistent. According to a post-launch monitoring (PLM) survey on consumer purchases of foods (spreads, salad dressings, milk- and yoghurt-type products) with added phytosterols in five European countries, the mean phytosterol intakes per household were 0.35-0.86 g/d. In the 95th percentile of the population, intakes ranged from 1.0 g/d in France to 3.7 g/d in The Netherlands; The Netherlands was the only country in which approximately 6 % of households were identified as potential "over consumers" (Willems *et al.*, 2013). These data indicating that overconsumption of phytosterols seems unlikely are in agreement with the results obtained in the mandatory PLM performed by Unilever covering the first year of marketing of enriched vegetable oil spreads; in that survey the median intakes of phytosterols for regular purchasers were 1.2-1.4 g/d, the 95th percentile intakes ranged from 2.2 g/d in France to 3.6 g/d in The Netherlands (Lea and Hepburn, 2006). On the other hand, a significantly higher mean phytosterol intake (2.45 g/d) was reported in a study performed on the Irish market. In total, 23 % of the respondents had mean phytosterol intakes higher than 3 g/d and the majority of consumers (58 %) had been consuming these products for more than one year (Hearty *et al.*, 2009). A study investigating the consumption of phytosterol-enriched foods in Belgium also identified 16% of consumers to have a phytosterol intake above 3 g/d (Sioen *et al.*, 2011).

2.2.2 Efficacy and Safety Aspects

The efficacy of consuming plant sterol/stanol-enriched foods regarding the lowering of plasma LDL-cholesterol is largely uncontested. However, although the relation between elevated LDL-cholesterol and an increased cardiovascular risk is equally established, direct evidence supporting an LDL-cholesterol mediated reduction of cardiovascular diseases has so far not been generated, neither for foods enriched with phytosteryl/-stanyl fatty acid esters (Plat *et al.*, 2012b; Ras *et al.*, 2015a), nor for phytosterol intake based on natural sources (Ras *et al.*, 2015b). Besides focusing on risk factor reduction such as changes in serum LDL-cholesterol concentrations, studies

evaluating changes in surrogate markers of atherosclerosis have been conducted. In most of these studies, no significant improvement in arterial health could be demonstrated; therefore, studies evaluating the effects of long-term interventions using plant sterol/stanol ester-enriched foods on clinical endpoints beyond cholesterol lowering are needed (Plat *et al.*, 2012b).

Regarding the safety of a regular consumption of high doses of phytosteryl/-stanyl esters resulting from the enrichment of foods, first of all there was concern on a reduced absorption of lipophilic dietary substances other than cholesterol. Indeed, reductions of serum *ß*-carotene, *α*-carotene, and lycopene concentrations were shown upon plant sterol supplementation (Plat and Mensink, 2005). This decrease can, however, be counteracted by increasing the consumption of vegetables and fruits (Plat and Mensink, 2005). Further, serum levels of vitamin A have been shown to be not affected (Gylling *et al.*, 2010).

Beyond the effect on *ß*-carotene levels, one recent study showed with the example of *Apc*^{Min} (*Adenomatous polyposis coli*) mice a gender-specific induction of intestinal adenoma formation in female mice having been fed a plant sterol-enriched diet (Marttinen *et al.*, 2013b), and induction of intestinal adenoma formation in both genders upon feeding a plant stanol ester-enriched diet (Marttinen *et al.*, 2013a). Even though the underlying mechanisms remained unclear, it was shown that the fed sterols and stanols were taken up by the enterocytes where they regulated cell signaling and gene expression.

Several studies demonstrated that plant sterols, in contrast to cholesterol, are able to cross the blood-brain-barrier. First of all, this phenomenon was shown for phytosterolemic patients due to their pathologically high serum plant sterol concentrations (Salen *et al.*, 1985). The phytosterolemic patients, however, did not show severe neurological dysfunctions. Since then, the accumulation of dietary-fed plant sterols and stanols in the brains of Watanabe rabbits (Fricke *et al.*, 2007), of dietary phytosterols in the brains of ABCG5 and ABCG8 deficient mice (Jansen *et al.*, 2006) as well as in the brains of C57BL/6NCrl mice (Vanmierlo *et al.*, 2012) was demonstrated. In the latter study, a plant sterol ester diet was fed to C57BL/6NCrl mice for six weeks, leading to significantly increased concentrations of plant sterols in serum, liver and in murine brains. Interestingly, blocking the intestinal sterol uptake for the next 6 months while feeding the mice with a plant stanol ester-enriched diet resulted in strongly reduced phytosterol levels in serum and liver; however, brain phytosterol levels were not affected, indicating the accumulation of phytosterols in the murine brain being stable. Campesterol was shown to cross the blood-brain barrier more efficiently than sitosterol. Overall, the phytosterols are thought to positively affect neuroinflammation, neurodegeneration, and disease progression based on experiments with animal models for different CNS disorders (Vanmierlo *et al*., 2015). However, certain phytosterol

metabolites such as glycosylated sterols are considered to be neurotoxic (Vanmierlo *et al.*, 2015). The detailed potential neuroprotective or neurotoxic impacts of such phytosterol accumulations have yet to be elucidated, as well as the situation in humans.

Another controversially discussed issue is the increased absorption of phytosterols, potentially resulting in their accumulation and subsequently the promotion of vascular diseases (Weingärtner *et al.*, 2008a). Concerns on potential pro-atherosclerotic effects of elevated plasma phytosterol levels are due to observations made for phytosterolemic patients who are predisposed to develop premature atherosclerosis (Lütjohann and von Bergmann, 2009). Further, it has been shown in healthy persons that phytosterol supplementation causes an increase in plasma phytosterol concentrations. Additionally, there are studies available showing a correlation between plasma phytosterol levels and cardiovascular risk (Glueck *et al.*, 1991; Rajaratnam *et al.*, 2000; Sudhop *et al.*, 2002; Assmann *et al.*, 2006; Silbernagel *et al.*, 2010; Weingärtner *et al.*, 2011). However, data are inconclusive, as there are also studies suggesting no association (Wilund, 2004; Pinedo *et al.*, 2006; Miettinen *et al.*, 2010) or even a reduced risk of cardiovascular events when plasma plant sterol concentrations are elevated (Fassbender *et al.*, 2008; Escurriol *et al.*, 2010; Weingärtner *et al.*, 2010). The interpretation of these observations is causing controversial discussions, as serum plant sterol concentrations have been shown to reflect intestinal cholesterol absorption that varies between individuals (Tilvis and Miettinen, 1986). Therefore, elevated plasma phytosterol levels might be considered only as markers of elevated cholesterol absorption, this in turn being the actual origin of increased cardiovascular risk (Plat *et al.*, 2012a; 2012b).

Plant sterols were shown to be present in atherosclerotic plaques of patients undergoing carotid endarterectomy (Miettinen *et al.*, 2005). The accumulation of plant sterols in human stenotic aortic valves has also been demonstrated (Helske *et al.*, 2008; Schött *et al.*, 2014), in the study by Helske *et al*. this finding was directly correlated to plasma phytosterol concentrations. Weingärtner *et al*. (2008b) studied the effects of food supplementation with phytosteryl esters in apolipoprotein E (ApoE) deficient mice and observed a correlation of increased plasma sterol concentrations with impaired endothelial function, ischemic brain injury, and atherogenesis. In humans, a correlation between consumption of phytosteryl ester-enriched margarine with increased phytosterol concentrations in plasma and aortic valves in patients with aortic stenosis has been described (Weingärtner *et al.*, 2008b). More recently, the concentrations of plant sterols in plasma and in aortic valve cusps of patients with severe aortic stenosis with and without coronary artery disease were compared; the campesterol-to-cholesterol ratio in plasma and the absolute concentrations of campesterol and sitosterol in aortic valve cusps of patients with concomitant coronary artery disease were shown to be significantly higher (Luister *et al.*, 2015).

Further, it has been reported that elevated plasma campesterol concentrations upon long-term consumption of phytosteryl ester-enriched foods correlate with an increase in retinal venular diameter (Kelly *et al.*, 2011). Bombo *et al.* (2013) fed a phytosteryl ester-enriched diet to $LDLR^{-}/^{-}$ mice and did not observe an absolute increase of phytosterol concentrations in the arterial wall compared to the control, concluding that dietary phytosterols do not accumulate in the vasculature. However, this finding is intensively discussed, as relative to cholesterol levels, the phytosterol contents actually increased (Lütjohann *et al.*, 2014). In this context of potential risks deriving from elevated plasma phytosterol concentrations due to dietary supplementation, the potential superiority of phytostanol-enriched foods is once more addressed, as plant stanols do not only reduce serum cholesterol levels but also those of plant sterols (Miettinen and Gylling, 2008).

However, based on the overall limited absorption rates of phytosterols/-stanols and resulting low plasma levels upon any kind of diet in comparison to cholesterol plasma concentrations, the atherogenecity of circulating phytosterols has to be far higher than that of cholesterol for exerting detrimental effects, compensating for the presumably beneficial effect of lowering LDL-cholesterol levels.

Another aspect should be taken into consideration when it comes to a risk-benefit assessment of phytosteryl/-stanyl ester-enriched foods: One of the mechanisms being discussed to be responsible for a potential pro-atherosclerotic effect of elevated phytosterol levels is not their presence *per se*, but rather their transformation into oxidation products.

2.3 Oxidation of Phytosterols

Due to their structural similarity, undesirable reactions known for cholesterol may also be expected in the case of phytosterols. A typical example is the formation of the so-called cholesterol oxidation products, i.e. keto, hydroxy and epoxy derivatives of cholesterol, a well-known group of substances studied in detail for many years. On the one hand, cholesterol oxidation products are crucial intermediates in mammalian metabolism, are enzymatically synthesized *in vivo*, and serve several regulatory purposes such as cholesterol homeostasis (Björkhem and Diczfalusy, 2002; Leonarduzzi *et al.*, 2002). On the other hand, they may be formed endogenously via non-enzymatic oxidation of cholesterol and may also be absorbed from the diet. In cholesterol-containing foods, cholesterol oxidation products can be formed via processing and storage (Leonarduzzi *et al.*, 2002). Elevated plasma levels of cholesterol oxidation products have been particularly correlated to atherogenic effects, and are also thought to be involved in other inflammatory processes such as neurodegeneration (Alemany *et al.*, 2014). Therefore, the occurrence in foods and the subsequent dietary intake not only of intact cholesterol but also of cholesterol oxidation products has been in the focus of recent research activities. An increased intake of dietary cholesterol oxidation products was shown to be associated with impaired hepatic function and lipid metabolism, and ultimately atherosclerotic progression in various animal models (Brown and Jessup, 1999; Meynier *et al.*, 2005; Staprans *et al.*, 2005; Ng *et al.*, 2008; Soto-Rodríguez *et al.*, 2009; Sasaki *et al.*, 2010; Sato *et al.*, 2012; Soto-Rodríguez *et al.*, 2012; Terunuma *et al.*, 2013). Taking into account the structural similarities between cholesterol and phytosterols and of the oxidation products expected to be formed thereof, studies on the formation, intake, and biological effects of phytosterol oxidation products have received increasingly growing scientific interest.

2.3.1 Formation of Phytosterol Oxidation Products

Phytosterol oxidation products can be formed by either non-enzymatic or enzymatic oxidation. Non-enzymatic oxidation reactions comprise photooxidation and autoxidation. In photooxidation singlet oxygen, mostly generated by photosensitizers, attacks one of the sides of the double bond in the B-ring (García-Llatas and Rodríguez-Estrada, 2011). Overall, the information on photooxidation reactions of phytosterols is limited. The most common oxidation mechanism is the non-enzymatic autoxidation of sterols (García-Llatas and Rodríguez-Estrada, 2011).

2.3.1.2 Autoxidation of Phytosterols

The autoxidation of phytosterols is a free radical mechanism that can be initiated in particular in the presence of heat, light, air, water, and transition metals (García-Llatas and Rodríguez-Estrada, 2011). The reaction starts with the abstraction of a reactive allylic hydrogen at C7 in the B-ring of Δ5-sterols, resulting in a radical molecule (Piironen *et al.*, 2000a; Oehrl *et al.*, 2001). The radical then reacts with triplet oxygen ($^3O_2$) and an instable 7-peroxy radical is formed. The second, rate-determining step comprises the addition of hydrogen of another sterol, yielding the more stable 7-hydroperoxide and an additional radical molecule (Figure 7) (Porter *et al.*, 1995; García-Llatas and Rodríguez-Estrada, 2011). The epimeric 7-hydroxyperoxides are the primary oxidation products.

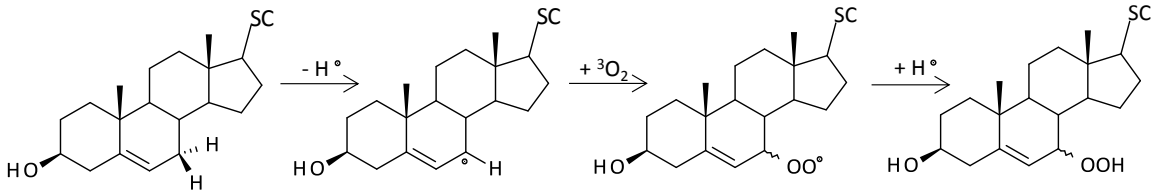

Figure 7. Chain sequence for the free radical autoxidation from sterol to hydroperoxysterol: [SC], side chain.

In the course of the oxidation reaction, the primary oxidation products degrade to the stable 7-hydroxy- and 7-ketosterols (Lampi *et al.*, 2002; Dutta, 2004). The formation of epoxysterols occurs by a bimolecular mechanism, as a non-oxidized sterol reacts with a hydroperoxy radical; the epoxides can be further hydrolyzed in acidic environment to form triols (García-Llatas and Rodríguez-Estrada, 2011).

In addition to oxidative modifications in the ring system, also the side chains are prone to oxidation reactions, however, to a minor extent. Owing to their tertiary nature, C20, C24, and C25 are most susceptible to the formation of hydroperoxides, finally leading to secondary side chain oxidation products (Yanishlieva *et al.*, 1980; Johnsson and Dutta, 2003; Johnsson *et al.*, 2003).

The secondary oxidation products may form further reaction products, yielding dimeric, oligomeric, and polymeric compounds as tertiary oxidation products. In addition, fragmented sterols are also proposed, resulting in mid-polar oxygenated and non-polar dimeric or oligomeric compounds. Structures for sterol dimers, such as disteryl ethers, have been proposed (Rudzinska *et al.*, 2010; Sosińska *et al.*, 2014). Besides, the secondary oxidation products can give rise to the formation of conjugated dienes and trienes after abstraction of the hydroxyl group at C3 (Lercker

and Rodriguez-Estrada, 2000; Dutta, 2004). Figure 8 gives an overview on the formation of the principal products of phytosterol oxidation.

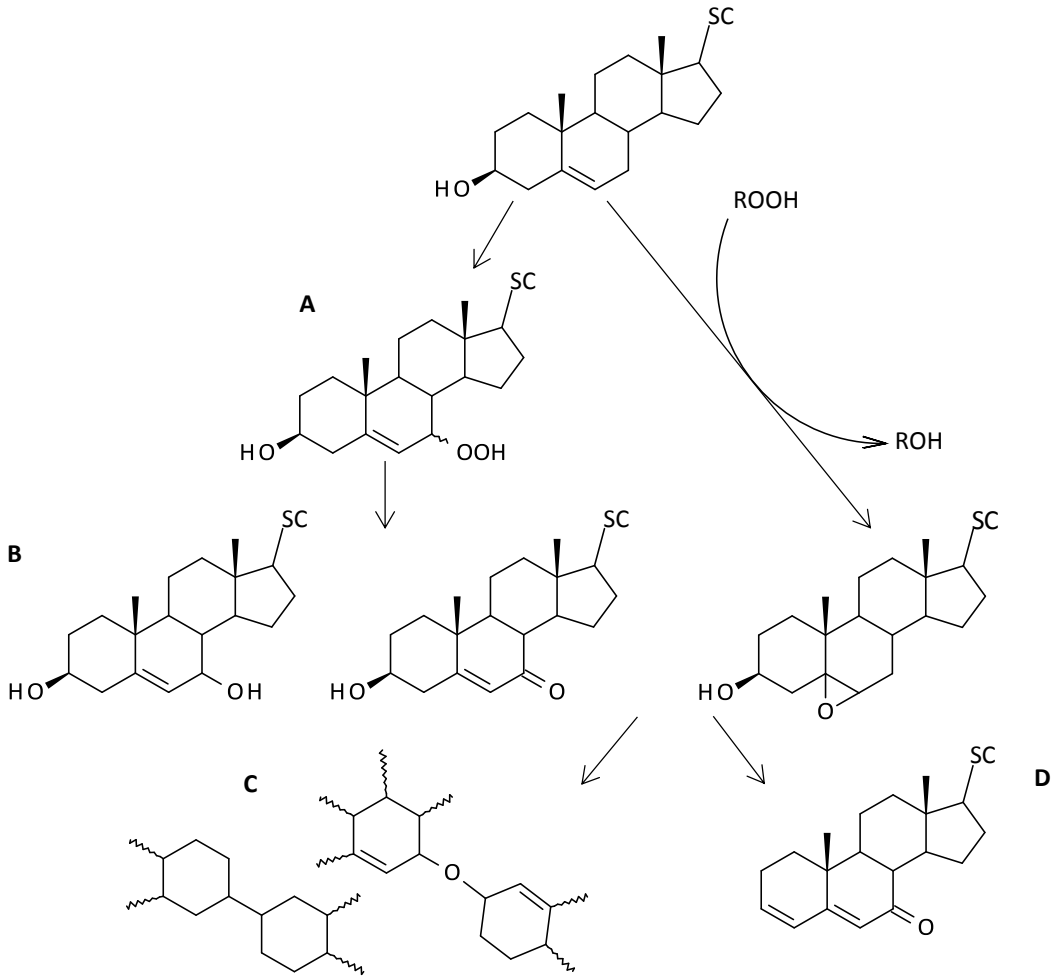

Figure 8. Schematic representation of the formation of the principal oxidation products of phytosterols during autoxidation: primary oxidation; formation of hydroperoxides (**A**), secondary oxidation; formation of hydroxy-, keto-, and epoxysterols (**B**), tertiary oxidation, formation of oligomers, dimers, polymers (**C**), degradation; formation of dienes, trienes (**D**).

However, large parts of the oxidation process of phytosterols still remain to be elucidated. Studies observing the losses of intact phytosterols and the concurrent formation of the secondary oxidation products demonstrated that the decreases of intact sterols exceeded by far the formed amounts of secondary oxidation products (Soupas *et al.*, 2007; Menéndez-Carreño *et al.*, 2008a). In a more comprehensive approach, stigmasterol was heated for 3 h at 180 °C, resulting in a loss of the intact sterol of 61 % (Menéndez-Carreño *et al.*, 2010). Polar, mid-polar, and non-polar oxidation products accounted for 39 % of this loss; the formation of dimers and polymers accounted for further 30 %. This means that there is a gap in the mass balance, leaving 31 % of the stigmasterol loss unexplained.

Even though they represent only a part of the autoxidation process, the polar secondary phytosterol oxidation products are the oxides that are characterized best, both in analytical and physiological regards. Thus, the widespread term “phytosterol oxidation products” (POP) commonly refers to this group of compounds. Structures of the most common and quantitatively dominating phytosterol oxidation products are shown in Figure 9.

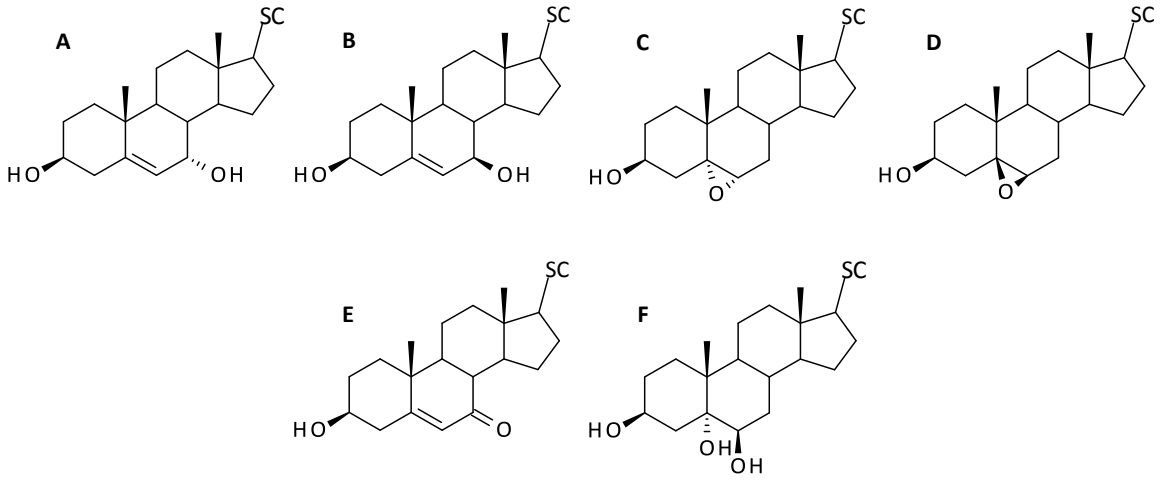

Figure 9. Structures of representative oxidative modifications of phytosterols: 7*α*-hydroxy (**A**), 7*ß*-hydroxy (**B**), 5,6*α*-epoxy (**C**), 5,6*ß*-epoxy (**D**), 7-keto (**E**), 3*ß*,5*α*,6*ß*-triol (**F**).

In contrast to phytosterols, phytostanols are commonly considered to be more stable due to their C5-C6 saturation. However, also saturated lipids have been described to undergo autoxidation, in particular at temperatures >100 °C; this is due to a decreasing selectivity of autoxidation at higher temperatures, as the formed hydroperoxides readily decompose to form hydroxy- and alkoxy radicals, being able to induce hydrogen abstraction also from saturated compounds (Swern *et al.*, 1948; Belitz *et al.*, 2001). Research on the oxidation of stanols is, however, scarce. Some structures have been proposed for saturated derivatives of cholesterol oxidized at C27, C28, and C29 upon incubation with rat liver mitochondrial enzymes (Aringer and Nordström, 1981). For free phytostanols, in addition to side chain oxidation, the generation of 3-keto compounds has been suggested (Dutta, 2004). The most significant advances concerning the identification of potential phytostanol oxidation products were achieved by research from Soupas *et al*. (2004b), who described gas chromatographic properties and electron impact (EI) mass spectra of several secondary oxidation products derived of thermo-oxidized sitostanol standard; the corresponding structures of the quantitatively most important sitostanol oxidation products are compiled in Figure 10.

Based on these data, the sitostanol oxides were quantitated in different heat-treated matrices enriched with either free phytostanols or phytostanyl fatty acid esters (Soupas *et al.*, 2004a; 2005;

2006; 2007). Thereby, 6*α*-hydroxysitostanol and 7*α*-hydroxysitostanol were identified as marker compounds for sitostanol oxidation. Still, oxidation reactions of sitostanol were observed to occur and proceed at a low rate, requiring higher temperatures (>100°C).

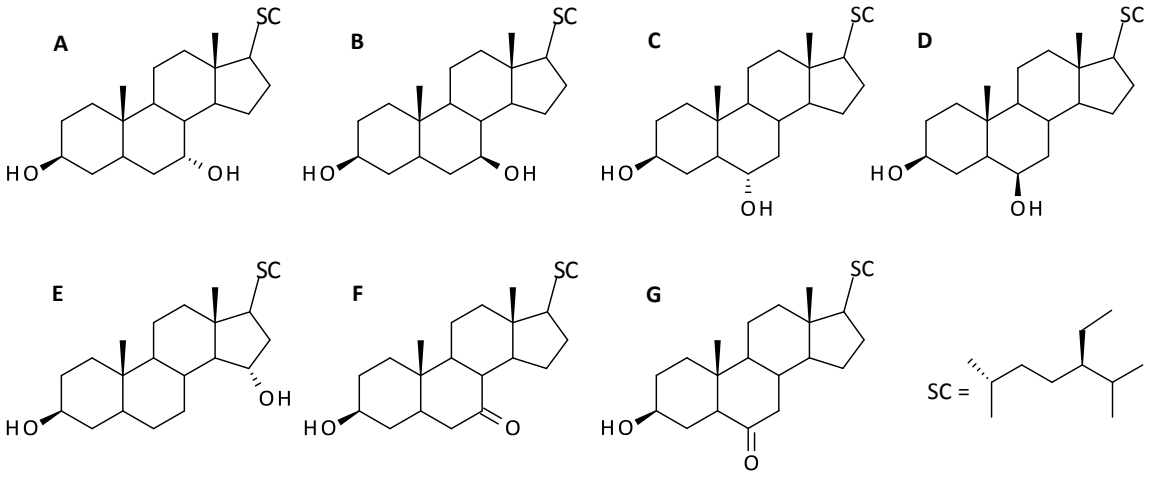

Figure 10. Structures of important sitostanol oxidation products: 7*α*-hydroxysitostanol (**A**), 7*ß*-hydroxysitostanol (**B**), 6*α*-hydroxysitostanol (**C**), 6*ß*-hydroxysitostanol (**D**), 15*α*-hydroxysitostanol (**E**), 7-ketositostanol (**F**), and 6-ketositostanol (**G**).

2.3.1.3 Endogenous Formation of Phytosterol Oxidation Products

The endogenous, enzymatic formation of phytosterol oxidation products has been shown in various *in vitro* experiments. In rat liver mitochondria and fractions, oxidations of both the sterol nucleus and the side chain of *ß*-sitosterol were observed; however, the conversion rates of *ß*-sitosterol were far below those of cholesterol (Subbiah and Kuksis, 1969; Aringer and Eneroth, 1973; 1974; Aringer *et al.*, 1976). In rat liver mitochondria, the side chain hydroxylation of campesterol occurred at a rate similar to cholesterol (Aringer *et al.*, 1976). In addition, as the microbial formation of cholesterol oxidation products in the gut has been observed in humans and rats, such transformation reactions are also being discussed for phytosterol oxidation products (Hwang and Kelsey, 1978; Fioriti *et al.*, 1970).

2.3.1.4 Autoxidation of Phytosteryl/-stanyl Fatty Acid Esters

The oxidative behavior of phytosterols esterified to fatty acids was shown to be different from that of free phytosterols. There are studies suggesting qualitative and quantitative differences in oxidation profiles between free and esterified phytosterols. Apparently, in particular at moderate temperatures at 100 – 150 °C, the autoxidation reactions as determined via peroxide values and secondary phytosterol oxidation products are more pronounced for esterified phytosterols

(Yanishlieva and Marinova, 1980; Yanishlieva-Maslarova *et al.*, 1982; Soupas *et al.*, 2005; Lehtonen *et al.*, 2011). At higher temperatures, the extent of formation of phytosterol oxidation products was comparable (Soupas *et al.*, 2005). As both, the phytosterol moiety and the fatty acid moiety of phytosteryl fatty acid esters are prone to oxidation reactions, the unsaturation degree of the fatty acid moieties may be of influence regarding phytosterol oxidation. Investigating the first sequence of autoxidation, the formation of hydroperoxides, Lehtonen *et al.* (2011) demonstrated with the example of cholesterol esterified to differently saturated fatty acids that esterification with a saturated fatty acid increased the formation of sterol hydroperoxides. However, due to the additional oxidation of the unsaturated fatty acid moieties, the formation of secondary sterol oxidation products was higher for esters of unsaturated fatty acids, the amounts increasing with increasing unsaturation. This is probably to be ascribed to a promotion of the oxidation of the steryl moiety by either intra- or intermolecular radical propagation (Smith, 1987). However, not much is known about the formation of secondary oxidation products of the fatty acid moieties. To date, only one approach for oxidized derivatives of sitosteryl oleate has been described allowing the investigation of secondary oxidative modifications of both, the fatty acid moiety and the steryl moiety of esters in their intact forms (Julien-David *et al.*, 2014). Although several oxidation products were quantitated in heat-treated enriched margarine, again, there was a pronounced gap between the determined losses of intact sitosteryl oleate and the formed amounts of sitosteryl oleate oxidation products.

2.3.2 Occurrence in Foods

2.3.2.1 Non-enriched Foods

Data on phytosterol oxidation products exist for various foods containing phytosterols/-stanols or their esters as naturally occurring constituents. The presence of phytosterol oxidation products in crude vegetable oils and their fate during refining has been analyzed (Bortolomeazzi *et al.*, 2003). The effects of heating of vegetable oils on the formation of phytosterol oxidation products have been studied in model experiments (Oehrl *et al.*, 2001; Johnsson and Dutta, 2006) as well as under industrial frying conditions (Dutta, 1997). Commercial potato crisps (Tabee *et al.*, 2008), potato chips prepared in different vegetable oils (Dutta and Appelqvist, 1997), and French fries prepared in these oils (Dutta, 1997) have been investigated. Sterol oxidation in infant milk formulas and milk cereals (Zunin *et al.*, 1998), and in ready-to-eat infant foods during storage (García-Llatas *et al.*, 2008) have also been studied. As examples, data on selected foods are summarized in Table 1.

Table 1. Contents of phytosterol oxidation products (POP) and corresponding oxidation rates determined in selected non-enriched foods and resulting intakes calculated on the basis of consumption data of the corresponding foods.

type of food	POP [mg/kg]	oxidation rate [%][a]	POP intake [mg/d][b]		reference
			median	95th percentile	
margarine					
63 % fat	13.3	0.4	0.1	0.7	Conchillo *et al.* (2005)
French fries					
oven, 225 °C, 15 min	1.2	0.5	0.08[c]	0.2[c]	Dutta (1997)
pre-fried samples	0.8	1.3	0.06[c]	0.1[c]	Dutta (1997)
restaurant samples	3.4	0.8	0.2[c]	0.5[c]	Dutta (1997)
potato crisps					Tabee *et al*. (2008)
high fat (>25 %)	1.1	0.6	0.02[c]	0.06[c]	Tabee *et al.* (2008)
low fat (<25 %)	1.2	0.8	0.03[c]	0.06[c]	Tabee *et al.* (2008)
chicken					
stir-fried, 6 min, 228 °C[d]	8.8[e]	3.7	0.5	1.3	Lin *et al*. (2016)
potatoes					
shallow-fried, 20 min, 195 °C[d]	11.3[e]	8.1	1.8	4.4	Lin *et al*. (2016)
steak					
shallow-fried, 15 min, 169 °C[d]	25.9[e]	9.8	1.1	3.5	Lin *et al*. (2016)
codfish,					
microwave, 5 min, 600 W[d]	1.3[e]	0.5	0.1	0.2	Lin *et al*. (2016)
muffins					
baking, 25 min, 140 °C	2.5	2.0	0.1	0.3	Lin *et al*. (2016)

[a] Calculated as percentage of POP with respect to the initial phytosterol content.
[b] Calculated on the basis of consumption data for adults among consumers only in Germany (EFSA), except for data labelled by footnote [c].
[c] Calculated on the basis of consumption data for adults among consumers only across European countries (EFSA).
[d] Heating times are the sum of a pre-heating time for heating the margarine and the main cooking time after addition of the ingredient.
[e] POP contents are the sum of the contents determined in the food and the contents determined in the residual fat.

The average content of phytosterol oxidation products in the heat-treated products French fries and potato crisps was around 1 mg/kg (except for the French fries obtained as restaurant samples); this content corresponds to an oxidation rate of approximately 0.8 %. Shallow-frying of potatoes in turn led to a significantly higher oxidation rate of 8 %. The highest contents of after heat treatment of foods (26 mg/kg) were determined for shallow-fried steak, corresponding to an oxidation rate of 9.8 %.

2.3.2.2 Enriched Foods

Information on the contents of phytosterol oxidation products in foods enriched with phytosteryl/-stanyl fatty acid esters is available for several matrices; the data are summarized in Table 2. The analyzed products differ regarding type of enrichment (free phytosterols/-stanols versus phytosteryl esters versus phytostanyl esters), degree of enrichment, and employed treatments (e.g. heat, storage, addition of ingredients). The contents of phytosterol oxidation products determined in conventionally pasteurized milk enriched with free phytosterols or phytosteryl esters were consistently around 2 mg/kg, corresponding to oxidation rates between 0.04 and 0.07 %. The effect of thermal processing on the formation of phytosterol oxidation products in milk was investigated using different heating techniques (Menéndez-Carreño *et al.*, 2008a). The detected contents ranged from 3.1 to 4.8 mg/kg; microwave heating at 900 W for 1.5 min yielded the highest amounts of oxidation products. However, the amounts of phytosterol oxides and the corresponding oxidation rates did not reflect the additionally determined losses of initial phytosterols. For example, heating in the Schaal oven resulted in similar amounts of oxidation products as electric heating, both procedures leading to oxidation rates of 0.1 %. At the same time, the determined loss of initial phytosterols was 4 % after heating in the Schaal oven, but 60 % after electric heating. This confirms the above-mentioned gap in mass balances based on the currently employed analytical procedures when focusing solely on the polar secondary phytosterol oxidation products.

The oxidation rates determined in commercially produced, non-heated margarines enriched with phytosteryl esters (Grandgirard *et al.*, 2004c; Conchillo *et al.*, 2005; Johnsson and Dutta, 2006; Husche *et al.*, 2011; Baumgartner *et al.*, 2013b; Lin *et al.*, 2016) were in the same order of magnitude as those in non-heated milk (Soupas *et al.*, 2006; Menéndez-Carreño *et al.*, 2008a). The effect of heating has been investigated in a liquid spread enriched with phytosteryl esters; treatment at 205 °C for 30 min resulted in a more than 10-fold higher content of phytosterol oxidation products compared to non-heated spreads, corresponding to an oxidation rate of 1.0 % (Unilever, 2003). This is in the same order of magnitude as oxidation rates determined in

experiments investigating the effect of pan-frying at 180 °C on the oxidation of sitosterol in rapeseed oil and liquid margarine enriched with phytosteryl esters (Soupas *et al.*, 2007).

The effects of storage were followed in a dark chocolate enriched with phytosteryl esters; after 5 months at 30 °C, the additionally formed amount of phytosterol oxidation products was low (Botelho *et al.*, 2014). On the other hand, storage of a phytostanyl ester-enriched spread resulted in the highest oxidation rates reported in non-heated enriched spreads (Rudzińska *et al.*, 2014). Another study investigated the effects of storing a milk-based fruit beverage enriched with free phytosterols for 6 months at different temperatures. Interestingly, storage at 4 °C led to higher contents in phytosterol oxidation products compared to storage at 24 °C and 37 °C. However, the overall increase of phytosterol oxidation products was low, and so were the corresponding oxidation rates (<0.1 %).

The data obtained upon storage of spread enriched with phytostanyl esters are surprising as they are based on the determination of phytosterol oxidation products, probably originating from a minor amount of free and esterified phytosterols, their presence being due to an incomplete hydrogenation process. Commonly, it is assumed that phytostanols and their fatty acid esters are less susceptible to oxidation reactions than phytosterols and the corresponding esters due to the completely saturated ring structure (Soupas *et al.*, 2004a; 2005). This was supported by the 10-fold lower oxidation rate, calculated on the basis of the determination of sitostanol oxidation products observed in pasteurized milk enriched with phytostanyl fatty acid esters compared to milk enriched to the same extent with phytosteryl fatty acid esters (Soupas *et al.*, 2006). Similarly, Baumgartner *et al.* (2013b) observed the same low oxidation rate for a phytostanyl ester-enriched margarine, however, on the basis of phytosterol oxidation products only, as no oxidation products of phytostanols were determined.

Table 2. Contents of phytosterol oxidation products (POP) determined in selected enriched foods and meals prepared with enriched foods, and resulting intakes calculated on the basis of a consumption of enriched foods corresponding to 3 g phytosterols per day.

type of food	treatment	POP [mg/kg]	oxidation rate [%][a]	POP intake [mg/d][b]	reference
milk					
free phytosterols (≙0.5 % phytosterols)	pasteurization (127 °C, 2 s)	2.2	0.04	1.3	Soupas *et al*. (2006)
phytosteryl esters (≙0.5 % phytosterols)	pasteurization (127 °C, 2 s)	2.0	0.04	1.2	Soupas *et al*. (2006)
phytostanyl esters (≙0.5 % phytostanols)	pasteurization (127 °C, 2 s)	0.2	0.004	0.1	Soupas *et al*. (2006)
phytosteryl/-stanyl esters (≙0.3 % phytosterols)	pasteurization	2.1	0.07	2.1	Menéndez-Carreño *et al*. (2008a)
phytosteryl/-stanyl esters (≙0.3 % phytosterols)	65 °C, 24 h	3.1	0.10	3.1	Menéndez-Carreño *et al*. (2008a)
phytosteryl/-stanyl esters (≙0.3 % phytosterols)	microwave (900 W, 1.5 min)	4.8	0.16	4.8	Menéndez-Carreño *et al*. (2008a)
phytosteryl/-stanyl esters (≙0.3 % phytosterols)	microwave (900 W, 2.0 min)	3.9	0.13	3.9	Menéndez-Carreño *et al*. (2008a)
phytosteryl/-stanyl esters (≙0.3 % phytosterols)	electric heating (15 min)	3.4	0.11	3.4	Menéndez-Carreño *et al*. (2008a)
margarine					
phytosteryl esters (≙8 % phytosterols)	-	68	0.09	2.6	Grandgirard *et al*. (2004)
phytosteryl esters (≙6 % phytosterols)	-	47	0.07	2.3	Conchillo *et al*. (2005)
phytosteryl esters	-	12	-	-	Johnsson and Dutta (2006)
phytosteryl esters	-	21	-	-	Husche *et al*. (2011)
phytosteryl esters (≙15 % phytosterols)	-	34	0.02	0.7	Baumgartner *et al*. (2013)
phytostanyl esters (≙15 % phytostanols)	-	5.4[c]	0.004	0.1	Baumgartner *et al*. (2013)
phytosteryl/-stanyl esters (≙7.5 % phytosterols)	-	11	0.01	0.4	Lin *et al*. (2016)
phytostanyl esters	-	255[c]	-	9.6[d]	Rudzinska *et al*. (2014)
phytostanyl esters	storage (6 weeks, 4 °C)	354[c]	0.12[d]	13.3[d]	Rudzinska *et al*. (2014)
phytostanyl esters	storage (6 weeks, 20 °C)	734[c]	0.61[d]	27.5[d]	Rudzinska *et al*. (2014)
liquid margarine					
phytosteryl/-stanyl esters (≙7.5 % phytosterols)	heating (205 °C, 30 min)	740	0.99	29.6	Unilever (2003)
phytosteryl esters (≙5 % phytosterols)	pan-frying (180 °C, 5 min)	291[c]	0.99	10.9	Soupas *et al*. (2007)
phytosteryl esters (≙5 % phytosterols)	pan-frying (180 °C, 10 min)	668[c]	1.3	25.1	Soupas *et al*. (2007)

Table 2. continued.

type of food	treatment	POP [mg/kg]	oxidation rate [%][a]	POP intake [mg/d][b]	reference
dark chocolate					
phytosteryl esters	-	69	-	2.9[d]	Botelho *et al*. (2014)
phytosteryl esters	storage (5 months, 30 °C)	71	0.003[d]	3.0[d]	Botelho *et al*. (2014)
milk-based fruit beverage					
free phytosterols/-stanols (≙0.67 % phytosterols)	pasteurization (100-115 °C, 15-30 s)	2.4	0.05	1.1	González-Larena *et al*. (2015)
free phytosterols/-stanols (≙0.67 % phytosterols)	storage (6 months, 4 °C)	3.8	0.07	1.7	González-Larena *et al*. (2015)
free phytosterols/-stanols (≙0.67 % phytosterols)	storage (6 months, 24 °C)	3.4	0.06	1.5	González-Larena *et al*. (2015)
free phytosterols/-stanols (≙0.67 % phytosterols)	storage (6 months, 37 °C)	3.7	0.06	1.7	González-Larena *et al*. (2015)
food prepared using enriched margarine					
phytosteryl/-stanyl esters (≙7.5 % phytosterols)					
cabbage	stir-frying (6 min, 260 °C)[e]	54	0.43	13.0	Lin *et al*. (2016)
chicken	stir-frying (6 min, 241 °C)[e]	236[g]	1.88	56.5	Lin *et al*. (2016)
onions	shallow-frying (11 min, 162 °C)[e]	21[g]	0.08	2.5	Lin *et al*. (2016)
potatoes	shallow frying (20 min, 199 °C)[e]	215[g]	2.96	88.8	Lin *et al*. (2016)
salmon	shallow-frying (15 min, 194 °C)[e]	170[g]	1.37	41.1	Lin *et al*. (2016)
beef	roasting (35 min, 140 °C)[e,f]	20[g]	0.35	10.4	Lin *et al*. (2016)
codfish	microwave (5 min, 600 W)	2.6[g]	0.02	0.6	Lin *et al*. (2016)
muffins	baking, (25 min, 140 °C)	4.3	0.07	2.1	Lin *et al*. (2016)
sponge cake	baking (60 min, 155 °C)	12	0.06	1.8	Lin *et al*. (2016)

[a] Calculated as percentage of POP with respect to the initial phytosterol content.
[b] Calculated on the basis of consumptions corresponding to 3 g phytosterol per day.
[c] Only phytosterol oxidation products were determined.
[d] Calculated on the basis of an experimentally determined degree of enrichment.
[e] Heating times are the sum of a pre-heating time for heating the margarine and the main cooking time after addition of the ingredient.
[f] Roasting had an additional initial shallow frying step.
[g] POP contents are the sum of the contents determined in the food and the contents determined in the residual fat.

A recent study investigated the formation of phytosterol oxidation products upon the preparation of different meals using an enriched margarine suitable for cooking and baking (Lin *et al.*, 2016). The extent of the formed amounts was shown to be strongly dependent on the food(s) prepared with the enriched margarine. For example, stir-fried chicken exhibited 4-times higher amounts of POP than stir-fried cabbage, under comparable frying conditions. Shallow-frying of potatoes led to the highest determined amounts of POP among all experiments, while baking a dough to which enriched margarine had been added led to the least formation of phytosterol oxidation products. Even if the absolute amounts of phytosterol oxidation products detected in this study for the preparation of meals using the enriched margarine were higher compared to the amounts formed in similar experiments using the non-enriched margarine, interestingly, the oxidation rates were observed to be higher for the non-enriched margarine (cf. Table 1).

The available data demonstrate the complexity of the processes underlying the oxidation of phytosterols/phytostanols and their esters added to foods. The determined concentrations of phytosterol oxidation products are the sums of keto-, hydroxy-, and epoxy-compounds. The interpretation of the data is hampered by the fact that the employed analytical methods are not standardized; thus, the actually covered phytosterol oxidation products may differ not only quantitatively but also qualitatively. Further, intra- and intermolecular reactions, e.g. the promotion of oxidation of the stanol moieties by oxidized fatty acid moieties may influence the formation of phytosterol oxidation products (Soupas *et al.*, 2005). Therefore, not only the initial phytosterol/phytostanol composition, but also the composition of the fatty acid moieties should actually be taken into account when assessing the oxidative potential of an enriched food and the potentially resulting loss of functional ingredients.

2.3.3 Estimation of Dietary Exposure to Phytosterol Oxidation Products

The available data on the occurrence of POPs in both, non-enriched and enriched foods, and the consumption of foods enriched with phytosteryl/-stanyl fatty acid esters have been used in two approaches to estimate the dietary exposure to phytosterol oxidation products.

One approach is based on (i) the use of the experimentally determined contents of phytosterol oxidation products in thermally treated enriched foods and (ii) the assumption that the upper daily intake of phytosterols/phytostanols of 3 g is achieved by consuming one of these foods. The daily intakes of phytosterol oxidation products resulting from the consumption of the respective serving sizes corresponding to 3 g phytosterols are given for the different enriched foods in Table 2. For non-heated foods (spreads, milk, dark chocolate, and milk-based fruit beverage), the intakes of phytosterol oxidation products range from 0.4 to 2.9 mg/d. Upon heating, the intake is

increased to 3.1 – 4.8 mg/d for milk and to 29.6 mg/d for liquid spread for cooking and baking. The intakes resulting from the preparation of true meals using an enriched margarine varied greatly, ranging from 0.6 mg/d upon microwave-heating of codfish to 88.8 mg/d upon the consumption of two portions of shallow-fried potatoes.

Another approach is based on (i) the use of data on the dietary exposure to phytosterols estimated from surveys on the consumption of enriched foods (Lea and Hepburn, 2006; Hearty *et al.*, 2009; Sioen *et al.*, 2011; Willems *et al.*, 2013) and (ii) the assumption of a minimum (0.1 %) and a maximum (1.0 %) oxidation rate. As shown in Table 3, the mean intakes of phytosterol oxidation products resulting from the application of this approach (0.35 – 2.45 mg/d for a minimum and 3.5 – 24.5 mg/d for a maximum oxidation rate) are in a similar order of magnitude as those determined on the basis of the previously mentioned estimate.

A comparison of the estimated intakes of phytosterol oxidation products from enriched foods to those resulting from non-enriched foods (Table 1) shows significantly higher intakes to be expected from the consumption of foods with added phytosterols/phytostanols and their esters. Despite oxidation rates partly being even higher in non-enriched foods, the higher initial amounts of phytosterols compensate for the lower oxidation rates, still leading to higher total amounts of oxidation products formed thereof and thus resulting in higher daily intakes. As shown in Table 2, this increase in intake is particularly pronounced for enriched foods subjected to heating processes.

In order to estimate the intake of phytosterols from multiple sources, a worst-case model simulating prospective phytosterol intake has been developed (Kuhlmann *et al.*, 2005), thereby assuming that the consumer does not follow the recommendations on the label. Using the German National Food Consumption Study, 0.3 – 2 g phytosterols were hypothetically added to the usual daily servings of ten different food products, selected from novel foods applications; the prospective phytosterol intake was calculated by stepwise accumulation of different functional foods in three enrichment scenarios. According to the worst-case in this model, an enrichment of 2 g phytosterols per proposed serving size would result in a maximum intake of 13 g/d. Assuming again oxidation rates of 0.1 and 1 %, respectively, this would result in dietary exposures to POP of 13 mg/d and 130 mg/d, respectively.

Table 3. Intake of phytosterol oxidation products (POP) based on consumption of enriched foods.

phytosterol intake [g/d]		POP intake [mg/d]				
		oxidation rate 0.1 %		oxidation rate 1.0 %		
mean	95th percentile	mean	95th percentile	mean	95th percentile	reference
0.35-0.86	1.06-3.70	0.35-0.86	1.06-3.70	3.5-8.6	10.0-37.0	Willems *et al*. (2013)[a,b]
0.24-0.96[d]	1.68-2.64	0.24-0.96	1.68-2.64	2.4-9.6	16.8-26.4	Lea *et al*. (2006)[a,b]
2.45	5.48	2.45	5.48	24.5	54.8	Hearty *et al*. (2009)[c]
1.51	4.20	1.51	4.10	15.1	42.0	Sioen *et al*. (2011)[c]

[a] Intake calculated on the basis of purchases per household.
[b] Data from The Netherlands, United Kingdom, Germany, and Belgium.
[c] Intake calculated on the basis of purchases per consumer.
[d] Data representing the median daily intake.

2.3.4 Uptake of Phytosterol Oxidation Products from the Diet

2.3.4.1 Animal Studies

Intragastric administration of two of the main classes of phytosterol oxidation products (7-keto- and epoxides; 5 mg in 1 mL of triolein) to mesenteric duct-cannulated adult male rats revealed that the lymphatic absorption rate of 7-keto-sitosterol (1.4 %) was similar to that of sitosterol (1.2 %). Epoxy-derivatives showed the highest lymphatic absorption rates (e.g. *α*-epoxy-sitostanol: 2.7 % and *ß*-epoxy-campestanol: 7.9 %), whereby campesterol oxidation products were generally better absorbed than the respective sitosterol derivatives (Grandgirard *et al.*, 1999).

Administration of an AIN-93G-based diet to thoracic duct-cannulated rats (2.5 g cholesterol/kg diet or 2.5 g cholesterol/kg diet + 2.5 g phytosterols or phytosterol oxidation products/kg diet) confirmed the low lymphatic absorption rates of phytosterols (sitosterol: 2.2 %, campesterol: 5.5 %) when compared to cholesterol (37.3 %). However, it revealed that the lymphatic absorption rates of oxidation products of sitosterol (9.1 %) and campesterol (15.9 %) were actually higher than those of the parent phytosterols (Tomoyori *et al.*, 2004).

A mixture of phytosterol oxidation products was fed to hamsters for 2 weeks and their concentrations were followed in plasma, aorta, liver, kidneys and heart (Grandgirard *et al.*, 2004a). At the two highest doses (500 mg/kg diet and 2500 mg/kg diet), phytosterol oxidation products were detectable in all investigated tissues. However, the proportion changed after intake: The levels of campesterol oxidation products were higher than those of the sitosterol oxidation products in plasma, while the amount of 7-ketositosterol, which was the dominating phytosterol oxidation product in the diet, was very low in blood. In contrast to plasma, sitostanetriol was the major phytosterol oxidation product detected in the tissues.

Similar differences of administered phytosterol oxidation products (1 g/kg diet) were observed in a 6-week feeding study with hamsters (Liang *et al.*, 2011). In the employed dietary mixtures of sitosterol and stigmasterol oxidation products, the 7-keto-derivatives dominated, whereas in plasma only the 7*α*- and 7*ß*-hydroxy-derivatives and in liver 7*α*- and 7*ß*-hydroxy- as well as the 5,6*α*- and 5,6*ß*-epoxides were detected.

2.3.4.2 Human Studies

The occurrence of oxidized plant sterols in human serum was first described for phytosterolemic patients (Plat *et al.*, 2001). Following this report, several studies reporting the presence of phytosterol oxidation products in plasma of healthy human subjects have been published. Taken

together, these studies indicate that POP determined in human plasma differ significantly in the amounts and type of oxidation (Table 4).

Table 4. Baseline levels of phytosterol oxidation products (POP) determined in human plasma/serum.

	POP in human plasma/serum [μM], year				
	2013[a]	2012[b]	2011[c]	2008[d]	2004[e]
7α-hydroxybrassicasterol	-	0.0007	-	-	-
7α-hydroxycampesterol	0.0002	0.006	0.0002	-	-
7α-hydroxystigmasterol	-	0.008	-	-	-
7α-hydroxysitosterol	0.0005	0.01	0.0004	0.11	-
7β-hydroxybrassicasterol	-	0.0006	-	-	-
7β-hydroxycampesterol	0.0008	0.004	0.0004	-	-
7β-hydroxystigmasterol	-	0.003	-	0.11	
7β-hydroxysitosterol	0.003	0.008	0.003	-	-
α-epoxysitostanol	-	-	-	-	0.01
β-epoxysitostanol	-	-	-	-	0.13
campestanetriol	-	-	-	-	0.01
sitostanetriol	-	-	-	-	0.09
7-ketocampesterol	0.001	0.002	0.001	-	-
7-ketostigmasterol	-	0.002	-	-	-
7-ketositosterol	0.006	0.004	0.007	-	0.01
total POP	0.011	0.05	0.012	0.22	0.26

[a] Baumgartner *et al*. (2013)
[b] Menéndez-Carreño *et al*. (2012)
[c] Husche *et al*. (2011)
[d] Menéndez-Carreño *et al*. (2008b)
[e] Grandgirard *et al*. (2004)

The earlier GC/MS-based studies only reported the presence of α- and β-epoxy- and triol-derivatives (Grandgirard *et al.*, 2004b) or the presence of 7α- and 7β-hydroxy-derivatives (Menéndez-Carreño *et al.*, 2008b). The largest spectrum of phytosterol oxidation products (in total: 11) was detected by applying GCxGC/TOF (Menéndez-Carreño *et al.*, 2012). Two studies based on isotope dilution GC/MS quantified six phytosterol oxidation products, 7-keto- and 7β-hydroxysitosterol being the major representatives (Husche *et al.*, 2011; Baumgartner *et al.*, 2013b). These studies reported similar ranges of the detected phytosterol oxidation products in two panels of 16 and 43 healthy volunteers, respectively; the determined concentration ranges of individual phytosterol oxidation products were 0.07 - 3.01 ng/ml serum (0.0002 – 0.007 μM) (Husche *et al.*, 2011) and 0.09 – 2.49 ng/ml plasma (Baumgartner *et al.*, 2013b).

There are only three studies available providing comparative data on the levels of phytosterol oxidation products before and after consumption of phytosteryl ester-enriched margarine. In the first study involving 16 human subjects consuming 3 g phytosterols/d via a margarine enriched with phytosteryl esters for 28 days, there were significant increases in the serum concentrations

of campesterol (from 2.82±1.44 μg/mL [7.0±3.6 μM] before to 4.19±1.55 μg/mL [10.5±3.9 μM] after the dietary intervention) and sitosterol (2.06±1.27 μg/mL [5.0±3.1 μM] before and 4.30±1.89 μg/mL [10.4±4.6 μM] after the dietary intervention) (Husche *et al.*, 2011). Among the detected phytosterol oxidation products, 7*ß*-hydroxysitosterol was the major representative in the consumed margarine (8.62±0.28 ng/mg). For this phytosterol oxidation product, a statistically significant increase (87 %) of the serum concentration from 1.20±0.54 ng/mL (0.003±0.001 μM) (before consumption of the margarine) to 2.24±1.25 ng/ml (0.005±0.003 μM) (after consumption of the margarine) was observed. In addition, there was a highly significant correlation between the serum levels of campesterol and the sum of 7-oxygenated campesterol (r^2=0.915; p<0.001) and sitosterol and the sum of 7-oxygenated sitosterol (r^2=0.915; p<0.001).

In a second randomized, double-blind cross-over study 43 healthy subjects consumed a margarine enriched with phytosteryl esters, a margarine enriched with phytostanyl esters and a control margarine, each of them for 4 weeks, separated by wash-out periods of four weeks; the consumption of the enriched margarines corresponded to intakes of 3 g/d of sterols and stanols, respectively (Baumgartner *et al.*, 2013b). Compared to control, the serum LDL-cholesterol concentrations were reduced after consumption of the phytosteryl ester-enriched (-8.1 %) and the phytostanyl ester-enriched margarines (-7.8 %). The consumption of the phytosteryl ester-enriched margarine did not result in changes of the fasting plasma concentrations of phytosterol oxidation products, the individual oxidation products ranging from 0.09 to 2.49 ng/ml (0.0002 – 0.006 μM) plasma before and from 0.09 to 2.35 ng/mL (0.0002 - 0.006 μM) plasma after the dietary intervention. On the other hand, the intake of the phytostanyl ester-enriched margarine reduced the fasting serum concentration of 7*ß*-hydroxycampesterol by 0.07 ng/mL compared with the control (~14 %) and the phytosteryl ester-enriched margarine (~15 %). The reason for the apparently inconclusive data regarding the concentrations of phytosterol oxidation products in plasma after consumption of enriched margarine obtained in the same laboratory using the same method remains unclear.

The second study revealed large variations in the baseline POP concentrations among the study subjects; however, they remained relatively stable over time. Fasting serum concentrations of (non-oxidized) sitosterol and campesterol did not correlate with fasting plasma concentrations of sitosterol and campesterol oxidation products during any of the three interventions (Baumgartner *et al.*, 2013a). Six subjects could be arbitrarily classified as having consistently low or high plasma POP concentrations. This differentiation into “low and high oxidizers” was also reflected in oxidized LDL concentrations. However, oxidative and anti-oxidative capacity markers, such as

iron/copper status, α-tocopherol concentrations and TEAC values could not explain these differences.

A subgroup (n=10) of the subjects that participated in the second study additionally consumed a mixed meal after the 28 day intervention period; the mixed meal was either non-enriched (control), phytosteryl ester-enriched, or phytostanyl ester-enriched, according to the respective composition of the margarine consumed during the previous 28 day intervention (Baumgartner *et al.*, 2015). After 4 h, a second, non-enriched mixed meal was consumed. As humans are in a non-fasting state for most part of the day, the effects of the intake of enriched meals on plasma concentrations of phytosterol oxidation products was investigated in a postprandial state. While the consumption of a non-enriched mixed meal and the consumption of the phytostanyl ester-enriched meal showed no effects, the consumption of the phytosteryl ester-enriched mixed meal led to significantly higher serum concentrations of 7*ß*-hydroxycampesterol and 7*ß*-hydroxysitosterol, however only after consumption of the second, non-enriched meal. Also, the concentrations of non-oxidized campesterol and sitosterol were increased. Interestingly, in plasma the concentration of 7*ß*-hydroxycampesterol was 2.2-fold higher than that of 7*ß*-hydroxysitosterol, while in the plant sterol ester-enriched mixed meal, 7*ß*-hydroxysitosterol was concentrated 4-fold in comparison to 7*ß*-hydroxycampesterol. This is further complicated by the fact that in the fasting control state, the plasma concentrations of 7*ß*-hydroxysitosterol were higher than those of 7*ß*-hydroxycampesterol. Based on these observations, the postprandial increase in serum concentrations of 7*ß*-hydroxysterols might be based either on a differential absorption of the oxidation products from the diet or on a distinct endogenous formation of certain species of phytosterol oxidation products, or on a combination of both effects.

In a recent study, the concentrations of the phytosterols campesterol and sitosterol and their oxidation products were determined in plasma and aortic valve cusps of patients with severe aortic stenosis (Schött *et al.*, 2014). The absolute and cholesterol-corrected levels of campesterol and sitosterol in plasma, in the aortic valve cusps, and between both compartments showed a strong correlation. In contrast, the correlation between the concentrations of the phytosterols and those of the corresponding phytosterol oxidation products in plasma and the correlation between the phytosterol oxidation products levels in plasma and those in aortic valve cusps were only weak. Moreover, the concentrations of plant sterols and those of their 7-oxidized metabolites in the tissue of aortic valve cusps significantly correlated. The authors speculated that the latter finding could relate to local inflammatory processes in atherosclerotic plaques and tissues, which generate free radicals and trigger oxidation processes.

The different routes of formation, i.e. enzyme-catalyzed vs. chemical oxidations, are expected to be reflected in differences between the spectra of endogenously formed phytosterol oxidation products and those ingested via the diet.

2.3.5 Biological Effects of Phytosterol Oxidation Products

2.3.5.1 Genotoxicity

Genotoxicity was assessed *in vitro* using a heat-treated, re-crystallized mixture of phytosterols containing approximately 30 % phytosterol oxidation products (Lea *et al.*, 2004). According to the results obtained from a bacterial mutation assay, a chromosome aberration assay and a micronucleus assay, phytosterol oxides are not considered to possess genotoxic potential. A study employing fractions isolated from thermo-oxidized *ß*-sitosterol confirmed that individual phytosterol oxidation products did not show mutagenic activity towards *Salmonella typhimurium* strains (Koschutnig *et al.*, 2010). No evidence of genotoxic effects *in vivo* was observed in a flow cytometer-based micronucleus assay in murine erythrocytes after intraperitoneal injection of mixtures of phytosterol epoxides or phytosterol triols (Abramsson-Zetterberg *et al.*, 2007).

2.3.5.2 Subchronic Toxicity

A 90-day feeding study in rats was performed using a heat-treated mixture of phytosterols containing approximately 30 % phytosterol oxidation products (Lea *et al.*, 2004). Rats were fed a control diet without added sterols or diets with either steryl esters (5.6 %) or steryl esters supplemented with 0.2, 0.6 or 1.6 % of this mixture of POP. There were no effects on behavior, food and water consumption, ophthalmoscopy, urinalysis and renal concentrating ability, gross necropsy and histopathology. At the highest dose tested, there were significant changes when compared to the control and the diet containing only steryl esters; they comprised a slight reduction in body weight (females), slight increases in the thrombocyte count (males) and decreases in the haemoglobin level, packed cell volume and mean corpuscular volume (females), slight decreases in the glucose level and increases in the albumin level as well as the albumin:globulin ratio (males), increases in *γ*-glutamyl transferase activity (females), reduced triglyceride and phospholipid levels (both sexes), and a slight increase in liver weight (females). None of these findings were supported by histopathological changes. According to these findings, a no adverse observed effect level (NOAEL) based on the mid dose (0.6 % of the mixture of POP in the diet) was established at an estimated dietary level of POP of 128 mg/kg/d for males and 144 mg/kg/d for females.

2.3.5.2 Cytotoxicity and Proinflammatory Potential

Numerous *in vitro* experiments incubating various cell types and cell lines with mixtures of oxides or synthesized pure oxides showed POP to exert cytotoxic effects, which are qualitatively similar to those observed for cholesterol oxidation products, but higher concentrations were required (>60 μM) (Adcox *et al.*, 2001; Maguire *et al.*, 2003; O'Callaghan *et al.*, 2010; 2013; Vejux *et al.*, 2012; Gao *et al.*, 2015). The assessment of markers indicative of inflammatory and/or apoptotic cellular mechanisms demonstrated a reduction of cell viability as well as the generation of oxidative stress and related processes thereof, 7*ß*-hydroxy- and 7-keto-derivatives exhibiting the highest cytotoxic potential among those oxides dominating in foods. However, not only the type of oxygenation seems to be of relevance, but also the structure of the phytosterol side chain was shown to determine the cytotoxic activity (Gao *et al.*, 2015). Also, a different behavior of mixtures of phytosterol oxidation products as compared to single substances should be considered (Gao *et al.*, 2015).

One study investigated the release of the cytokines tumor necrosis factor *α* (TNF*α*), interleukin (IL)-8 and IL-10 in the intestinal epithelial cell line Caco-2 upon addition of 7-ketocholesterol and 7-ketostigmasterol at a concentration of 60 μM (Alemany *et al.*, 2013). It was shown that 7-ketostigmasterol significantly increased the release of the three above-mentioned cytokines. Moreover, the amounts of the pro-inflammatory mediators TNF*α* and IL-8 released upon incubation with 7-ketostigmasterol were significantly higher than those secreted upon addition of 7-ketocholesterol. A further experiment was performed, using the same substances and concentrations, investigating the corresponding proteome changes in Caco-2 cells (Laparra *et al.*, 2015). For example, incubation with 7-ketostigmasterol induced increases in proteins associated with inflammatory response, cell proliferation and cell signaling processes, whereas proteins participating in immune response, and cell survival processes were decreased.

One *in vivo* study investigated the effect of mixtures of sitosterol oxidation products or stigmasterol oxidation products after being injected at a concentration of 5 μM into mealworms (Meyer *et al.*, 1998). In accordance with the *in vitro* observations, the administered phytosterol oxidation products were shown to be cytotoxic, thereby inducing an increase in mealworm mortality, but their activities were five times lower than those of cholesterol oxidation products.

2.3.5.3 Pro-Atherogenic Effects

In vivo-generated as well as dietary cholesterol oxidation products have been shown to be closely associated to atherosclerotic processes (Leonarduzzi *et al.*, 2002; Staprans *et al.*, 2005; Alemany *et al.*, 2014). Regarding potential pro-atherogenic effects of phytosterol oxides, Yang *et al*. (2013)

analyzed *in vitro* the effect of sitosterol and sitosterol oxidation products on the acetylcholine (ACh)-induced relaxation of rat aortae, a marker of vascular health, by isometric tension measurements. Whereas sitosterol did not impair vasorelaxation, sitosterol oxidation products significantly attenuated ACh-mediated relaxation at a concentration of 1 μg/mL (2.3 μM). This effect observed *in vitro* was considered by the authors to be an indicator for a pro-atherogenic potential of phytosterol oxidation products and was ascribed to an increased production of reactive oxygen species (ROS).

However, data on this issue are inconclusive when considering the available *in vivo* experiments. An *in vivo* study in ApoE-deficient mice could not establish a correlation between dietary administered POP (0.2 g/kg diet) for nine weeks and serum cholesterol concentrations as well as the size of atherosclerotic plaques when compared to a phytosterol-supplemented diet (0.2 g/kg diet) (Tomoyori *et al.*, 2004). Recently, Weingärtner *et al.* (2015) investigated the vascular effects of intraperitoneal injections of 1 mg/d cholesterol, sitosterol, 7*ß*-hydroxycholesterol, and 7*ß*-hydroxycholesterol in ApoE$^{-/-}$ mice for four weeks compared to a control group. Only 7*ß*-hydroxysitosterol application resulted in an increase of the respective plasma levels; additionally, this was closely associated with an increased production of ROS in the aortic tissue. However, the other outcome parameters that were considered such as endothelial function and early atherosclerosis were not affected. A recent study by Plat *et al.* (2014b) investigated the effects of a western-like diet containing 0.25 % cholesterol compared to the same diet in which 10 % of the cholesterol had been replaced by cholesterol oxidation products or dietary POP in LDLR$^{+/-}$ mice for 35 weeks. Concerning the lesion size, no differences could be observed among the three groups, confirming the results obtained by the above-mentioned study by Tomoyori *et al* (2004). However, there was a significantly higher proportion of severe atherosclerotic lesions not only in the mice having been fed the diet containing cholesterol oxidation products but also in the group having received the phytosterol oxidation products. Luister *et al.* (2015) compared the levels of oxidized phytosterols in plasma and aortic valve cusps in patients with severe aortic stenosis with and without concomitant coronary artery disease. Campesterol oxidation products in aortic valve cusps and oxidized sitosterol-to-cholesterol ratio in plasma were higher in patients with concomitant coronary artery disease, supporting the notion of a pro-atherogenic potential of POP. As in these patients also the campesterol-to-cholesterol ratio in plasma as well as the absolute concentrations of campesterol and sitosterol in aortic valve cusps were increased (cf. section 2.2.2), local endogenous oxidation processes might be the origin of the phytosterol oxidation products.

2.3.5.4 Loss of Anti-Atherogenic Properties

Besides potential pro-atherogenic effects, a lower anti-atherogenic potency of phytosterols due to oxidation when compared to non-oxidized phytosterols is being discussed. Such an effect would be of particular relevance considering the functionality and efficacy of phytosterol-enriched foods.

Tomoyori *et al.* (2004) showed that ApoE-deficient mice had elevated cholesterol oxidation product serum levels after having been fed a diet containing POP (0.2 g/kg diet) for nine weeks in comparison to a phytosterol-fed control group (0.2 g/kg diet). Furthermore, Liang *et al.* (2011) fed diets containing either 1 g/kg phytosterols (sitosterol/stigmasterol) or 1 g/kg of the corresponding oxidation products to male hamsters for six weeks. The aortic plaque size and aortic cholesterol levels were reduced in the animals having received the phytosterol diets when compared to a control group; these effects were not observed upon consumption of the diets containing POP. In addition, aortic contractions in response to ACh stimulation were significantly reduced in the phytosterol-fed group if compared to the animals fed a control diet. In turn, the aortae of the animals having consumed POP exhibited contractions as strong as the control group, indicating that oxidation results in a loss of the cardio-protective properties of phytosterols.

2.3.5.5 Potential Impact of Phytosterol Oxidation Products on Intestinal Cholesterol Transporters

The NPC1L1 protein is known to play an essential role for active uptake and absorption of cholesterol in the intestine. An inhibition of cholesterol uptake via this transporter by phytosterols/-stanols has been discussed as one of the mechanisms underlying their cholesterol-lowering properties (de Smet *et al.*, 2012). Incubating Caco-2 cells with either 7-ketocholesterol or 7-ketostigmasterol (60 μM) showed no effect on the expression of NPC1L1 protein compared to non-treated cells (Alemany *et al.*, 2013). This is in agreement with data in hamsters in which neither the dietary administration of phytosterols (1 g sitosterol/stigmasterol per kg diet) nor that of the corresponding oxidation products (1 g/kg) for six weeks resulted in an altered expression of this transporter (Liang *et al.*, 2011). Both studies also investigated the potential impact of POP on the active secretion of cholesterol back into the intestinal lumen, a process that is mediated by the two half transporters ABCG5 and ABCG8 (de Smet *et al.*, 2012). In the *in vitro* study, a downregulation of ABCG5 mRNA was induced by both 7-ketocholesterol and 7-ketostigmasterol (Alemany *et al.*, 2013). In the *in vivo* study in hamsters this effect was observed not only for POP but also for the intact phytosterols (Liang *et al.*, 2011). A downregulation of ABCG5 would lead to an increased intracellular cholesterol concentration and thus an enhanced availability of

cholesterol for esterification and incorporation into chylomicrons; however, in both studies no effect on the expression of ABCG8 mRNA was observed. Taken together, the data on the impact of phytosterol oxidation products on the active intestinal transport of cholesterol are still limited.

2.3.5.6 Potential Impact of Phytosterol Oxidation Products on the Hydrolysis of Phytosteryl Esters

The essential pre-condition for the cholesterol lowering properties of phytosterols added to foods as their fatty acid esters is the intestinal cleavage of the ester bonds. In a mechanistic study Julien-David *et al.* (2008) determined the impact of oxidation on the *in vitro* activity of pancreatic cholesterol esterase using sitosteryl oleate and the oxidation products 7-ketositosteryl oleate and sitosteryl-9,10-dihydroxystearate as substrates. As shown for 7-ketositosteryl oleate, the oxidation of the sterol moiety led to an increased affinity to the cholesterol esterase and a faster conversion when compared to sitosteryl oleate. In contrast, the oxidative modification of the fatty acid moiety leading to sitosteryl-9,10-dihydroxystearate resulted in an almost complete loss of hydrolysis. In addition, in the presence of sitosteryl-9,10-dihydroxystearate the hydrolysis rate of sitosteryl oleate was significantly decreased.

2.4 Analytical Methods

2.4.1 Analysis of Intact Phytosteryl/-stanyl Fatty Acid Esters

Currently, a number of methodologies for the analysis of naturally occurring or added phytosterols/-stanols in foods are available. The most common methods involve extraction of the lipid fraction from the sample material using non-polar solvents. Subsequent alkaline hydrolysis liberates the sterols from their esters, and the free sterols can be extracted as part of the unsaponifiable matter. Frequently, a further clean-up of the extract is applied, via TLC, SPE, or column chromatography, followed finally by chromatographic analysis of the individual compounds (Piironen *et al.*, 2000a; Abidi, 2001; Toivo *et al.*, 2001; Moreau *et al.*, 2002; Lagarda *et al.*, 2006; Inchingolo *et al.*, 2014). While a determination of the total plant sterol content may be appropriate in certain cases, the loss of information concerning the fatty acid composition as well as the phytosterol and phytostanol pattern within the esters is a drawback of these approaches. However, a qualitative and quantitative analysis of phytosteryl/-stanyl fatty acid esters in their intact forms is not trivial.

2.4.2.1 Sample Preparation

As far as complex matrices are concerned, it is advisable to isolate the intact esters prior to chromatographic analysis to avoid matrix interferences. In order to analyze intact steryl esters in cocoa butter, Kamm *et al.* (2001) used the on-line coupling of LC and GC for LC-based pre-separation of the steryl esters from interfering matrix lipids and subsequent GC-based analysis of the intact esters. Employing the same instrumental approach, Miller *et al.* (2003) analyzed γ-oryzanol, a mixture of phytosteryl ferulates, in rice lipids. Similarly, for the analysis of intact phytostanyl fatty acid esters in enriched lipid-based foods, Barnsteiner *et al.* (2011) applied an on-line LC-GC-based methodology using a phytostanyl ester-enriched margarine as example. Prior to on-line LC-GC analysis, the lipids were extracted using organic solvents, such as a mixture of *n*-hexane and methyl *tert*-butyl ether (MTBE) for the extraction of lipids from the enriched margarine (Barnsteiner *et al.*, 2011). Alternatively, for fat-based matrices, solid phase extraction procedures have been described for efficiently separating intact phytosteryl and phytostanyl fatty acid esters from matrix di- and triglycerides in plant lipid extracts using aminopropyl-modified silica material (Oelschlägel *et al.*, 2012; Esche *et al.*, 2012; Firl, 2016). Owing to a reduced polarity of the aminopropyl material compared to pure silica, it enables a separation of the esters and triglycerides. Based on establishing Van-der-Waals interactions between the propyl groups and

the triglycerides, the triglycerides are being retained, thus allowing a selective elution of the phytosteryl/-stanyl fatty acid esters (Oelschlägel *et al.*, 2012).

For protein-based food matrices in turn, such as skimmed milk-drinking yoghurts enriched with phytosteryl and/or phytostanyl fatty acid esters, a miniaturized Weibull-Stoldt methodology based on acid hydrolysis of the protein matrix was developed, followed by extraction of the intact esters using organic solvents (Barnsteiner *et al.*, 2011; 2012).

As far as enriched foods are concerned, there are also products available exhibiting both, high fat and protein content. For these types of matrices, Esche *et al*. (2013a) described a combination of (i) acid digestion according to Barnsteiner *et al.* (2011; 2012), (ii) subsequent lipid extraction and (iii) on-line LC-GC analysis for analysis of intact phytosteryl/-stanyl fatty acid esters in enriched milk and cheese-based spread.

2.4.2.2 Chromatographic Analysis

The variability regarding sterols/stanols as well as fatty acid moieties of the ester preparations used for food enrichment and the resulting multitude of compounds with a high degree of structural similarities constitutes a challenge for chromatographic separations.

Several GC-based approaches have been described; due to the molecular weights of the esters and the resulting low volatility, non-polar, temperature stable GC phases such as dimethylpolysiloxane were frequently used. However, the use of such non-polar stationary columns did not result in sufficient resolution of steryl fatty acid esters (Evershed *et al.*, 1987; Kamm *et al.*, 2001; Gunawan *et al.*, 2010), neither did the application of more polar stationary phases (50 % phenyl – 50 % methyl polysiloxane) (Gordon and Griffith, 1992b; Gordon and Miller, 1997; Caboni *et al.*, 2005). Barnsteiner *et al*. (2011; 2012) reported a capillary gas chromatography (GC)-based separation and quantitation using an intermediately polar and temperature stable trifluoropropylmethyl polysiloxane stationary phase of either intact plant stanyl fatty acid esters or of mixtures of plant stanyl and steryl fatty acid esters. A detailed compositional analysis of individual plant steryl/stanyl fatty acid esters contained in enriched foods could thus be performed for the first time. Subsequently, the methodology has been successfully applied to the investigation of phytosteryl and phytostanyl fatty acid esters in several enriched foods and in natural plant matrices, as well as in feces after dietary consumption of enriched foods (Esche *et al.*, 2012; 2013a; 2013c; Lubinus *et al.*, 2013). Though, under the employed GC conditions esters of saturated and monounsaturated fatty acids of the same chain length could not be resolved. A very recent approach described a very good high temperature GC-

based separation of fatty acid esters of cholesterol and phytosterols employing a ionic liquid capillary column (Hammann and Vetter, 2015). However, no quantitation was performed, and phytostanyl esters were not included either. Overall, due to the high boiling points of intact phytosteryl/-stanyl fatty acid esters and due to the presence of reactive double bonds in the fatty acid moieties, thermal degradations during high-temperature GC are a critical disadvantage of this chromatographic technique. Therefore, LC-based approaches seem to be more appropriate for the analysis of these compounds.

First attempts to analyze intact phytosteryl fatty acid esters extracted from plant materials and plasma using reversed phase LC were reported in the 1980s and early 1990s (Billheimer *et al.*, 1983; Kuksis *et al.*, 1986; Evershed *et al.*, 1987; Gordon and Griffith, 1992b). Billheimer *et al.* (1983) were the first to report a separation of molecular species of steryl fatty acid esters utilizing a C18 column, but the chromatographic resolution was not satisfactory and, owing to the UV-detector employed, the sensitivity of the method was low. The following early approaches also suffered from incomplete resolution, long analysis times, and low sensitivities. In order to improve the analytical performance of LC-based methodologies, the use of mass selective detectors has proven to be effective, helping to differentiate between co-eluting esters and to enhance sensitivity. Caboni *et al.* (2005) employed positive electrospray ionization (ESI) after addition of ammonium acetate to the mobile phase to enhance the electrospray ionizability of the rather apolar phytosteryl fatty acid esters extracted from wheat. As it is typical for soft ionization techniques like ESI, adducts of the intact molecules were formed upon ionization. Besides, the mass spectra of the steryl esters exhibited a fragment $[M\text{-}FA+H]^+$, corresponding to the protonated sterol nucleus after loss of the fatty acid, as base peak. Esters with different sterol nuclei that could not be separated via LC were distinguished by extraction of ions corresponding to the respective $[M\text{-}FA+H]^+$ fragments. A very recent approach described the analysis of several phytosteryl fatty acid esters in different plant matrices via ESI-MS/MS after LC pre-separation on an RP C4 column, allowing a full separation of esters of stearic, oleic, linoleic, and linolenic acid sharing the same sterol nucleus (Firl, 2016). The transitions of ammoniated molecular adduct ions $[M+NH_4]^+$ to the fragment ions $[M\text{-}FA+H]^+$ were detected in order to differentiate esters of different sterol nuclei. However, due to the occurrence of isotopologues the transitions were revealed to be not fully specific.

Direct infusion-based approaches with ESI, detecting transitions of ammoniated molecular ions to $[M\text{-}FA+H]^+$ product ions have also been described for the quantitative analysis of phytosteryl fatty acid esters in *Arabidopsis* plants (Wewer *et al.*, 2011), and for a qualitative analysis of phytosteryl esters in corn and enriched spreads (Hailat and Helleur, 2014). Kalo and Kuuranne (2001)

qualitatively recorded precursor ion electrospray tandem mass spectra of phytosteryl esters after direct infusion. However, despite the simplicity of such approaches, as it has already been described for cholesteryl fatty acid esters (Yu *et al.*, 2014), the applicability of direct flow injection without previous chromatographic separation might be restricted to the detection of only a limited number of steryl and/or stanyl fatty acid esters due to their high structural similarity. Therefore, this approach often serves only qualitative purposes.

In contrast to the use of ESI in combination with dopants, atmospheric pressure chemical ionization (APCI) of steryl esters does not yield molecular ions in considerable amounts. Due to an in-source fragmentation of the esters, fragments corresponding to $[M\text{-}FA+H]^+$ have been described to be formed as base peaks, being characteristic of the esterified sterol nucleus (Mezine *et al.*, 2003; Rudell *et al.*, 2011; Ishida, 2014). Based on this fragmentation, Ishida (2014) differentiated and quantified fatty acid esters of stigmasterol, campesterol and sitosterol in tobacco leaves after a non-aqueous LC separation on a C18 column, employing selected ion monitoring (SIM). Mezine *et al.* (2003) used LC-APCI-MS with subsequent ion extraction to quantify phytosteryl fatty acid esters in enriched spreads and beverages. Applying the same method, the authors also reported a qualitative analysis of a mixture of phytosteryl and phytostanyl fatty acid esters in an enriched spread. Apparently, the detection of individual esters via their respective $[M\text{-}FA+H]^+$ fragment was not specific enough for a quantitation of such complex mixtures because of an insufficient chromatographic separation of the esters on a hexyl-phenyl column and a co-elution of matrix triglycerides.

The lack of commercially available phytosteryl and phytostanyl fatty acid esters is another problem encountered with the establishment of appropriate chromatographic methodologies. Barnsteiner et al. (2011; 2012) described facile procedures for the preparation of phytosteryl and phytostanyl fatty acid esters. They were based on either enzyme-catalyzed transesterification of fatty acid methyl esters and phytostanols using *C. rugosa* lipase or *C. antarctica* lipase B, or on chemical synthesis via heat-catalyzed esterification in a nitrogen atmosphere.

2.4.2 Analysis of Phytosterol Oxidation Products

The principles of the existing analytical methodologies for the determination of phytosterol oxidation products rely on those described for cholesterol oxidation products (Piironen *et al.*, 2000a). Owing to the analytical capabilities, the focus has almost exclusively been put on the secondary polar oxidation products of phytosterols, determined, if ester-derived, after cleavage of the ester bonds. Generally, the development of methodologies for the determination of sterol

oxides is challenging due to their overall low concentrations in potentially complex food matrices, polarity differences between the individual oxygenated species, and their low stability. The qualitative and quantitative analysis of phytosterol oxidation products is further complicated, as, owing to the increased number of substrates, they comprise an even greater number and variety of structurally similar compounds. This results in exhibiting challenges regarding chromatographic separation as well as mass spectrometric differentiation. As there is no standardized procedure existing, various approaches are available based on (i) lipid extraction and saponification or transesterification, (ii) isolation and purification via column chromatography, thin layer chromatography (TLC), or SPE, (iii) derivatization, and (iv) detection via HPLC- or GC-based techniques (Dutta, 2004; Guardiola *et al.*, 2004; Vanmierlo *et al.*, 2013).

2.4.2.1 Sample Preparation

Lipid Extraction

If food samples to be investigated are not already present as lipid matrix, the lipids are usually extracted from the sample. Very commonly, mixtures of chloroform and methanol are used according to Folch *et al.* (1957) or according to Bligh and Dyer (1959), which, however, are described to form emulsions in some food matrices, making subsequent extractions and phase separations difficult (Schmarr *et al.*, 1996). Alternatively, combinations of *n*-hexane and *iso*-propanol are other frequently used solvent systems (Dutta and Appelqvist, 1997; Dutta, 1997), as originally described by Hara and Radin (1978). Further modifications or combinations of these methods have been described, such as for example Soxhlet extraction (Schmarr *et al.*, 1996). As concluded in a very recent publication, where several lipid extraction methodologies for the subsequent determination of phytosterol oxidation products in different matrices were tested, extraction conditions need to be selected carefully, as the performance of a methodology might depend on the type of matrix (Menéndez-Carreño *et al.*, 2016). Thus, the authors applied the Folch extraction to meat products, the Bligh and Dyer method to fish samples, and the method described by Hara and Radin to vegetable and potato samples. Lipids from bakery products, in turn were extracted via acid hydrolysis in order to release phytosterols from steryl glycosides. For the analysis of eggs, acid hydrolysis of the freeze-dried sample material was shown to be optimum, followed by automated Soxhlet extraction using diethyl ether/petroleum ether. However, as diethyl ether is prone to form peroxides (Little *et al.*, 1979), this solvent should be used with caution regarding the analysis of sterol oxidation products. Interestingly, Dionisi *et al.* (1998) observed with the example of cholesterol oxidation products in milk powder direct

saponification without prior lipid extraction being the best choice as upon the application of these methods the least amounts of artifacts were formed.

Saponification / Transesterification

In order to hydrolyze the lipid matrix, saponification or transesterification is usually employed. It serves not only to remove the quantitatively dominating triglycerides, but also liberates the oxidized sterol moieties. Hot and cold saponification are possible approaches, with hot saponification, however, having been described to favor artifact formation and degradations of 7-ketocholesterol and the labile 5,6-epoxycholesterols (Tsai and Hudson, 1984; Guardiola *et al.*, 2004; Park *et al.*, 1996). Therefore, cold saponification was used by most of the groups working on phytosterol oxide analysis (Conchillo *et al.*, 2005; Soupas *et al.*, 2007; Menéndez-Carreño *et al.*, 2008a; Ubhayasekera and Dutta, 2009), although this may require reaction times up to 20 h. Transesterification, in contrast, provides significantly milder conditions in combination with short reaction times. In addition, soap formation as occurring in saponified lipid extracts is avoided, and for cholesterol oxidation products it was demonstrated that artifact formation does not occur (Schmarr *et al.,* 1996). Modifications of a transesterification method described by Schmarr *et al.* (1996) for the analysis of cholesterol oxidation products have been described and adapted also to the analysis of phytosterol oxidation products in lipid matrices (Johnsson and Dutta, 2006; Tabee *et al.*, 2008; Rudzińska *et al.*, 2014). After alkaline hydrolysis or transesterification, appropriate organic solvents can be used for extraction of the unsaponifiable material containing mainly non-oxidized free sterols and their oxidized derivatives; upon transesterification, also fatty acid methyl esters will be co-extracted.

Enrichment and Purification

The main goal of subsequent purification steps is the separation of the abundant non-oxidized sterols from the low-concentrated sterol oxides in order to avoid interferences in the course of chromatographic analysis. Several procedures have been described, mainly LC-based (Louter, 2004; Menéndez-Carreño *et al.*, 2016), TLC-based (Nourooz-Zadeh and Appelqvist, 1992; Conchillo *et al.*, 2005), and SPE-based (Dutta and Appelqvist, 1997; Johnsson and Dutta, 2006; Soupas *et al.*, 2007). However, SPE-based approaches are most commonly used in recent years, being relatively cheap and minimizing the exposure of the oxides to oxygen compared to TLC-based techniques (Dutta and Appelqvist, 1997; Guardiola *et al.*, 2004).

Derivatization

For both, gas chromatographic and liquid chromatographic analyses, sterol oxidation products are usually derivatized. For GC-analysis, free hydroxyl groups require derivatization in order to improve the volatility of the oxides, which is most commonly accomplished by the formation of trimethylsilyl (TMS) ether derivatives (Guardiola *et al.*, 2004; Louter, 2004). Silylation further improves the stability of the analytes, the chromatographic resolution, and the response to the flame ionization detector (FID) (Schmarr *et al.*, 1996; Guardiola *et al.*, 2004). As additional advantage, EI mass spectra of silylated derivatives are easier to interpret due to a reduced fragmentation (Louter, 2004). For silylation, different time/temperature conditions are described in literature, as well as different silylation reagents (Guardiola *et al.*, 2004). With respect to HPLC analysis, the attachment of a functional group is common in order to increase peak resolution and the compound detector response (Vanmierlo *et al.*, 2013).

2.4.2.2 Chromatographic Analysis

Similar to the intact phytosteryl and phytostanyl fatty acid esters, commercially available reference compounds for phytosterol oxidation products are lacking, further complicating in particular the development of reliable and reproducible chromatographic methods. Both, syntheses (Zhang *et al.*, 2005; Kenny *et al.*, 2012; O'Connell *et al.*, 2012; Gao *et al.*, 2013; O'Callaghan *et al.*, 2013) and isolation procedures via preparative HPLC (Koschutnig *et al.*, 2010) have been described for the generation of individual phytosterol oxidation products. In several studies, already described EI mass spectra of mainly silylated phytosterol oxidation products served as basis for the identification of the oxides.

Sterol oxidation products are most commonly analyzed by gas chromatography, coupled to an FID or a mass spectrometer. Whereas the use of elevated temperatures as required for GC analysis might actually additionally challenge analyses of the thermolabile sterol oxidation products, GC is still usually preferred as it possesses much better separation capacity, in particular as derivatization procedures considerably enhance the thermostability of the oxides and thus ensure good analytical recoveries (Louter, 2004; Grün and Besseau, 2016). Further, the possibility of coupling GC to FID enables a highly sensitive and linear detection and quantitation of the oxides (Louter, 2004). Thereby, the existing analytical methodologies mostly cover the 7-keto-, 7-hydroxy-, 5,6-epoxy- and triol-derivatives of the various phytosterols. As in the majority of both natural and enriched foods sitosterol is the dominating sterol, oxygenated derivatives formed thereof are chromatographically separated and determined in most studies. Depending on the original phytosterol composition, this is frequently complemented by oxides of campesterol,

stigmasterol and/or brassicasterol. In addition, Johnsson *et al*. (2003) and Johnsson and Dutta (2003) established GC/MS methodologies for the characterization of side chain oxidation products of stigmasterol, sitosterol and campesterol that, however, were reported to occur only in minor amounts.

The employed capillary columns are normally coated with non-polar stationary phases, most frequently consisting of 5 % phenyl groups (Lampi *et al.*, 2002; Apprich and Ulberth, 2004; Louter, 2004; Soupas *et al.*, 2004a; González-Larena *et al.*, 2011; Derewiaka and Obiedziński, 2012; Barriuso *et al.*, 2015). In a recently developed methodology, a more polar stationary phase was used with a proportion of 14 % phenyl groups (Menéndez-Carreño *et al.*, 2016). However, depending on the number of target analytes, co-elutions can be hardly avoided (Apprich and Ulberth, 2004). The use of MS detectors helps differentiating overlapping peaks and is therefore getting increasingly important. Still, MS-based quantitation usually requires calibration of the individual analytes due to pronounced differences in responses between the analytes, whereas the use of FID reduces the need for external calibration (Guardiola *et al.*, 2004; Louter, 2004). This is particularly relevant in the view of the lack of commercially available reference compounds. Johnsson and Dutta (2005) connected a non-polar 5 % phenyl coated to a mid-polar 35 % phenyl-coated stationary phase for improving the chromatographic separation of both side chain- and ring-oxygenated phytosterols; however, some co-elutions still occurred.

As far as GC-based analyses are concerned, also the injection technique may be a point of critical evaluation, as cool-on-column injections seem to be more appropriate for the labile sterol oxides and therefore are frequently applied (Lampi *et al.*, 2002; Louter, 2004; Soupas *et al.*, 2004a; Menéndez-Carreño *et al.*, 2016). Despite the higher temperatures accompanying the use of a split/splitless injector, derivatization seems to sufficiently enhance the stability of the analytes; therefore, this injection technique has also been employed on several occasions (Bortolomeazzi *et al.*, 2003; Azadmard-Damirchi and Dutta, 2009; Oehrl *et al.*, 2001; Derewiaka and Obiedziński, 2012).

Despite the advantage of being able to operate at room temperature, HPLC analyses are less frequently established. Owing to a limited separation power, LC-based approaches, usually in combination with an APCI source, have predominantly been applied to the analysis of oxidation products of cholesterol (Manini *et al.*, 1998; Razzazi-Fazeli *et al.*, 2000; Raith *et al.*, 2005), as these are less numerous than oxidation products of mixtures of phytosterols. Though, Kemmo *et al*. developed HPLC-based approaches employing a silica phase allowing the quantitative determination of oxidation products of stigmasterol via HPLC-UV and HPLC separation and fluorescence (FL) detection (Kemmo *et al.*, 2005) as well as via HPLC-APCI-MS (Kemmo *et al.*,

2007); the use of HPLC-APCI-MS also allowed the MS-based differentiation of individual oxidation products from complex phytosterol mixtures after appropriate chromatographic separation (Kemmo *et al.*, 2008). Very recently, another approach allowing the additional determination of non-polar and mid-polar oxidation products together with polar oxidation products of different phytosterols and cholesterol based on liquid chromatography – atmospheric photoionization – mass spectrometry (LC-APPI-MS) using a diol-bonded analytical column was described (Grün and Besseau, 2016).

However, the described sequences for sample preparation and subsequent chromatographic analysis, be it GC or HPLC, are based on off-line techniques for the isolation and purification of phytosterol oxidation products, and the procedures are time-consuming and carry the risks of sample losses, degradation of the labile oxides and artifact formation. Hence, the stability of the oxides during sample preparation and chromatographic analysis should always be monitored.

Besides the development of analytical approaches targeting the polar secondary oxidation products of phytosterols, first attempts to isolate fractions containing dimers, trimers, and tetramers via high performance size exclusion chromatography have been described (Lampi *et al.*, 2009; Struijs *et al.*, 2010; Lehtonen *et al.*, 2012) and structures for sterol dimers have been proposed upon APCI-MS and coordination ion spray-MS analyses (Struijs *et al.*, 2010; Rudzinska *et al.*, 2010) and additionally assessed by nuclear magnetic resonance spectroscopy (NMR) (Sosińska *et al.*, 2013; 2014). Further, apolar secondary oxidation products and degradation products of phytosterols in refined vegetable oils and of thermo-oxidized stigmasterol standard have been characterized via SPE-GC/MS (Bortolomeazzi *et al.*, 2003; Menéndez-Carreño *et al.*, 2010) and more recently via Diol-LC-APPI-MS (Grün and Besseau, 2016). As the determination of oxidized phytosterol moieties after cleavage of the ester bonds neglects potential oxidation reactions of the fatty acid moieties, a very recent study described a first approach to analyze intact oxidized derivatives of sitosteryl oleate via LC-ES-MS/MS, determining the MS/MS transitions of lithiated adducts of the oxidized esters (Julien-David *et al.*, 2014). Some more mechanistic analytical approaches additionally focused on the formation and decomposition of hydroperoxides, investigating phytosterol hydroperoxides via LC-FL after post-column derivatization with diphenyl-1-pyrenylphosphine (Säynäjoki *et al.*, 2003; Kemmo *et al.*, 2005) and via LC-APCI-MS (Kemmo *et al.*, 2007). Hydroperoxides of both, the steryl moiety and the fatty acid moieties of intact stigmasteryl oleate in a saturated lipid matrix could be determined after SPE clean-up and subsequent HPLC-DAD/ELSD analysis (Lehtonen *et al.*, 2012).

2.4.2.3 On-Line LC-GC

The on-line coupling of liquid chromatography and gas chromatography has proven to be an elegant tool for diverse analytical approaches requiring extensive sample preparation prior to chromatographic analysis of the individual compounds from complex samples (Grob and Lanfranchi, 1989). Using this technique, the LC part performs a selective clean-up, concentration and/or fractionation of the sample, replacing laborious sample pre-treatment, usually based on normal phase (NP) LC separation (Hyötyläinen and Riekkola, 2004). Furthermore, the facility to monitor the separation on-line via the LC-detector allows the appropriate LC-conditions to be optimized quickly and precisely. Subsequently, the fraction(s) of interest can be accurately cut and transferred on-line to the GC by switching a transfer valve (Grob, 2000; Hyötyläinen and Riekkola, 2004). This allows analyses being performed in a closed and fully automatable system. These features offer highly reproducible analyses as risks of sample losses and contaminations are minimized, accompanied by low detection limits due to the on-line transfer as all solute material contained in the LC-fraction is transferred to the GC (Grob, 2000; Hyötyläinen and Riekkola, 2003). Therefore, the main application area of on-line LC-GC is considered to be the quantitation of a limited number of compounds present at low levels in a complex matrix (Janssen *et al.*, 2004). The principal challenges that are to be solved are an expensive initial set-up, the establishment and optimization of appropriate LC- and GC conditions as well as relatively complicated interface techniques to be handled (Dugo *et al.*, 2003). The latter are necessary as the LC fractions to be transferred are typically several hundreds of microliters, while conventional GC allows just a few microliters; therefore, the need of specially designed interfaces is mandatory for removal of the eluent (Herrero *et al.*, 2009). Commonly, on-column, loop-type, and vaporizer interfaces are used. On-column and loop-type interfaces are usually applied with retention gap techniques and/or concurrent solvent evaporation, respectively, performed in the GC capillary column by means of a pre-column system in combination with an early solvent vapor exit placed between the pre-column and the analytical column (Grob, 2000; Hyötyläinen and Riekkola, 2003). Further, the vaporizer interface is a widely used technique in on-line coupling of LC and GC. Most applications involving the vaporizer interface make use of a programmable temperature vaporizer (PTV) or a hot vaporizing chamber (Hyötyläinen and Riekkola, 2003). The PTV interface is used when large volumes of injection are needed (Herrero *et al.*, 2009). There are several ways to perform the transfer: PTV solvent split, PTV large volume splitless transfer, PTV vapor overflow, PTV vapor overflow transfer with or without splitting and various modifications of these techniques (Hyötyläinen and Riekkola, 2003). They allow the elimination of the solvent prior to the GC capillary column, preventing contamination of the column. In this case, the installation of a solvent vapor exit is not necessary. As aqueous eluents as used in reversed phase (RP) LC are

unsuitable for direct transfer to GC, RPLC-GC is much less used. The problems related to RPLC-GC coupling can be solved either by phase switching techniques before introduction of the LC-fraction to the GC, or by the design of special interface techniques (Hyötyläinen and Riekkola, 2004; Toledano *et al.*, 2012a; 2012b).

The on-line coupling of LC and GC has been used for several analytical purposes; recent work focused in particular on the analysis of phytosterols, phytosteryl/phytostanyl fatty acid and ferulic acid esters in oils (Grob and Lanfranchi, 1989; Grob *et al.*, 1992; Lechner *et al.*, 1999; Toledano *et al.*, 2012a), fats (Kamm *et al.*, 2001), cereals (Miller *et al.*, 2003; Esche *et al.*, 2013e), and nuts (Esche *et al.*, 2013b), as well as on the analysis of phytosterols and phytosteryl/-stanyl fatty acid esters in enriched foods (Barnsteiner *et al.*, 2011; Esche *et al.*, 2013a).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

The following chemicals were used:

Acetic anhydride (≥99 %)	Sigma-Aldrich, Steinheim, Germany
Acetone (SupraSolv)	Merck, Darmstadt, Germany
N,O-Bis(trimethylsilyl)trifluoroacetamide + 1 % trimethylchlorosilane (BSTFA/TMCS)	Sigma-Aldrich, Steinheim, Germany
tert-Butanol (>99.7 %)	Sigma-Aldrich, Steinheim, Germany
Chloroform (AnalaR Normapur)	VWR International, Darmstadt, Germany
Cholesteryl linolenate (≥85 %)	Sigma-Aldrich, Steinheim, Germany
Cholesteryl oleate (≥98 %)	Sigma-Aldrich, Steinheim, Germany
Cholesteryl palmitate (≥98 %)	Sigma-Aldrich, Steinheim, Germany
Cholesteryl stearate (≥98 %)	Sigma-Aldrich, Steinheim, Germany
Citric acid, anhydrous (for synthesis)	Merck, Darmstadt, Germany
Copper sulfate pentahydrate (p.A.)	Merck, Darmstadt, Germany
Diethyl ether (extra pure)	Sigma-Aldrich, Steinheim, Germany
Dichloromethane (p.A., 95 %)	Sigma-Aldrich, Steinheim, Germany
Ethyl acetate (Rotisolv, LC-MS)	Carl Roth GmbH, Karlsruhe, Germany
5,6*α*-Epoxycholesterol (≥95 %)	Sigma-Aldrich, Steinheim, Germany
5,6*ß*-Epoxycholesterol (≥95 %)	Sigma-Aldrich, Steinheim, Germany
Fatty acids and fatty acid methyl esters 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 22:0, 22:1 (analytical grade)	Sigma-Aldrich, Steinheim, Germany, Carl Roth GmbH, Karlsruhe, Germany
n-Hexane (AnalaR Normapur)	VWR International, Darmstadt, Germany
n-Hexane (HiPerSolv Chromanorm)	VWR International, Darmstadt, Germany
Hydrochloric acid (25 %)	Sigma-Aldrich, Steinheim, Germany
7*ß*-Hydroxycholesterol (≥95 %)	Sigma-Aldrich, Steinheim, Germany
7-Ketocholesterol (≥90 %)	Sigma-Aldrich, Steinheim, Germany
7-Ketostigmasterol (92 %)	Steraloids, Newport, RI, USA
Magnesium sulfate (anhydrous)	Sigma-Aldrich, Steinheim, Germany
Methanol (HiPerSolv Chromanorm)	VWR International, Darmstadt, Germany

Methyl *tert*-butyl ether	Evonik Industries AG, Essen, Germany
Potassium hydroxide (≥85 %)	Sigma-Aldrich, Steinheim, Germany
Potassium permanganate (p.A.)	Merck, Darmstadt, Germany
iso-Propanol (HiPerSolv, Chromanorm)	VWR International, Darmstadt, Germany
Pyridine (99.8 %)	Sigma-Aldrich, Steinheim, Germany
beta-Sitosterol, with ca. 10 % campesterol (~75 %)	Acros Organics, Morris Plains, NJ, USA
Sodium bicarbonate (>99 %)	Sigma-Aldrich, Steinheim, Germany
Sodium methoxide (reagent grade, 95 %)	Sigma-Aldrich, Steinheim, Germany
Sodium methoxide (30 % in methanol)	Merck, Darmstadt, Germany
Sodium sulfate (anhydrous)	VWR International, Darmstadt, Germany
Stigmastanol (95 %)	Sigma-Aldrich, Steinheim, Germany
Stigmasterol (~95 %)	Sigma-Aldrich, Steinheim, Germany
Water (Chromasolv, LC-MS Ultra)	Sigma-Aldrich, Steinheim, Germany

MTBE and diethyl ether were distilled prior to use.

3.1.2 Plant stanol/sterol and stanyl/steryl fatty acid ester mixtures

A mixture of phytosteryl and phytostanyl fatty acid esters (Vegapure® 95E; 2.7 % free sterols/stanols and 96.5 % phytosteryl/phytostanyl esters with an ester profile of 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) was provided by Cognis GmbH (Illertissen, Germany).

A mixture of phytostanyl fatty acid esters (STAEST-115; 96.8 % phytostanyl esters with an ester profile of 55.9 % sitostanyl-18:1, 18.4 % sitostanyl-18:2, 8.1 % sitostanyl-18:3, 5.7 % campestanyl-18:1, 4.1 % sitostanyl-16:0, 1.9 % campestanyl-18:2, 1.5 % sitostanyl-18:0, 1.3 % sitostanyl-20:1, 0.8 % campestanyl-18:3, 0.5 % sitostanyl-20:0, 0.4 % sitostanyl-22:1, 0.4 % campestanyl-16:0, 0.3 % sitostanyl-22:0, 0.2 % sitostanyl-16:1, 0.2 % campestanyl-18:0, 0.1 % campestanyl-20:1,

0.1 % campestanyl-20:0, 0.04 % campestanyl-22:1, 0.03 % campestanyl-22:0, and 0.03 % campestanyl-16:1) was provided by Raisio Group (Raisio, Finland).
Mixtures of soy sterols (Generol® 122 N) and of wood stanols (Reducol® Stanol Powder) were provided by Cognis GmbH (Illertissen, Germany).

3.1.3 Enzyme Preparations

Lipase B acrylic resin from *Candida antarctica*	Sigma-Aldrich, Steinheim, Germany
Lipase from *Candida rugosa*	Sigma-Aldrich, Steinheim, Germany

3.1.4 Syntheses of Reference Compounds

Individual phytosteryl and phytostanyl fatty acid esters were synthesized according to previously described procedures (Barnsteiner *et al.*, 2011; 2012). Syntheses of steryl/-stanyl esters of palmitoleic acid, behenic acid, and erucic acid were enzymatically catalyzed.

5,6*ß*-Epoxysitosteryl acetate and 5,6*ß*-epoxycampesteryl acetate were synthesized according to a procedure previously described by Kenny *et al.* (2012) for the synthesis of 5,6*ß*-epoxydihydrobrassicasteryl acetate.

3.1.5 Food Materials

The analyzed enriched foods are compiled in Table 5.

Table 5. Overview on the investigated enriched foods.

food	no.	manufacturer	country of purchase	enrichment	other ingredients
skimmed milk-drinking yoghurt					
"Emmi Benecol strawberry"	1	Emmi, Luzern (CH)	D	3.0 % phytostanols	2.1 % fat, 3.0 % protein
	2				
soft cheese-style spread					
"Benecol – Soft Cheese style spread"	1	McNeill Nutritionals Ltd., Wokingham (UK)	UK	4.0 % phytostanols	13.9 % fat, 8.1 % protein
	2				
margarine					
"Benecol – Margaryna roślinna o zwartości trzech czwartych tłuszczu "	1	Bunge Polsa Sp. Z.o.o. Karczew (PL)	PL	8.0 % phytostanols	60 % fat
"Benecol voi & rypsiölyi"	2	Raisio (FIN)	FIN	8.0 % phytostanols	60 % fat
"Becel pro.activ"	3-7	Unilever (D)	D	12.5 % plant sterol esters	40 % fat
"Fruit d'Or pro-activ Cuisson & Tartine"	8	Unilever (F)	F	12.5 % plant sterol esters	60 %fat
"Deli Reform active"	9	Walter Rau Lebensmittelwerke GmbH (D)	D	12.5 % plant sterol esters	39 % fat
"Bellasan activ"	10	Walter Rau Lebensmittelwerke GmbH (D)	D	12.5 % plant sterol esters	39 % fat

3.2 Methods

3.2.1 Analysis of Intact Phytosteryl and/or Phytostanyl Fatty Acid Esters

3.2.1.1 Lipid Extraction

The extraction of lipids from skimmed milk-drinking yoghurts as well as from the soft cheese-style spread was performed according to a previously described method (Barnsteiner *et al.*, 2011; Esche *et al.*, 2013a). First, the drinking yoghurt was homogenized by vigorous shaking and the soft cheese-style spread by using an electric blender. Three hundred microliters of the internal standard (IS) cholesteryl linolenate [2.5 mg/mL in *n*-hexane/MTBE (3:2, v/v)] were put into a reaction vessel, and the solvent was removed by a gentle stream of nitrogen. One hundred milligrams of drinking yoghurt or 75 mg of soft cheese-style spread, a magnetic stir bar, 400 μL of bi-distilled water, and 1 mL of hydrochloric acid (25 %) were added, and the sealed vessel was sonicated until the internal standard was dispersed; the completeness of the dispersion was examined visually. The digestion was performed at 130 °C for 45 min. After cooling to room temperature, 1 mL of bi-distilled water was added, and the solution was filtered using a 0.45 μm membrane filter. The vial and the filter were washed three times with 5 mL of bi-distilled water until the pH of the filtrate was neutral. The lipids were extracted three times with 5 mL of *n*-hexane/MTBE (3:2, v/v), and the combined extracts were dried with sodium sulfate. An aliquot of the drinking yoghurt extract was dried under nitrogen, re-dissolved in the same volume of ethyl acetate (EtOAc) and directly used for UHPLC-MS analysis. The soft cheese-style spread extract was further subjected to solid phase extraction.

For lipid extraction from the margarine samples, a previously published method was used (Barnsteiner *et al.*, 2011). Thirty mg of the sample were weighed into a vessel, 300 μL of the internal standard cholesteryl linolenate or cholesteryl palmitate [2.5 mg/mL in *n*-hexane/MTBE (3:2, v/v)], 5 mL of *n*-hexane/MTBE (3:2, v/v) and sodium sulfate were added and sonicated for 1 min. The solution was filtered through a 0.45 μm membrane filter. The vessel and the filter were washed twice with 5 mL of *n*-hexane/MTBE (3:2, v/v), and the combined extracts were used for solid phase extraction.

3.2.1.2 Solid Phase Extraction

A 500 μL aliquot of the lipid extracts of the margarines and the soft cheese-style spread was dried via a gentle stream of nitrogen and re-dissolved in the same volume of *n*-hexane. One hundred and twenty μL of the lipid extract was loaded onto a Strata NH_2, 55 μm, 70 Å, 400 mg/3 mL SPE

cartridge (Phenomenex, Aschaffenburg, Germany) previously conditioned with 2 x 3 mL of *n*-hexane, and the stanyl/steryl fatty acid esters were eluted with 2 x 3 mL of *n*-hexane/diethyl ether (Et_2O) (99:1, v/v). After evaporation of the solvent by a gentle stream of nitrogen, the residue was dissolved in 150 μL of EtOAc, filtered through a 0.2 μm membrane filter, and subjected to UHPLC-MS analysis.

3.2.1.3 UHPLC-APCI-MS Analysis of Intact Phytostanyl Fatty Acid Esters in Enriched Foods

Samples were analyzed by injecting 0.8 μL into a 1290 Infinity Series UHPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a Surveyor MSQ Plus single quadrupole mass-selective detector, using an atmospheric pressure chemical ionization (APCI) source (Dionex Softron GmbH, Germering, Germany). UHPLC separation was carried out on a 150 mm x 2.10 mm i.d., 1.7 μm, 100 Å, Kinetex C8 column (Phenomenex, Aschaffenburg, Germany) at 50 °C and a flow rate of 0.58 mL/min. The mobile phase was composed of MeOH and water with the following gradient: from 90 % to 95 % MeOH within 1.2 min and holding until 3 min; from 95 % to 100 % MeOH between 3 and 12 min, and back to the initial 90 % from 12 to 15 min. The MS parameters were set as follows: ionization mode: APCI (positive); probe temperature: 400 °C; N_2 nebulizer pressure: 45 psi; cone voltage: 80 V; coronal discharge: 8 μA. Instrument control and data acquisition were performed by Xcalibur software 2.0.7, Thermo MSQ 2.0 (Thermo Scientific, Austin, TX), Chromeleon software 6.80 (Dionex Corporation, Sunnyvale, CA) in combination with Agilent Instrument Control Framework (Agilent Technologies, Waldbronn, Germany). Mass spectra of synthesized reference compounds were recorded in full scan mode at a mass range of *m/z* 100-750. For quantitative analyses the MS detector was adjusted to monitor positive ions in the selected ion monitoring (SIM) mode with a mass window of 0.2 at *m/z* 369.3, 385.3, and 399.3, respectively.

The identities of the phytostanyl fatty acid esters extracted from enriched foods were confirmed via comparison of relative retention times to those of synthesized standard compounds in the respective SIM mode. Stanyl fatty acid esters were quantitated by generating five-point calibration functions with 0.1, 0.26, 0.43, 0.6, and 0.76 mg of total esters (STAEST-115)/mL, except for campestanyl-20:0, campestanyl-20:1, and campestanyl-18:0 which were quantitated via three-point calibration functions with 0.43, 0.6, and 0.76 mg of total esters (STAEST-115)/mL. Each calibration point was analyzed in triplicate. Linear regression was confirmed in ratio of areas (area stanyl ester/area IS) and amounts (amount stanyl ester/amount IS).

3.2.1.4 UHPLC-APCI-MS Analysis of Intact Phytosteryl and Phytostanyl Fatty Acid Esters in Complex Mixtures

Samples were analyzed by injecting 0.8 μL into a 1290 Infinity Series UHPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a 6430 triple quadrupole mass selective detector (Agilent Technologies, Waldbronn, Germany), equipped with an APCI source (Agilent Technologies, Waldbronn, Germany). The UHPLC conditions were as described above (cf. 3.2.1.3). The MS was operated in the SIM mode, using the second quadrupole, and parameters were set as follows: ionization mode, APCI (positive); drying gas temperature, 350 °C; vaporizer temperature, 350 °C; drying gas (N_2) flow, 9 l/min; nebulizer pressure, 60 psi; capillary voltage, 3000 V; coronal discharge, 8 μA; fragmentor voltage, 70 V; cell accelerator voltage, 7V; resolution, unit. Instrument control and data acquisition were performed by MassHunter workstation software B.06.00 (Agilent Technologies, Waldbronn, Germany). Mass spectra of synthesized reference compounds were recorded in full scan mode at a mass range of *m/z* 150 – 800. For the quantitative determination of sitosteryl and campesteryl fatty acid esters, the MS detector scanned in a first chromatographic run in the selected ion monitoring (SIM) mode with a dwell time of 150 ms at *m/z* 369.2, 397.2, and 383.2. For the quantitative analysis of brassicasteryl, campestanyl, stigmasteryl, and sitostanyl fatty acid esters, the MS detector operated in a second chromatographic run in the SIM mode scanning with a dwell time of 40 ms at *m/z* 369.2, 381.2, 385.2, 395.3, 399.2, 665.4, and 679.5.

The identities of the phytosteryl and phytostanyl fatty acid esters extracted from enriched margarines were confirmed via comparison of relative retention times to those of synthesized reference compounds in the respective SIM mode. Steryl and stanyl fatty acid esters were quantitated by generating five-point calibration functions with 0.1, 0.2, 0.3, 0.4, and 0.5 mg of total esters (Vegapure 95E)/mL, except for campestanyl-22:0 and sitostanyl-22:0 which were quantified via three-point calibration functions with 0.3, 0.4, and 0.5 mg of total esters (Vegapure 95E)/mL. Each calibration point was analyzed in triplicate.

3.2.1.5 Validation of the UHPLC-APCI-MS Approaches

Limits of Detection and Limits of Quantitation

The instrumental limits of detection (LOD) and limits of quantitation (LOQ) were determined according to the method of Vogelgesang and Hädrich (1998).

Linearity

Linear regression of the five-point calibration curves was confirmed in ratio of areas (area of steryl/stanyl ester / area of IS) and amounts (amount of steryl/stanyl ester / amount of IS).

Recovery Rates

For the phytostanyl ester-enriched foods, recovery rates were determined by spiking non-enriched products with known amounts of the plant stanyl ester mixture STAEST-115. To 100 mg of skimmed milk yogurt (0.1 % fat), 30 mg of margarine (45 % fat) and 75 mg of cream cheese (13 % fat) were added 5 mg, 4.5 mg and 4.9 mg of the phytostanyl ester mixture, respectively.
Recovery rates for the phytosteryl/-stanyl ester enriched margarine were determined by spiking 30 mg of a non-enriched margarine (45 g fat per 100 g) with known amounts of the phytosteryl/phytostanyl ester mixture Vegapure 95E at three different levels (3.0, 3.7, and 4.5 mg, corresponding to 10, 12.3, and 15 % of phytosteryl fatty acid esters, respectively) in triplicate. Subsequently, the spiked samples were subjected to the extraction procedures as described above. The absence of inherently present phytosteryl/phytostanyl fatty acid esters (i.e. <LOD) was demonstrated by subjecting the non-enriched margarine to the analytical procedure.

Repeatability and Reproducibility

Two packages of enriched margarine were analyzed in triplicate on three different days by one operator to determine the repeatability of the method. Reproducibility was assessed by the additional triplicate analysis of each package by a second operator. As additional quality control measures, the performance of the instrument was regularly confirmed by assessing the responses obtained for the calibration solutions and by analyzing and quantitating the same margarine sample each time a new batch of samples was subjected to LC-MS analysis. The stability of the samples, i.e. the target analytes in EtOAc, was demonstrated by repeated analyses of the calibration solutions over a period of 4 weeks.

3.2.2 Analysis of Phytosterol/Phytostanol Oxidation Products

During all sample preparations, potential oxidation of lipids was minimized by wrapping transparent labware with aluminum foil. The use of glassware was avoided in order to prevent a loss of analytes due to adsorption; transesterification, extraction, and washing steps were performed in 15 mL polypropylene centrifuge tubes.

3.2.2.1 Preparation of Reference Compounds

Reference compounds of 5,6*α*-epoxy-, 7-keto-, 7*α*-hydroxy-, and 7*ß*-hydroxysterols, and of hydroxysitostanols were obtained via a semi-preparative HPLC-separation of thermo-oxidized sterol standard compounds, based on the principles of a method described by (Kemmo *et al.*, 2005). Briefly, 15 mg of sterol/stanol standard were heated at 180 °C for 180 min in an open 11 mL glass vial in a ventilated oven, and subsequently cooled in a dessicator.

For preparation of sitostanol hydroxides, the oxidized sitostanol standard was dissolved in 5 mL of *n*-hexane/Et_2O (9:1, v/v) using a sonicator and purified using a Supelclean LC-Si 500 mg/3 mL SPE cartridge (Sigma-Aldrich, Steinheim, Germany) according to a previously described procedure (Lampi *et al.*, 2002; Soupas *et al.*, 2004b). After activation of the silica with 5 mL of *n*-hexane, 1 mL of the oxidized standard was loaded onto the SPE cartridge. The cartridge was washed with 5 mL of *n*-hexane/Et_2O (9:1, v/v), followed by 5 mL of *n*-hexane/Et_2O (1:1, v/v). The stanol oxidation products were subsequently eluted with 5 mL of acetone. The acetone fractions of three SPE purifications were combined and the solvent was removed via a gentle stream of nitrogen and subjected to semi-preparative HPLC separation.

All other oxidized sterol standards were directly dissolved in 1200 μL *n*-hexane/*iso*-propanol (96:4, v/v) using a sonicator. The separation was carried out using a Dionex HPLC system (UltiMate 3000 series, Dionex Softron GmbH, Germering, Germany) equipped with a wavelength detector-3100 adjusted to 206 nm. The sample (1 mL) was injected onto a Nucleosil 50-5 column (250 x 8 mm, 5 μm; CS-Chromatographie, Germany) tempered at 30 °C. A linear gradient was used with *n*-hexane and *iso*-propanol at 3.5 mL/min; the amount of *iso*-propanol was raised from 4 % to 12 % within 30 min. This resulted in the separation of 5,6*α*-epoxy-, 7-keto-, 7*α*-hydroxy-, and 7*ß*-hydroxysterols. The purities and identities of the substances were confirmed via GC/FID and GC/MS of TMS derivatives according to mass spectra from the literature (Dutta and Appelqvist, 1997; Dutta, 1997; Lampi *et al.*, 2002; Apprich and Ulberth, 2004; Conchillo *et al.*, 2005). Further, two different hydroxysitostanols could be separated. The GC/MS spectrum of the first compound as silylated derivative was identical to that reported for silylated 6*α*-hydroxysitostanol (Soupas *et al.*, 2004b). The second compound was identified as 7-hydroxysitostanol based on comparison of the GC/MS spectrum of the TMS-derivative to mass spectra described by (Soupas *et al.*, 2004b), the identity of the epimer could not be assigned. 5,6*ß*-Epoxysitosteryl acetate and 5,6*ß*-epoxycampesteryl acetate were synthesized according to a procedure previously described by Kenny *et al*. (2012) for the synthesis of 5,6*ß*-epoxydihydrobrassicasteryl acetate. All reference compounds were stored under nitrogen at -10 °C.

3.2.2.2 Thermo-Oxidation of Stigmasterol

Stigmasterol standard (9 mg) was weighed into an 11 mL glass vial and heated in an oven at 180 °C for 60 min. The sample was cooled in a desiccator and dissolved in 1 mL of *n*-hexane/*iso*-propanol (96:4, v/v). An aliquot of 500 μL was evaporated to dryness and acetylated using 1 mL pyridine and 100 μL acetic anhydride (room temperature, 12 h), based on a protocol of Meneghetti *et al.* (1987). After acetylation, the reagents were removed by a gentle stream of nitrogen and the residue was dissolved in 2 mL *n*-hexane/MTBE/*iso*-propanol (80:20:0.3, v/v/v) for on-line LC-GC analysis.

3.2.2.3 Thermo-Oxidation of a Phytosteryl Ester-Enriched Margarine

For thermo-oxidation of a phytosteryl ester-enriched margarine, 4 g ± 0.1 g were weighed into a 250 mL glass bottle (5.52 cm i.d. at the bottom); this resulted in a surface area in contact with air of 24 cm^2 of the melted margarine. The bottle was sealed with perforated aluminum foil that was fixed with a hose clamp and the samples were heated in an oven at 180 °C for 30, 60, 90, and 150 min, respectively. After cooling, the samples were dissolved in 15 mL of MTBE and immediately aliquoted to 11 mL vials; the aliquots corresponded to 250 mg ± 10 mg margarine.

3.2.2.4 Sample Preparation of Enriched Margarines

For analysis of the non-heated margarine, 250 mg sample were directly weighed into 11 mL vials and subjected to further analysis. To the samples, 5,6*ß*-epoxycholesterol and 7-ketocholesterol were added as internal standards (IS). The amounts of internal standards (0.037 mg – 0.268 mg 5,6*ß*-epoxycholesterol (IS_1), 0.015 mg – 0.15 mg 7-ketocholesterol (IS_2)) were adjusted to the expected amounts of phytosterol oxidation products in the sample. After removal of the solvents via a gentle stream of nitrogen, the phytosterol oxidation products were extracted according to a transesterification procedure described by (Rudzińska *et al.*, 2014). Briefly, 1 mL of MTBE and 2 mL of sodium methoxide (10 % in methanol) were added to the dried samples. 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 a spatula tip full of citric acid were added and the phytosterol oxidation products were extracted twice with 5 mL and 4 mL chloroform, respectively. The combined organic phases were washed with 3x5 mL bi-distilled water, evaporated to dryness 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 1 – 3 mL of *n*-hexane/MTBE/*iso*-propanol (80:20:0.3, v/v/v), depending on the expected

concentrations of phytosterol oxidation products in the sample, and subjected to on-line LC-GC analysis.

3.2.2.5 On-Line LC-GC Analysis

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/-stanols relative to the respective internal standards; the Rfs had been determined using reference compounds.

3.2.2.6 Validation of the On-Line LC-GC Approach

Limits of Detection and Limits of Quantitation

The instrumental LOD and LOQ were determined according to the method of Vogelgesang and Hädrich (1998) for selected representatives of acetylated stigmasterol and cholesterol oxidation products.

Linearity and Working Range

Five point calibration curves were established in the range from 2 μg/mL to 360 μg/mL for acetylated 5,6α-epoxysitosterol, 7β-hydroxysitosterol, and from 2 μg/mL to 350 μg/mL for acetylated 5,6β-epoxycholesterol, 7-ketocholesterol, and 7-ketostigmasterol. Three replicates of each concentration were performed. The equations of the calibration curves were determined by linear regression analysis of the peak area (y) versus the concentration of the analyte (x).

Matrix Effect

The potential matrix effect on GC analysis was evaluated using thermo-oxidized stigmasterol (n=3) that was dissolved in 1 mL of *n*-hexane/*iso*-propanol (96:4, v/v); after addition of the internal standards, aliquots of 150 μl were both (i) directly acetylated and analyzed via on-line LC-GC/FID and (ii) spiked to 250 mg of non-heated and heated non-enriched margarine, respectively, subjected to the sample preparation procedure, acetylated and analyzed via on-line LC-GC/FID. The amounts of stigmasterol oxidation products determined in the directly acetylated thermo-oxidized stigmasterol standard versus the quantitated amounts of the stigmasterol oxidation products spiked to matrix were calculated.

Recovery Rates

Recovery rates for the whole method were determined by spiking 250 mg of a non-enriched margarine (45 % fat) with known amounts of representative stigmasterol oxidation products at three different levels in the range of 20-510 μg in triplicate. Subsequently, the samples were

taken through the entire extraction procedure as described above (cf. 3.2.2.4 and 3.2.2.5). The non-enriched margarine was also subjected to the sample preparation procedure in order to confirm the absence of phytosterol oxidation products.

Repeatability and Reproducibility

In order to cover the full range of analyte concentrations, three different margarine samples (non-heated, heated for 90 min, heated for 150 min) corresponding to the low, medium and high levels determined for each analyte, respectively, were chosen. The samples were analyzed in triplicate on three days by one operator to determine the repeatability. Reproducibility was assessed by the additional triplicate analysis of each of the three samples by a second operator. A 10-fold injection of a margarine sample revealed very good instrumental repeatability, as for all compounds the relative standard deviation was <6 %.
As additional quality control measures, the performance of the instrument was regularly confirmed by injection of thermo-oxidized stigmasterol as reference sample.

3.2.3. Complementary Analysis of Enriched Margarines Before and After Heat Treatments

3.2.3.1 Heating Experiments

Microwave Heating

Margarine (1 g, accuracy of ± 0.1 mg) was weighed into a 250 mL glass bottle (5.52 cm i.d. at the bottom, resulting surface area in contact with air 24 cm^2). The bottle was sealed with a filter disk and the samples were heated for 4 min in a microwave at 800 W. After cooling to room temperature, the sample was dissolved in chloroform and transferred to a graduated flask with a final volume of 20 mL.

Pan-Frying

Margarine (1 g, accuracy of ± 0.1 mg) was placed in the middle of a Teflon-coated frying pan (Tefal, diam. 12 cm). The temperature of the enriched margarines was recorded every 30 s with an infrared thermometer (MeasuPro IRT20) during the heating procedure to assure similar temperature profiles for both margarines. The heating process was repeated several times and was shown to be reproducible. After 9 min of heating, the pan was removed from the cooker and cooled to room temperature. Subsequently, the heated sample was dissolved in chloroform and transferred to a graduated flask with a final volume of 20 mL.

Oven-Heating in a Bottle

Margarine (1 g, accuracy of ± 0.1 mg) was weighed into a 250 mL glass bottle (5.52 cm i.d. at the bottom, resulting surface area in contact with air 24 cm^2). The bottle was sealed with perforated aluminum foil that was fixed with a hose clamp and heated in an oven for 20 min at 200 °C after pre-heating the oven at 200 °C for 40 min. After cooling to room temperature, the sample was dissolved in chloroform and transferred to a graduated flask with a final volume of 20 mL.

Oven-Heating in a Casserole

Margarine (2.9 g, accuracy of ± 0.1 mg) was greased in a casserole (surface area 546.25 cm^2) and heated in an oven for 20 min at 200 °C after pre-heating the oven at 200 °C for 40 min. After cooling to room temperature, the sample was dissolved in chloroform and transferred to a graduated flask with a final volume of 50 mL.

All heat treatments were performed in duplicate.

3.2.3.2 Sample Preparation and UHPLC-APCI-MS Analysis

Either 30 mg (accuracy of ± 0.1 mg) of the non-heated margarine or an aliquot of the heated samples corresponding to 30 mg of originally used margarine was put into a vessel, the solvent was removed under a gentle stream of nitrogen if necessary, and 300 μL of the internal standard cholesteryl palmitate [2.5 mg/mL] was added. Subsequently, the samples were prepared according to sections 3.2.1.1 and 3.2.1.2. UHPLC-APCI-MS analyses were carried out using the 6430 triple quadrupole mass selective detector (Agilent Technologies, Waldbronn, Germany). All other parameters were set as described in section 3.2.1.4; for quantitative analysis of the margarine enriched with phytostanyl fatty acid esters, the MS detector was adjusted to monitor positive ions in the selected ion monitoring (SIM) mode with a dwell time of 40 ms at *m/z* 369.3, 385.3, and 399.3, respectively.

3.2.3.3 Sample Preparation for On-Line LC-GC Analysis

For analyses of the non-heated margarine, 150 mg material (accuracy of ± 0.1 mg) was directly weighed into 11 mL vials and subjected to further analysis. For analyses of the heated margarines, aliquots corresponding to 150 mg of originally used margarine for analysis of phytosterol oxidation products were placed into 11 mL vials. To the samples, 0.034 mg of 5,6*ß*-epoxycholesterol (IS_1) and 0.036 mg of 7-ketocholesterol (IS_2) were added as internal standards; to the margarine enriched with phytosteryl/-stanyl fatty acid esters that was heated in the oven in the casserole, 0.12 mg and 0.13 mg of IS_1 and IS_2 were added, respectively. The transesterification

for extraction of the phytosterol oxidation products and the subsequent acetylation were performed as described above (cf. 3.2.2.4). The residues were dissolved in 0.5-1 mL of *n*-hexane/MTBE/*iso*-propanol (80:20:0.3, v/v/v), depending on the expected concentrations of phytosterol oxidation products in the sample, and subjected to on-line LC-GC analysis according to the established and validated parameters (cf. 3.2.2.5).

3.2.3.4 Statistical Analysis

IBM SPSS Statistics (version 23) was used for statistical analyses. The impact of the heating procedures was evaluated by one-way ANOVA. Levels of significance: $p<0.001$: highly significant (***); $p<0.01$: very significant (**); $p<0.05$: significant (*). Statistically significant differences between the means were identified by Tukey's HSD post hoc test ($p<0.05$).

4 RESULTS AND DISCUSSION

4.1 Method Development for the Analysis of Intact Phytosteryl and/or Phytostanyl Fatty Acid Esters in Enriched Foods

Suitable analytical methods allowing the elucidation of the individual intact phytosteryl/-stanyl fatty acid esters are of scientific interest for several reasons. First of all, knowing the detailed qualitative and quantitative composition of these esters in enriched foods is the essential basis for authenticity assessments as required by the aforementioned regulations. An additional aspect reinforcing the importance of being able to characterize the ester preparations on an individual basis in their intact forms are the potential differences between individual phytosteryl/-stanyl esters concerning their cholesterol-lowering efficacies due to differences regarding intestinal hydrolysis. Further, as previously discussed, for a comprehensive characterization of the effects of oxidation processes on enriched foods, a differential detection of the individual intact esters is required.

Due to the thermal degradations of the intact esters in the course of GC-based analysis, as for example described by (Barnsteiner *et al.*, 2011; 2012), LC-based approaches seem to be more advisable. Despite the described progress (cf. 2.4.1), the presently available LC-based methodologies do only allow the quantitative investigation of mixtures containing only phytosteryl fatty acid esters on an individual basis. However, for those ester preparations commonly used for the enrichment of foods, i.e. either mixtures of phytostanyl fatty acid esters or even more complex mixtures of both, phytostanyl and phytosteryl fatty acid esters, appropriate methodologies enabling a quantitative determination of the individual intact esters are lacking.

4.1.1 Analysis of Phytostanyl Fatty Acid Esters in Enriched Foods via UHPLC-APCI-MS

There is a variety of foods available on the European market enriched with mixtures containing solely phytostanyl fatty acid esters. Therefore, as a first step, a thermally non-destructive, rapid, and sensitive chromatographic method based on UHPLC-separation in combination with MS-detection for the quantitation of intact phytostanyl fatty acid esters was developed. As mass spectrometer, a Dionex MSQ single quad instrument was used. Based on the fatty acid profiles of those oils authorized as sources, stanyl esters of long chain fatty acids (C16-C22) were expected to occur in the enriched foods (Figure 11). This was further ascertained by previous GC-based research, where several long chain fatty acid esters of sitostanol and campestanol were identified in phytostanyl ester-enriched foods (Barnsteiner *et al.*, 2011).

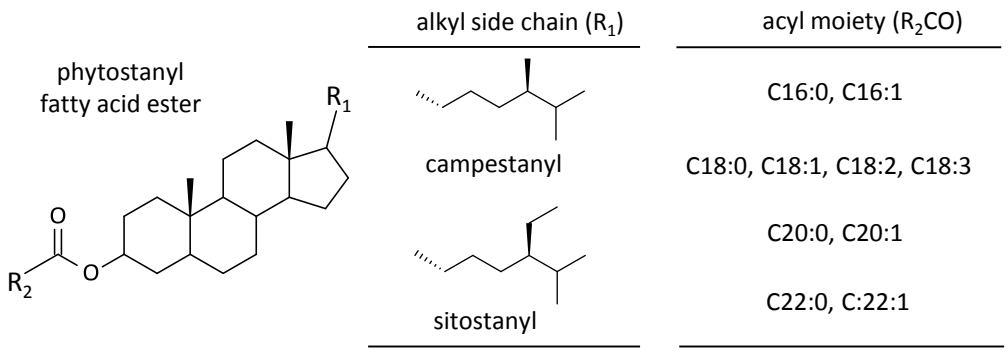

Figure 11. Structural variability of campestanyl and sitostanyl fatty acid esters occurring in phytostanyl ester-enriched foods.

The suitability of the approach should be demonstrated using different types of enriched foods reflecting the diversity in potential matrices (skimmed milk-drinking yogurt, margarine, and soft cheese-style spread).

4.1.1.1 Method Development and Optimization

As first step of the method development, commercially available cholesteryl esters were used to investigate the principle chromatographic behavior of steryl fatty acid esters. Using positive-ion APCI-MS, an intense formation of the fragment ion $[M\text{-}FA+H]^+$, resulting from the loss of the fatty acid due to an in-source fragmentation of the esters, has been described for both phytosteryl and phytostanyl fatty acid esters (Mezine *et al.*, 2003; Rudell *et al.*, 2011; Ishida, 2014). Therefore, in order to establish suitable LC conditions, the MS detector was operated in the positive-ion APCI SIM mode at *m/z* 369.3, corresponding to the mass of the $[M\text{-}FA+H]^+$ fragment ion of cholesteryl esters. Former efforts to establish RP-LC-based analyses of individual plant steryl fatty acid esters mainly used C18 columns (Billheimer *et al.*, 1983; Evershed *et al.*, 1987; Gordon and Griffith, 1992a; 1992b; Kuksis *et al.*, 1986; Caboni *et al.*, 2005; Ishida, 2014). Another study employed a hexyl phenyl column; however, this column also did not yield sufficient separations (Mezine *et al.*, 2003). In the present study, at first a C8 column with a particle size of 5 μm was tested, revealing a promising separation of cholesteryl esters of fatty acids that were expected to be the major esterified fatty acids in the enriched foods (Figure 12 A). Though, the resolution of the palmitic acid ester and the oleic acid ester was incomplete. This would have been a particular problem with regard to the analysis of enriched foods as the fatty acid mixtures used for the esterification of the phytostanols have previously been described to show a clear dominance of linoleic acid compared to palmitic acid (Barnsteiner *et al.*, 2011). Transferring the methodology to a UHPLC system, using a C8 column with sub 2 μm particles, significantly improved peak shapes, sensitivity, and the chromatographic separation. Besides a shortened analysis time, most notably, a

satisfactory resolution of the peak pair of cholesteryl-16:0 and cholesteryl-18:1 could thus be achieved (Figure 12 B).

In contrast to the approach described by Mezine *et al.* (2003), a MeOH/H_2O step gradient was chosen instead of a linear gradient of acetonitrile in water; the gradient elution improved the chromatographic separation and the use of methanol also provided a much better resolution and peak shape. In addition, an enhanced ionization efficiency has been ascribed to methanol as solvent in LC-APCI-MS applications due to a reduced proton affinity when compared to acetonitrile (Kostiainen and Kauppila, 2009).

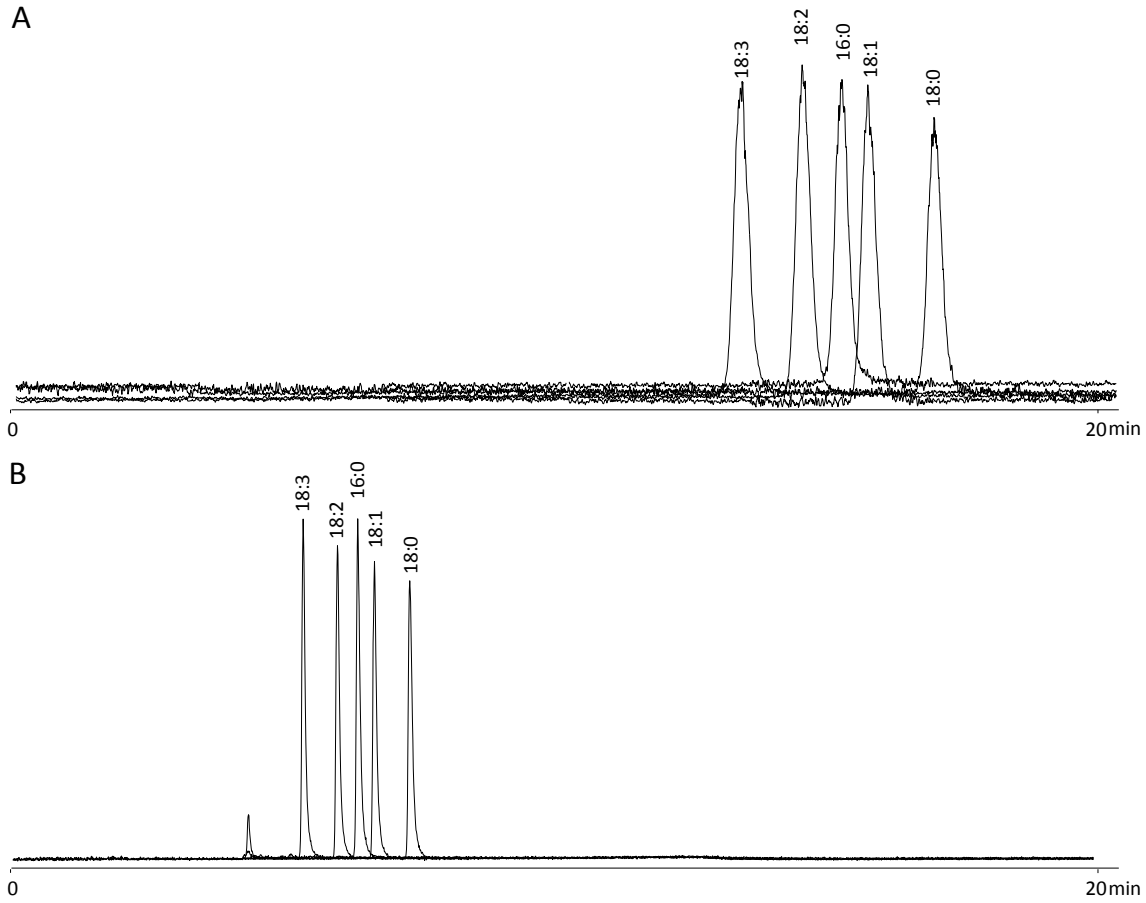

Figure 12. RP-HPLC separation of cholesteryl fatty acid esters: Overlay of APCI-SIM chromatograms on a 5 μm C8 column (Dionex Ultimate 3000, MN Licrospher RP8, 250 mm x 3 mm i.d.; 90 % MeOH in H_2O to 95 % MeOH in 2 min, 95 % MeOH until 5 min, to 100 % MeOH between 5 and 20 min; 0.8 mL/min, 5 μL injection volume; column temperature, 60 °C) (**A**) and of APCI-SIM chromatograms on a 1.7 μm C8 column (**B**) at *m/z* 369.3, corresponding to $[M\text{-}FA+H]^+$ (for conditions, cf. 3.2.1.3).

Based on the established conditions, the separation of sitostanyl and campestanyl fatty acid esters as to be expected in phytostanyl ester-enriched foods was investigated using synthesized reference compounds of stanyl esters of fatty acids occurring in the vegetable oils authorized as sources (SCF, 2000; 2003a; 2003b). The MS detector was operated in the positive-ion APCI SIM

mode, at *m/z* 385.3 for campestanyl fatty acid esters, and at *m/z* 399.3 for sitostanyl fatty acid esters. The 1.7 μm C8 column was shown to enable a separation of all stanyl fatty acid esters consisting of the same phytostanol nucleus within a rather short analysis time (12 min). The achieved resolution factors were all higher than 1. Those esters exhibiting different stanol nuclei were further distinguished via the selection of masses corresponding to the fragment ions $[M\text{-}FA+H]^+$ in the SIM mode. Overlays of the chromatograms obtained for the synthesized campestanyl and sitostanyl fatty acid esters are shown in Figure 13.

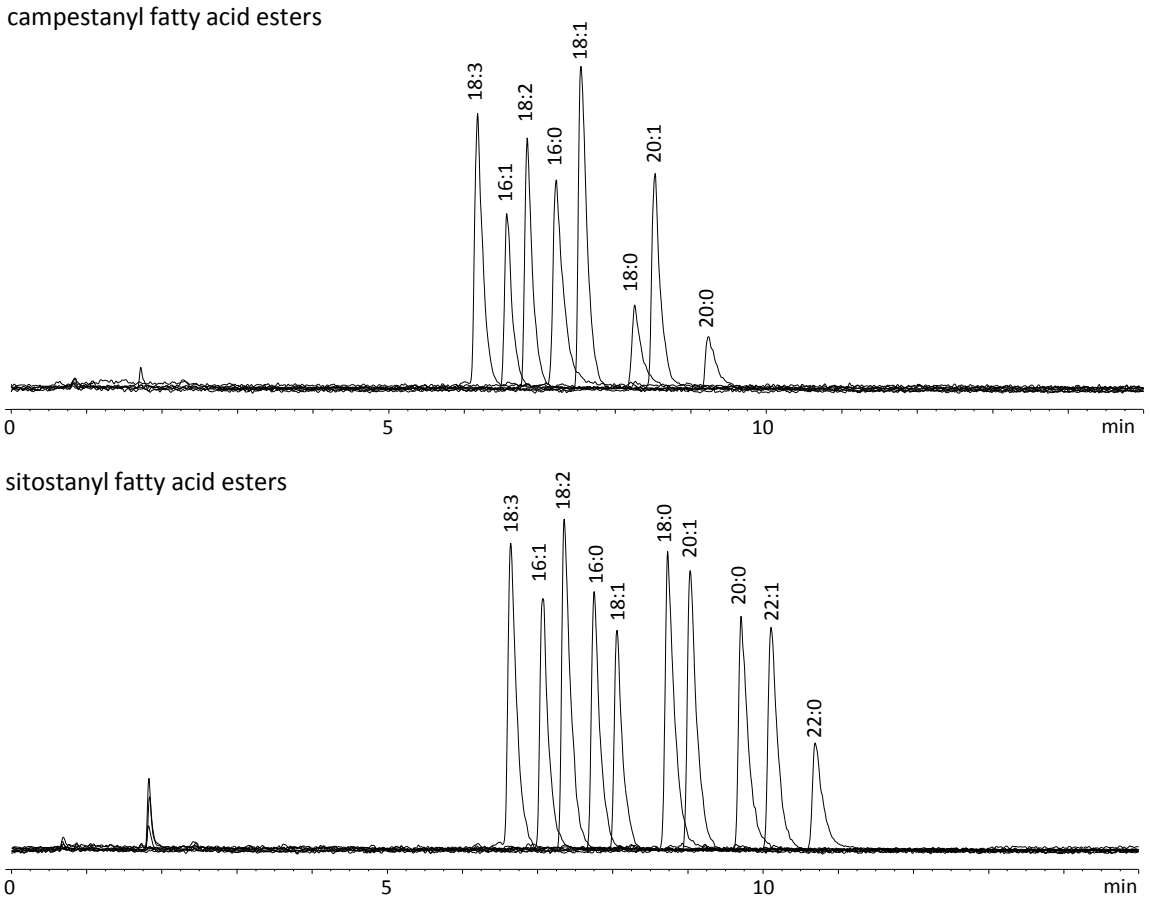

Figure 13. RP-UHPLC based separation of synthesized phytostanyl fatty acid esters on a 1.7 μm C8 column: Overlay of APCI-SIM chromatograms of campestanyl fatty acid esters at *m/z* 385.2 and of APCI-SIM chromatograms of sitostanyl fatty acid esters at *m/z* 399.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ions.

The order of elution was impacted by both the chain length and the degree of saturation of the esterified fatty acids: the lower the carbon number and the higher the number of double bonds, the shorter was the retention time, analogous to the separation observed for the cholesteryl esters. Additionally, campestanyl fatty acid esters eluted earlier than the corresponding sitostanyl fatty acid esters. The observed retention behavior is similar to elution profiles described for phytosteryl/-stanyl fatty acid esters on reversed phases (Billheimer *et al.*, 1983; Evershed *et al.*, 1987; Mezine *et al.*, 2003; Caboni *et al.*, 2005).

Full scan APCI mass spectra of the synthesized reference compounds were recorded under the established LC-conditions; as examples, the positive ion full scan mass spectra of sitostanyl-18:0 and sitostanyl-18:3 are shown in Figure 14.

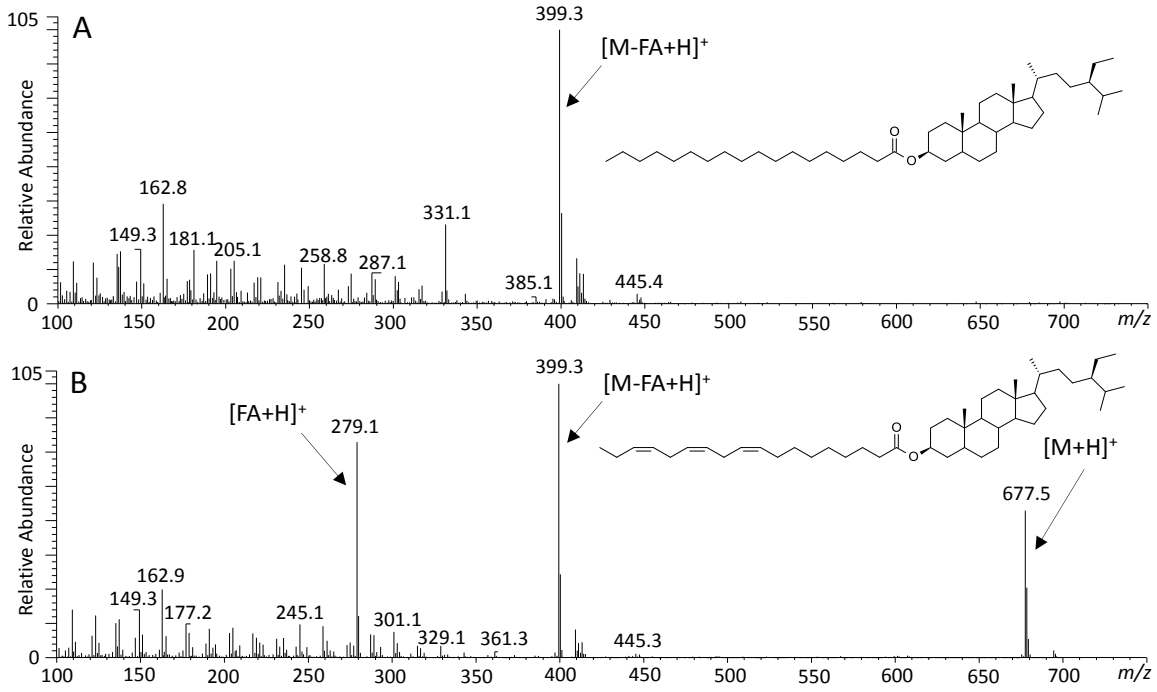

Figure 14. APCI-MS full scan spectra of sitostanyl-18:0 (**A**) and sitostanyl-18:3 (**B**): $[M+H]^+$, protonated molecular ion; [FA], fatty acid (for conditions, cf. 3.2.1.3).

The fragment ion $[M-FA+H]^+$ used for detection of the standard compounds was shown to constitute the base peak of all stanyl fatty acid ester spectra; for the employed MS instrumentation, a cone voltage of 80 V turned out to be optimum for the formation of this ion. Protonated molecular ions $[M+H]^+$ were only observed in spectra of stanols esterified to unsaturated fatty acids; however, intensities were low for stanyl esters of mono-unsaturated fatty acids, whereas poly-unsaturation favored the formation of molecular ions. Likewise, the extent of the formation of a characteristic fragment ion $[FA+H]^+$ correlated with the number of double bonds in the fatty acid moiety.

4.1.1.2 UHPLC-APCI-MS Quantitation

As the fragment ion $[M-FA+H]^+$ occurred in high abundance in the mass spectra of all esters, the respective quantitation of campestanyl fatty acid esters (*m/z* 385.3) and sitostanyl fatty acid esters (*m/z* 399.3) in SIM mode was based on this ion. The employed internal standard cholesteryl linolenate was also detected in SIM mode at *m/z* 369.3, the mass accordingly corresponding to the fragment ion $[M-FA+H]^+$. The chromatographic usefulness of cholesteryl linolenate as internal

standard was confirmed via injection and subsequent selected ion monitoring at *m/z* 385.3 and *m/z* 399.3. The compound exhibited a fragment ion at *m/z* 385, indicating a cleavage of the acyl moiety. Owing to the shorter retention time of the internal standard, this fragment ion did not impair campestanyl ester quantitation and therefore its concentration dependency was not further pursued. In contrast, the cheaper and easier available cholesteryl palmitate was not sufficiently separated from campestanyl linolenate and was therefore not considered appropriate as internal standard. A compilation of chromatographic data and selected ions for individual phytostanyl fatty acid esters is shown in Table 6.

Table 6. Relative retention times (RRT), and selected ions of individual phytostanyl fatty acid esters.

no.	stanyl fatty acid ester	RRT[a,b]	$[M\text{-}FA+H]^+$ *m/z*[b]
1	campestanyl-18:3	1.165	385.3
2	campestanyl-16:1	1.240	385.3
3	campestanyl-18:2	1.291	385.3
4	campestanyl-16:0	1.362	385.3
5	campestanyl-18:1	1.421	385.3
6	campestanyl-18:0	1.553	385.3
7	campestanyl-20:1	1.613	385.3
8	campestanyl-20:0	1.742	385.3
9	sitostanyl-18:3	1.252	399.3
10	sitostanyl-16:1	1.334	399.3
11	sitostanyl-18:2	1.376	399.3
12	sitostanyl-16:0	1.459	399.3
13	sitostanyl-18:1	1.505	399.3
14	sitostanyl-18:0	1.651	399.3
15	sitostanyl-20:1	1.707	399.3
16	sitostanyl-20:0	1.836	399.3
17	sitostanyl-22:1	1.893	399.3
18	sitostanyl-22:0	2.019	399.2

[a] Retention times relative to cholesteryl linolenate (Kinetex C8, 1.7 μm).
[b] Relative retention times and mass spectra were determined using synthesized reference compounds.

In order to compensate for varying detector responses of distinct stanyl fatty acid esters, external five-point-calibration functions were generated, calibrating the response ratio of each individual stanyl fatty acid ester and the internal standard. Only for campestanyl-18:0, -20:0, and -20:1 three-point-calibration functions were used, because of the lack of responses for these components in the calibration mixtures at the lowest concentrations.

4.1.1.3 Method Validation

The developed approach was validated in terms of LOD, LOQ, linearity, application to different matrices, repeatability, reproducibility, and recovery rates. The determined limits of detection

and limits of quantitation of the individual plant stanyl fatty acid esters occurring in the mixture STAEST-115 together with the characteristics of their respective calibration curves are given in Table 7. For campestanyl fatty acid esters, the LOD and LOQ were in the same order of magnitude as those achieved for the previously described GC-based approach (Barnsteiner *et al.*, 2011). In contrast, for sitostanyl esters (except for palmitate), the LOD and LOQ obtained via the UHPLC-analysis were considerably lower; this reflects the increased thermal degradation of these later eluting compounds in the course of high temperature GC. The coefficients of correlation (r^2) were in the range of 0.9914 – 0.9997, confirming a linear relationship of each plant stanyl fatty acid ester within the respective concentration range in comparison to the internal standard. Area ratios and amount ratios of the investigated plant stanyl esters were consistently within the range of the calibration.

Table 7. LOD, LOQ, and characteristics of calibration curves of individual phytostanyl fatty acid esters.

stanyl fatty acid ester	LOD [μg/mL][a]	LOQ [μg/mL][a]	slope	intercept	r^2
campestanyl-18:3	0.09	0.28	0.2728	0.0023	0.9996
campestanyl-18:2	0.10	0.30	0.2189	0.0040	0.9996
campestanyl-16:0	0.06	0.17	0.3397	0.0024	0.9984
campestanyl-18:1	0.11	0.32	0.2250	0.0120	0.9997
campestanyl-18:0	0.10	0.28	0.4259	-0.0011	0.9936
campestanyl-20:1	0.07	0.21	0.2977	-0.0004	0.9994
campestanyl-20:0	0.10	0.25	0.5455	-0.0007	0.9914
sitostanyl-18:3	0.05	0.16	0.1994	0.0109	0.9959
sitostanyl-18:2	0.09	0.26	0.1595	0.0228	0.9992
sitostanyl-16:0	0.04	0.13	0.3162	0.0108	0.9989
sitostanyl-18:1	0.07	0.21	0.1644	0.0872	0.9988
sitostanyl-18:0	0.06	0.17	0.3347	0.0043	0.9990
sitostanyl-20:1	0.08	0.25	0.1788	0.0040	0.9927
sitostanyl-20:0	0.04	0.10	0.3442	0.0026	0.9962
sitostanyl-22:1	0.05	0.15	0.1470	0.0018	0.9979
sitostanyl-22:0	0.03	0.09	0.3043	0.0024	0.9923

[a] LOD and LOQ were determined using the plant stanyl fatty acid mixture "STAEST-115" and are expressed as μg/mL of injection volume, determined on the basis of 0.8 μL injection volume; LOD, LOQ, and calibration curves were not determined for campestanyl-16:1 and sitostanyl-16:1, as these compounds could not be detected in any of the samples analyzed.

Plant Stanyl Fatty Acid Esters in Skimmed Milk-Drinking Yogurts

Skimmed milk-drinking yogurts were selected to test the suitability of the developed UHPLC-APCI-MS methodology because they are examples for protein-based, low-fat enriched dairy products. For this food category a method allowing the fast extraction of intact fatty acid stanyl esters has been described, based on acid digestion of the protein matrix (Barnsteiner *et al.*, 2011). No

degradation or hydrolysis of the esters has been observed during this workup step (Barnsteiner *et al.*, 2011; Esche *et al.*, 2013a). Due to the fat content of only 2.1 % in this type of product, no interferences with lipid matrix constituents during chromatographic analysis were expected. Preliminary LC-MS experiments revealed a pronounced signal suppression caused by co-eluting triglycerides only if the ratio of triglycerides and plant stanyl fatty acid esters in the injection solutions was higher than 1. EtOAc was chosen as injection solvent as the stanyl ester extracts could not be completely dissolved in more polar media. The SIM chromatograms for campestanyl and sitostanyl fatty acid esters extracted from skimmed milk-drinking yogurts are shown in Figure 15.

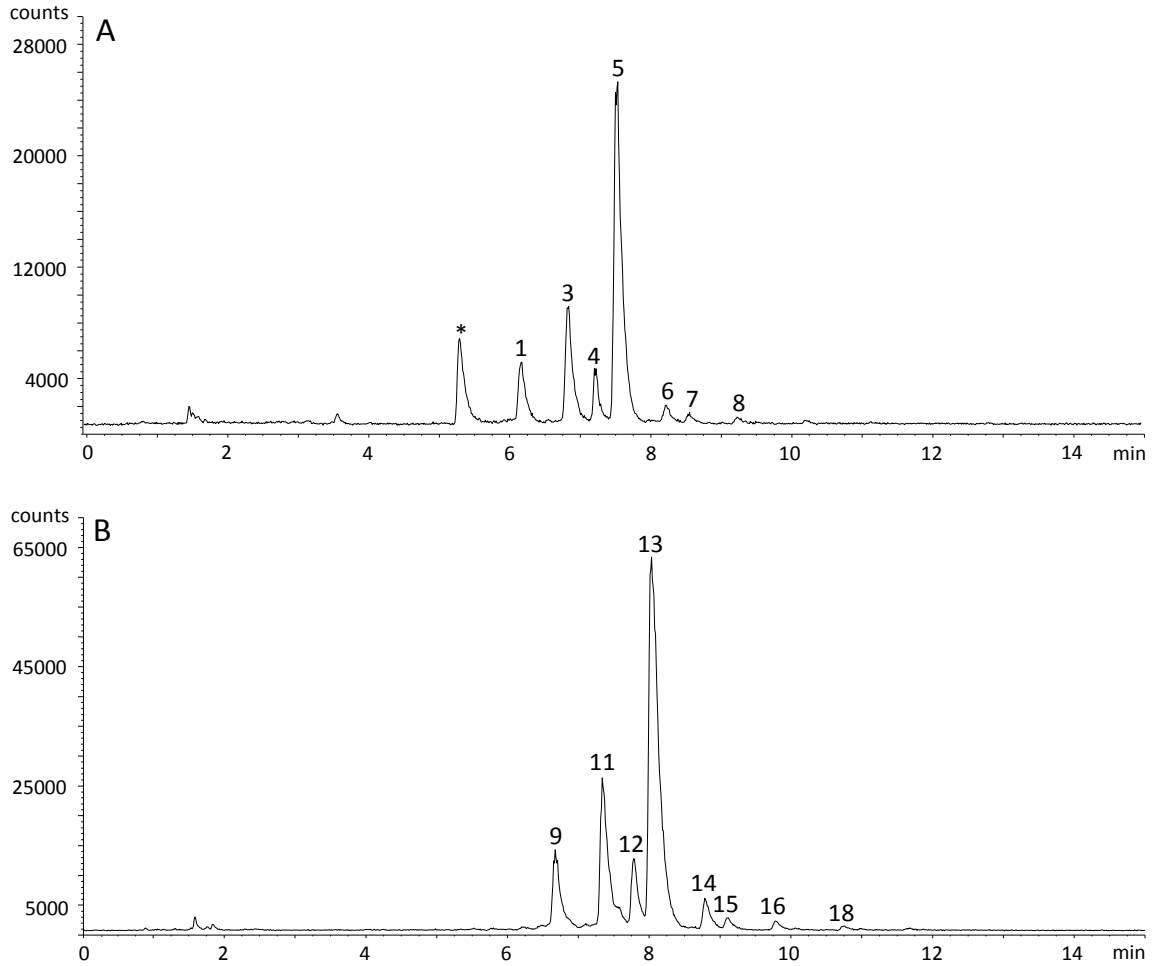

Figure 15. UHPLC-APCI-MS analysis of phytostanyl fatty acid esters extracted from phytostanyl ester-enriched skimmed milk-drinking yogurt. SIM chromatogram of campestanyl fatty acid esters at *m/z* 385.3 (**A**), and SIM chromatogram of sitostanyl fatty acid esters at *m/z* 399.3 (**B**), corresponding to the respective $[M\text{-}FA+H]^+$ fragment ions. The peak numbering is in accordance with that in Table 6. The asterisk in panel (A) is derived from the internal standard cholesteryl-18:3. For conditions, cf. 3.2.1.3.

The detailed compositions of phytostanyl fatty acid esters and the total contents of phytostanyl esters determined in skimmed milk-drinking yogurts are given in Table 8 and Table 9.

Table 8. Repeatability and reproducibility data for the UHPLC-APCI-MS analysis of individual phytostanyl fatty acid esters in phytostanyl ester-enriched skimmed milk-drinking yogurt (no. 1).

stanyl fatty acid ester	operator I			operator II	mean	CV [%]
[g/kg]	day 1	day 2	day 3	day 1		
campestanyl-18:3	0.91 ± 0.05[a]	0.94 ± 0.04	0.86 ± 0.04	0.88 ± 0.02	0.90 ± 0.05[b]	5.1
campestanyl-18:2	2.11 ± 0.02	2.14 ± 0.05	2.09 ± 0.01	2.14 ± 0.03	2.12 ± 0.04	1.7
campestanyl-16:0	0.49 ± 0.01	0.48 ± 0.00	0.49 ± 0.02	0.48 ± 0.01	0.49 ± 0.01	2.1
campestanyl-18:1	6.53 ± 0.12	6.77 ± 0.02	6.49 ± 0.12	6.65 ± 0.09	6.60 ± 0.13	2.0
campestanyl-18:0	0.18 ± 0.01	0.20 ± 0.02	0.20 ± 0.03	0.18 ± 0.01	0.19 ± 0.02	8.7
campestanyl-20:1	0.12 ± 0.02	0.14 ± 0.02	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	11.0
campestanyl-20:0	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.01	0.06 ± 0.01	7.8
sitostanyl-18:3	3.22 ± 0.16	3.22 ± 0.13	3.33 ± 0.07	3.31 ± 0.06	3.27 ± 0.11	3.3
sitostanyl-18:2	7.92 ± 0.12	7.86 ± 0.08	7.86 ± 0.19	7.84 ± 0.05	7.87 ± 0.11	1.4
sitostanyl-16:0	1.72 ± 0.03	1.71 ± 0.04	1.89 ± 0.17	1.80 ± 0.06	1.78 ± 0.11	6.2
sitostanyl-18:1	25.85 ± 0.36	26.00 ± 0.33	25.95 ± 0.43	26.06 ± 0.23	25.97 ± 0.31	1.2
sitostanyl-18:0	0.76 ± 0.02	0.79 ± 0.04	0.79 ± 0.06	0.75 ± 0.01	0.77 ± 0.04	4.7
sitostanyl-20:1	0.42 ± 0.01	0.41 ± 0.05	0.37 ± 0.03	0.47 ± 0.04	0.42 ± 0.05	11.3
sitostanyl-20:0	0.20 ± 0.02	0.20 ± 0.01	0.21 ± 0.02	0.19 ± 0.02	0.20 ± 0.02	8.4
sitostanyl-22:0	0.07 ± 0.00	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	17.6
total esters	50.57 ± 0.76	50.98 ± 0.13	50.82 ± 0.24	51.05 ± 0.20	50.86 ± 0.40	0.8

[a] Values represent the mean ± standard deviation (n=3).
[b] Values represent the mean of all analyses ± standard deviation (n=12).

Table 9. Repeatability and reproducibility data for the UHPLC-APCI-MS analysis of individual phytostanyl fatty acid esters in phytostanyl ester-enriched skimmed milk-drinking yoghurt (no. 2).

stanyl fatty acid ester	operator I			operator II	mean	CV [%]
[g/kg]	day 1	day 2	day 3	day 1		
campestanyl-18:3	0.81 ± 0.07	0.82 ± 0.03	0.89 ± 0.04	0.88 ± 0.05	0.85 ± 0.06	7.0
campestanyl-18:2	2.05 ± 0.05	2.12 ± 0.10	2.10 ± 0.01	1.99 ± 0.00	2.07 ± 0.07	3.4
campestanyl-16:0	0.48 ± 0.04	0.48 ± 0.00	0.50 ± 0.05	0.46 ± 0.03	0.48 ± 0.03	5.8
campestanyl-18:1	6.59 ± 0.09	6.57 ± 0.11	6.83 ± 0.15	6.67 ± 0.06	6.67 ± 0.14	2.1
campestanyl-18:0	0.19 ± 0.02	0.18 ± 0.00	0.20 ± 0.02	0.19 ± 0.03	0.19 ± 0.02	10.5
campestanyl-20:1	0.14 ± 0.02	0.14 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	10.4
campestanyl-20:0	0.07 ± 0.01	0.07 ± 0.00	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	11.8
sitostanyl-18:3	3.17 ± 0.05	3.27 ± 0.06	3.33 ± 0.09	3.21 ± 0.01	3.25 ± 0.08	2.5
sitostanyl-18:2	7.94 ± 0.20	8.01 ± 0.13	8.13 ± 0.05	7.91 ± 0.08	8.00 ± 0.14	1.8
sitostanyl-16:0	1.82 ± 0.06	1.87 ± 0.04	1.66 ± 0.06	1.79 ± 0.01	1.79 ± 0.09	5.1
sitostanyl-18:1	26.41 ± 0.30	26.43 ± 0.37	26.79 ± 0.61	26.86 ± 0.70	26.62 ± 0.49	1.9
sitostanyl-18:0	0.74 ± 0.01	0.74 ± 0.05	0.73 ± 0.01	0.80 ± 0.03	0.75 ± 0.04	5.1
sitostanyl-20:1	0.46 ± 0.05	0.45 ± 0.04	0.32 ± 0.01	0.49 ± 0.02	0.43 ± 0.07	17.3
sitostanyl-20:0	0.21 ± 0.03	0.20 ± 0.01	0.22 ± 0.01	0.21 ± 0.02	0.21 ± 0.02	8.0
sitostanyl-22:0	0.10 ± 0.01	0.09 ± 0.03	0.09 ± 0.00	0.11 ± 0.01	0.09 ± 0.02	19.0
total esters	51.17 ± 0.57	51.45 ± 0.59	51.99 ± 0.83	51.78 ± 0.77	51.60 ± 0.68	1.3

[a] Values represent the mean ± standard deviation (n=3).
[b] Values represent the mean of all analyses ± standard deviation (n=12).

Oleic acid esters dominated followed by esters of linoleic, linolenic, and palmitic acid, whereas the fatty acids consisting of 20 and 22 carbon atoms were only esterified to a minor extent; this pattern indicated rapeseed oil as fatty acid source for esterification (Codex Alimentarius, 1999). The calculated amounts of total phytostanols were in good agreement with the labelling ("plant stanol 3.0 %"). In addition, both the individual ester contents and percentage distributions determined via the presented approach are corresponding to data obtained via GC/FID on intact plant stanyl fatty acid esters in yogurt drinks of the same type and brand (Barnsteiner *et al.*, 2011). Phytostanyl esters of palmitoleic acid could not be identified, and sitostanyl-22:1 was also not detectable in the present work; however, a direct comparison of the two approaches, in that respect, is not possible due to co-elutions of esters of saturated and monounsaturated fatty acids in the GC-based method. Two bottles from different batches were analyzed in triplicate on three days by one operator, revealing excellent repeatability of the method. A quantitation of comparable ester contents after analysis of the samples by a second operator demonstrated the reproducibility of the method. This was supported by excellent coefficients of variation of 0.8 and 1.3 % for the quantitation of the total esters; only for some low-concentrated esters, the coefficients of variation were >10 %.

Plant Stanyl Fatty Acid Esters in Enriched Margarines

Margarines enriched with plant stanyl fatty acid esters were selected as example for fat-based food matrices. In this case, prior removal of interfering lipid matrix constituents was required. Recently, aminopropyl-based solid phase extraction was shown to be a suitable tool for the selective separation of plant stanyl fatty acid esters from other lipids (Esche *et al.*, 2012; Oelschlägel *et al.*, 2012). Hence, a rapid and efficient SPE-based pre-separation was established, using aminopropyl-modified silica cartridges and a mixture of *n*-hexane/diethyl ether (99:1, v/v) for the selective elution of phytostanyl fatty acid esters. Table 10 shows the quantitative data obtained after investigation of a commercial margarine on three different days and additionally by a second operator; the results were good regarding both overall repeatability and reproducibility with a CV of 1.4 %. Similar to the analyses of the skimmed milk-drinking yogurt, the quantitatively minor esters revealed higher CVs. The calculated contents of total phytostanols were in very good agreement with the amounts declared on the label. In accordance with the pattern of the esters detected in skimmed milk-drinking yogurts, the distribution of the plant stanyl fatty acid esters determined in margarine suggested rapeseed fatty acids having been used for esterification. In addition to the compounds determined in skimmed milk-drinking yogurts, sitostanyl-22:1 could also be detected and quantitated, accounting for approximately 0.3 % of total esters in the margarine sample.

Table 10. Repeatability and reproducibility data for the UHPLC-APCI-MS analysis of individual phytostanyl fatty acid esters in phytostanyl ester-enriched margarine (no. 1).

stanyl fatty acid ester	operator I			operator II	mean	CV [%]
[g/kg]	day 1	day 2	day 3	day 1		
campestanyl-18:3	2.52 ± 0.05[a]	2.42 ± 0.02	2.67 ± 0.01	2.71 ± 0.08	2.58 ± 0.13[b]	5.1
campestanyl-18:2	8.12 ± 0.22	7.99 ± 0.42	8.15 ± 0.02	8.50 ± 0.03	8.19 ± 0.28	3.4
campestanyl-16:0	2.06 ± 0.06	2.12 ± 0.23	2.09 ± 0.07	2.18 ± 0.03	2.12 ± 0.12	5.5
campestanyl-18:1	27.15 ± 0.27	27.48 ± 0.80	27.73 ± 0.27	27.09 ± 0.36	27.36 ± 0.49	1.8
campestanyl-18:0	0.65 ± 0.05	0.66 ± 0.06	0.71 ± 0.02	0.72 ± 0.08	0.68 ± 0.06	8.5
campestanyl-20:1	0.62 ± 0.12	0.52 ± 0.15	0.55 ± 0.02	0.58 ± 0.04	0.57 ± 0.10	17.4
campestanyl-20:0	0.25 ± 0.05	0.24 ± 0.06	0.26 ± 0.03	0.27 ± 0.03	0.25 ± 0.04	15.1
sitostanyl-18:3	5.81 ± 0.21	5.32 ± 0.21	5.57 ± 0.08	5.33 ± 0.17	5.51 ± 0.26	4.6
sitostanyl-18:2	18.84 ± 0.18	18.56 ± 0.32	18.30 ± 0.29	19.03 ± 0.54	18.68 ± 0.42	2.3
sitostanyl-16:0	4.73 ± 0.08	5.08 ± 0.63	5.05 ± 0.18	5.17 ± 0.19	5.01 ± 0.34	6.8
sitostanyl-18:1	66.54 ± 1.10	68.10 ± 1.25	68.53 ± 1.15	66.52 ± 0.35	67.42 ± 1.29	1.9
sitostanyl-18:0	1.85 ± 0.12	1.78 ± 0.17	1.90 ± 0.06	1.70 ± 0.11	1.81 ± 0.13	7.2
sitostanyl-20:1	0.93 ± 0.06	1.15 ± 0.11	1.09 ± 0.06	1.09 ± 0.13	1.07 ± 0.12	11.1
sitostanyl-20:0	0.51 ± 0.07	0.54 ± 0.11	0.55 ± 0.02	0.52 ± 0.03	0.53 ± 0.06	11.0
sitostanyl-22:1	0.53 0.12	0.45 0.11	0.47 0.07	0.53 0.07	0.50 0.09	18.0
sitostanyl-22:0	0.23 ± 0.05	0.20 ± 0.05	0.25 ± 0.00	0.27 ± 0.05	0.24 ± 0.05	20.0
total esters	141.34 ± 1.82	142.62 ± 3.17	143.88 ± 1.43	142.2 ± 0.60	142.51 ± 1.95	1.4

[a] Values represent the mean ± standard deviation (n=3).
[b] Values represent the mean of all analyses ± standard deviation (n=12).

Plant Stanyl Fatty Acid Esters in Soft Cheese-Style Spread

The soft cheese-style spread was selected for further method validation because it contains both considerable amounts of protein and fat. Consequently, a combination of the acid digestion applied to the skimmed milk-drinking yogurts and the solid phase extraction employed for removal of triglycerides in the margarine sample was used for this type of product. This allowed such a complex food matrix also to be analyzed quickly and without further methodological modifications. The distributions of the plant stanyl fatty acid esters analyzed in two packages of soft cheese-style spread resembled those determined in drinking yogurts and margarine, once more indicating rapeseed oil as fatty acid source and at the same time confirming the reliability of the established analytical approach. Table 11 and Table 12 present the quantitative data for two packages of soft cheese-style spread obtained by triplicate analyses on three days and by an additional second operator, their overall low deviations proving good repeatability and good reproducibility. However, as previously observed, for the lower concentrated phytostanyl esters, coefficients of variations of 10 to 17 % were revealed. The calculated amounts of total phytostanols matched the contents declared on the package labeling.

Table 11. Repeatability and reproducibility data for the UHPLC-APCI-MS analysis of individual phytostanyl fatty acid esters in phytostanyl ester-enriched soft cheese-style spread (no. 1).

stanyl fatty acid ester	operator I			operator II	mean	CV [%]
[g/kg]	day 1	day 2	day 3	day 1		
campestanyl-18:3	1.20 ± 0.03[a]	1.20 ± 0.03	1.14 ± 0.03	1.18 ± 0.04	1.18 ± 0.04[b]	3.1
campestanyl-18:2	2.68 ± 0.11	2.67 ± 0.11	2.49 ± 0.06	2.69 ± 0.06	2.63 ± 0.12	4.4
campestanyl-16:0	0.67 ± 0.03	0.56 ± 0.08	0.54 ± 0.02	0.64 ± 0.07	0.60 ± 0.07	12.4
campestanyl-18:1	8.37 ± 0.11	8.17 ± 0.21	8.38 ± 0.24	8.40 ± 0.04	8.33 ± 0.17	2.0
campestanyl-18:0	0.24 ± 0.01	0.29 ± 0.01	0.31 ± 0.03	0.36 ± 0.01	0.30 ± 0.05	15.8
campestanyl-20:1	0.17 ± 0.01	0.20 ± 0.02	0.16 ± 0.02	0.19 ± 0.03	0.18 ± 0.02	13.1
campestanyl-20:0	0.07 ± 0.00	0.08 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	16.5
sitostanyl-18:3	4.49 ± 0.03	4.54 ± 0.04	4.59 ± 0.12	4.52 ± 0.01	4.53 ± 0.07	1.5
sitostanyl-18:2	10.98 ± 0.05	10.93 ± 0.04	10.90 ± 0.43	11.06 ± 0.10	10.97 ± 0.20	1.8
sitostanyl-16:0	2.56 ± 0.09	2.59 ± 0.03	2.41 ± 0.06	2.57 ± 0.09	2.53 ± 0.10	3.9
sitostanyl-18:1	36.19 ± 0.34	35.99 ± 0.33	37.48 ± 0.52	36.09 ± 0.46	36.44 ± 0.73	2.0
sitostanyl-18:0	1.11 ± 0.03	1.10 ± 0.04	1.02 ± 0.04	1.11 ± 0.07	1.08 ± 0.05	4.9
sitostanyl-20:1	0.66 ± 0.07	0.72 ± 0.00	0.65 ± 0.07	0.67 ± 0.05	0.67 ± 0.06	8.3
sitostanyl-20:0	0.39 ± 0.05	0.38 ± 0.03	0.27 ± 0.02	0.35 ± 0.03	0.35 ± 0.05	15.6
sitostanyl-22:0	0.13 ± 0.02	0.15 ± 0.02	0.11 ± 0.01	0.13 ± 0.01	0.13 ± 0.02	16.1
total esters	69.91 ± 0.35	69.56 ± 0.32	70.53 ± 1.21	70.04 ± 0.38	70.01 ± 0.68	1.0

[a] Values represent the mean ± standard deviation (n=3).
[b] Values represent the mean of all analyses ± standard deviation (n=12).

Table 12. Repeatability and reproducibility data for the UHPLC-APCI-MS analysis of individual phytostanyl fatty acid esters in phytostanyl ester-enriched soft cheese-style spread (no. 2).

stanyl fatty acid ester	operator I			operator II	mean	CV [%]
[g/kg]	day 1	day 2	day 3	day 1		
campestanyl-18:3	1.29 ± 0.05[a]	1.17 ± 0.00	1.15 ± 0.09	1.17 ± 0.06	1.19 ± 0.07[b]	6.1
campestanyl-18:2	2.76 ± 0.15	2.79 ± 0.08	2.72 ± 0.10	2.78 ± 0.06	2.76 ± 0.08	3.0
campestanyl-16:0	0.64 ± 0.15	0.66 ± 0.02	0.68 ± 0.07	0.59 ± 0.00	0.67 ± 0.08	11.9
campestanyl-18:1	8.30 ± 0.14	8.15 ± 0.05	8.70 ± 0.20	8.46 ± 0.06	8.41 ± 0.24	2.9
campestanyl-18:0	0.32 ± 0.07	0.31 ± 0.01	0.37 ± 0.03	0.29 ± 0.02	0.32 ± 0.04	13.5
campestanyl-20:1	0.17 ± 0.00	0.17 ± 0.02	0.16 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	9.6
campestanyl-20:0	0.07 ± 0.00	0.07 ± 0.00	0.09 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	13.5
sitostanyl-18:3	5.10 ± 0.24	4.68 ± 0.06	4.52 ± 0.04	4.84 ± 0.11	4.76 ± 0.23	4.9
sitostanyl-18:2	11.28 ± 0.01	11.14 ± 0.02	11.34 ± 0.17	11.30 ± 0.08	11.26 ± 0.12	1.0
sitostanyl-16:0	2.45 ± 0.07	2.63 ± 0.07	2.50 ± 0.09	2.59 ± 0.21	2.55 ± 0.13	5.2
sitostanyl-18:1	36.81 ± 0.55	36.14 ± 0.18	37.59 ± 0.36	36.48 ± 0.20	36.75 ± 0.65	1.8
sitostanyl-18:0	1.05 ± 0.04	1.11 ± 0.04	1.08 ± 0.06	1.00 ± 0.09	1.06 ± 0.07	6.8
sitostanyl-20:1	0.60 ± 0.08	0.70 ± 0.04	0.50 ± 0.02	0.72 ± 0.05	0.63 ± 0.11	16.8
sitostanyl-20:0	0.30 ± 0.03	0.28 ± 0.01	0.28 ± 0.02	0.27 ± 0.02	0.28 ± 0.02	7.9
sitostanyl-22:0	0.15 ± 0.02	0.13 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	9.9
total esters	71.29 ± 0.33	70.15 ± 0.17	71.84 ± 0.40	70.94 ± 0.36	71.03 ± 0.72	1.0

[a] Values represent the mean ± standard deviation (n=3).
[b] Values represent the mean of all analyses ± standard deviation (n=12).

For the three matrices, recovery rates were determined for individual phytostanyl fatty acid esters. The respective amounts of the plant stanyl ester mixture "STAEST-115" were added to non-enriched foods, and they were subjected to the corresponding working-up procedure. The resulting recovery rates are compiled in Table 13.

Table 13. Recovery rates of individual phytostanyl fatty acid esters spiked to non-enriched skimmed milk-drinking yoghurt, soft cheese-style spread, and margarine.

steryl / stanyl fatty acid ester	recovery [%]		
	drinking yoghurt[a]	soft cheese-spread[b]	margarine[c]
campestanyl-18:3	97.0 ± 2.5[d]	98.2 ± 2.5	98.8 ± 1.0
campestanyl-18:2	97.2 ± 0.9	95.7 ± 0.6	96.7 ± 3.1
campestanyl-16:0	100.7 ± 2.6	100.5 ± 0.3	98.8 ± 4.6
campestanyl-18:1	96.6 ± 1.2	95.1 ± 1.2	94.0 ± 1.0
campestanyl-18:0	96.8 ± 0.1	97.3 ± 0.7	98.0 ± 2.5
campestanyl-20:1	106.4 ± 3.7	101.8 ± 7.5	104.8 ± 1.6
campestanyl-20:0	97.2 ± 0.1	99.0 ± 0.8	95.7 ± 3.4
sitostanyl-18:3	97.2 ± 2.3	99.0 ± 0.5	99.0 ± 0.3
sitostanyl-18:2	97.5 ± 1.1	96.5 ± 1.1	97.4 ± 1.4
sitostanyl-16:0	96.7 ± 3.1	100.3 ± 0.7	96.7 ± 3.0
sitostanyl-18:1	98.0 ± 0.4	95.6 ± 0.7	97.1 ± 2.4
sitostanyl-18:0	98.7 ± 1.6	99.7 ± 0.7	97.3 ± 3.6
sitostanyl-20:1	99.0 ± 0.2	98.2 ± 1.5	99.3 ± 1.7
sitostanyl-20:0	98.6 ± 2.2	98.0 ± 0.9	95.1 ± 1.3
sitostanyl-22:1	99.4 ± 1.6	100.2 ± 1.0	99.0 ± 1.4
sitostanyl-22:0	95.9 ± 2.9	99.9 ± 1.7	98.1 ± 1.5
total esters	97.7 ± 0.5	96.4 ± 0.3	97.2 ± 1.4

[a] Blank samples were spiked with 5.0 mg of a phytostanyl fatty acid ester mixture.
[b] Blank samples were spiked with 4.9 mg of a phytostanyl fatty acid ester mixture.
[c] Blank samples were spiked with 4.5 mg of a phytostanyl fatty acid ester mixture.
[d] Values represent the mean ± standard deviation (n=3).

The recovery rates of the individual esters were throughout between 94 and 106.4 % for all three analytical approaches. For total esters, also very good recoveries were determined, ranging from 96.4 % for the soft cheese-style spread to 97.7 % for the skimmed milk-drinking yoghurt, thus confirming the stability of the analytes during all of the working-up procedures and the applicability of the methodology to different matrices and varying concentrations of the analytes.

4.1.2 Analysis of Phytosteryl and Phytostanyl Fatty Acid Esters via UHPLC-APCI-MS

Among the ester mixtures that are used for preparation of the enriched foods, about two thirds are phytosteryl/-stanyl ester mixtures that may contain up to 20 % stanyl esters (SCF, 2003b; EFSA, 2008). Therefore, the available methodologies, enabling only a quantitative analysis of individual intact phytosteryl fatty acid esters, would neglect a substantial proportion of these mixtures, underlining the need for an appropriate analytical approach covering individual esters in complex mixtures. However, chromatographic separations and also mass spectrometric differentiations are certainly further complicated as the number of structurally similar compounds increases. The expected structural variability of the ester mixtures is illustrated in Figure 16.

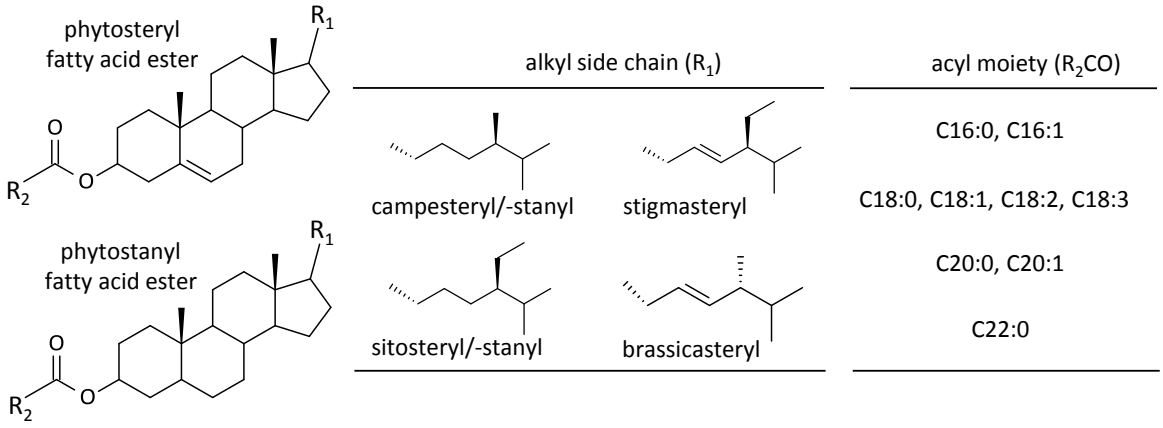

Figure 16. Structural variability of phytosteryl and phytostanyl fatty acid esters in phytosteryl/-stanyl ester-enriched foods.

As the first-time use of sub 2 μm particles led to a significantly improved the chromatographic performance with regard to mixtures of phytostanyl fatty acid esters, the objective was to establish a methodology for the analysis of both, phytosteryl and phytostanyl fatty acid esters, using the previously developed approach as basis. For these investigations, an Agilent 6430 triple quadrupole mass spectrometer was available as MS instrument.

4.1.2.1 Method Development and Optimization

The initial idea was to extend the concept of detecting esters of different stanol nuclei based on their $[M\text{-}FA+H]^+$ fragment ions to the simultaneous detection of phytosteryl fatty acid esters and phytostanyl fatty acid esters as occurring in complex mixtures. In order to differentiate esters of the same sterol/stanol, the UHPLC-based method was tested regarding the separation of individual phytosteryl fatty acid esters. Indeed, the use of the 1.7 μm C8 column and MeOH/H_2O as eluent resulted in an elution of individual phytosteryl fatty acid esters analogous to that of

phytostanyl fatty acid esters. Exemplarily, the separation of synthesized sitosteryl esters of those fatty acids occurring in the vegetable oils authorized as sources for the esterification process (SCF, 2000; 2003a; 2003b) is shown in Figure 17; similarly to the cholesteryl and phytostanyl esters, the lower the carbon number and the higher the number of double bonds of the fatty acid, the shorter was the retention time of the ester. As again the base peak of each positive ion APCI mass spectrum recorded for synthesized reference compounds of phytosteryl fatty acid esters corresponded to the respective $[M\text{-}FA+H]^+$ fragment, this ion could be used for detection and quantitation of these compounds in the SIM mode.

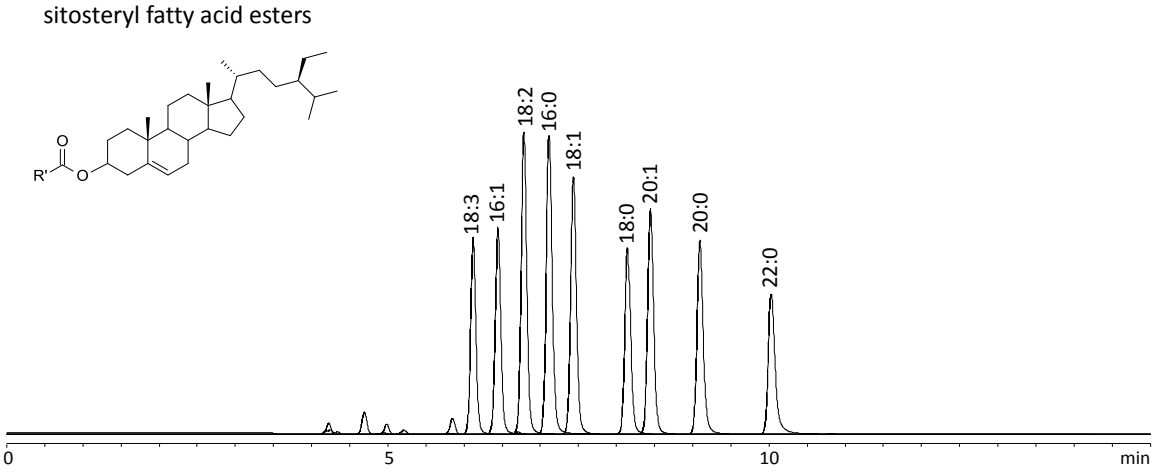

Figure 17. RP-UHPLC based separation of synthesized sitosteryl fatty acid esters on a 1.7 μm C8 column: Overlay of APCI-SIM chromatograms at *m/z* 397.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (for conditions, cf. 3.2.1.4).

However, the full scan APCI mass spectra of the phytosteryl fatty acid esters also revealed the presence of isotope peaks 1 and 2 relative mass units higher than the nominal mass corresponding to the $[M\text{-}FA+H]^+$ fragment. As illustrated as example in Figure 18 for the full scan spectrum of sitosteryl-18:2, isotope peaks at m/z 398.2 and m/z 399.2 were formed in addition to the base peak at m/z 397.2 corresponding to the nominal mass of the $[M\text{-}FA+H]^+$ fragment ion. Due to the saturation of the sterol nucleus, the relative mass of the $[M\text{-}FA+H]^+$ fragment ion of phytostanyl fatty acid esters is 2 mass units higher than that of the $[M\text{-}FA+H]^+$ fragment ion of the respective phytosteryl fatty acid esters with the same side chain. Consequently, in the presence of the corresponding phytosteryl fatty acid esters, the use of the $[M\text{-}FA+H]^+$ fragment ion for the detection of phytostanyl fatty acid esters leads to the additional detection of phytosteryl fatty acid esters exhibiting the same side chain, potentially causing an overlapping of peaks. Therefore, appropriate chromatographic conditions were required in order to effectively compensate for this effect.

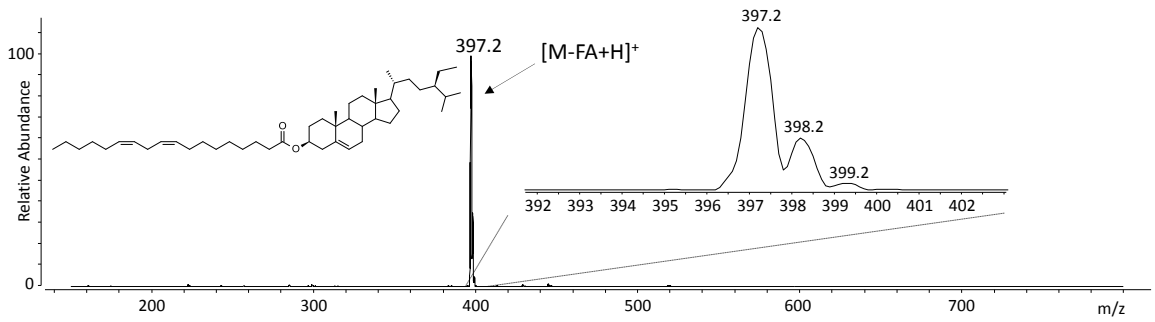

Figure 18. APCI-MS full scan spectrum of sitosteryl-18:2: [FA], fatty acid (for conditions, cf. 3.2.1.4).

Accordingly, the chromatographic separation of individual sitosteryl/sitostanyl fatty acid esters and of campesteryl/campestanyl fatty acid esters, respectively, was investigated. The overlay of synthesized reference compounds of sitosteryl and sitostanyl fatty acid esters shown in Figure 19 demonstrates the suitability of the 1.7 μm C8 column. As the saturation of the sterol nucleus led to longer retention of the stanyl esters on the employed column, the established chromatographic conditions enabled a separation of individual sitosteryl and sitostanyl fatty acid esters. The elution of individual campesteryl and campestanyl fatty acid esters was analogous to that of the sitosteryl and sitostanyl fatty acid esters.

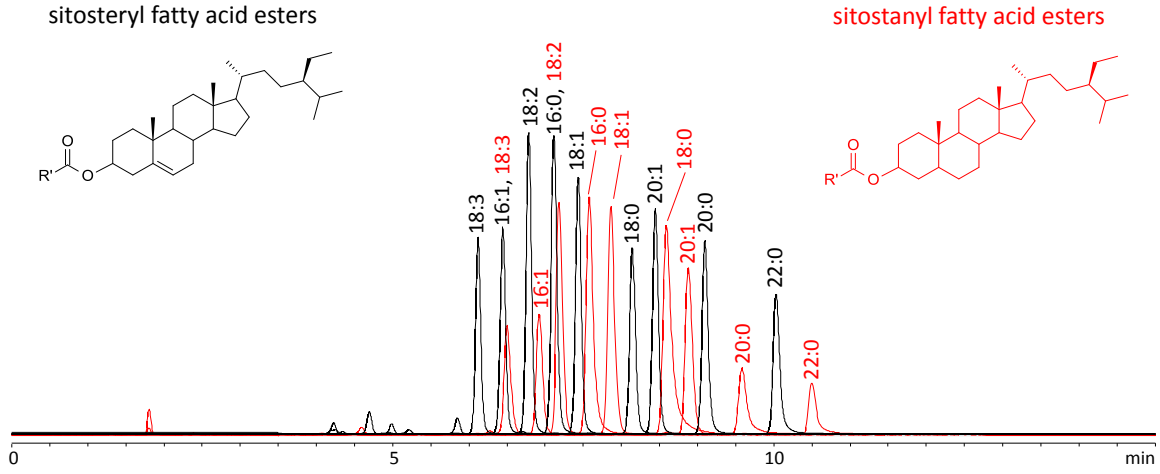

Figure 19. RP-UHPLC based separation of synthesized phytosteryl and phytostanyl fatty acid esters on a 1.7 μm C8 column: Overlay of APCI-SIM chromatograms corresponding to the $[M\text{-}FA+H]^+$ fragment ions of sitosteryl fatty acid esters (black) at *m/z* 397.2 and sitostanyl fatty acid esters (red) at *m/z* 399.2. For conditions, cf. 3.2.1.4.

Thus, this chromatographic separation offered a first basis for the detection and quantitation of phytostanyl fatty acid esters via their $[M\text{-}FA+H]^+$ fragment ion, even in the presence of phytosteryl fatty acid esters. However, sitosteryl-16:0 and sitostanyl-18:2, and sitosteryl-16:1 and sitostanyl-18:3, respectively, were not separated. The co-elutions observed for these phytostanyl linoleic

and linolenic acid esters would seriously hamper correct peak assignment and quantitation. Therefore, the analytical approach had to be complemented by a third strategic element: As already observed, phytostanyl esters of unsaturated fatty acids may also form stable protonated molecular ions $[M+H]^+$ using APCI, in addition to the $[M-FA+H]^+$ fragment ion. Accordingly, a comprehensive testing of different mass spectrometric parameters was performed for the novel MS instrumentation; this finally revealed that adjusting the APCI source to high temperatures together with a corona current of 8 μA and a fragmentor voltage of 70 V leads to the formation of rather intense molecular ions $[M+H]^+$ for phytostanyl esters of unsaturated fatty acids. Exemplarily, in Figure 20 the full scan spectrum of sitostanyl linoleic acid ester is depicted, with the $[M+H]^+$ ion showing a relative intensity of approximately 45 %. The absolute intensity of this ion was below the intensities of the $[M-FA+H]^+$ fragment ions of the stanyl esters of saturated fatty acids; for example, the intensity of the $[M+H]^+$ ion of sitostanyl-18:2 was approximately 30 % lower than that of the $[M-FA+H]^+$ fragment ion of sitostanyl-18:0.

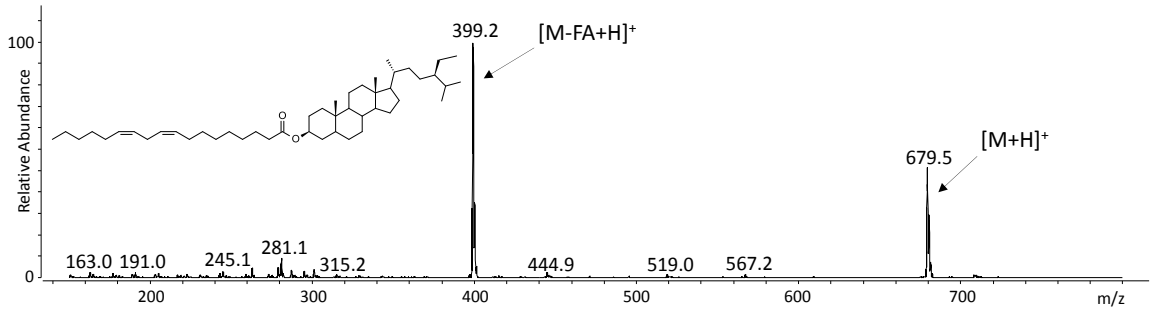

Figure 20. APCI-MS full scan spectrum of sitostanyl-18:2: $[M+H]^+$, protonated molecular ion, [FA], fatty acid (for conditions, cf. 3.2.1.4).

This distinct fragmentation enabled the alternative detection and quantitation of phytostanyl linoleic and linolenic acid esters in the SIM modes corresponding to the masses of their respective molecular ions, regardless of isotope peaks of phytosteryl esters. As sitostanol and campestanol are the only naturally occurring saturated plant sterols, this strategy for the differentiation of chromatographically non-resolved pairs of steryl fatty acid esters and stanyl esters of unsaturated fatty acids sharing an isotopic sterol/stanol nucleus is applicable to any mixture of these components.

The use of ESI would have enabled a formation of e.g. ammoniated molecular ions $[M+NH_4]^+$ after the addition of salt. For qualitative approaches or in cases of only a limited number of analytes, the detection of transitions to $[M-FA+H]^+$ product ions would provide improved sensitivity and specificity, even allowing the differentiation of steryl fatty acid esters following direct flow injection without chromatographic pre-separation (Wewer *et al.*, 2011; Hailat and Helleur, 2014).

However, due to the high number of analytes that was targeted in the present study and considering the high degree of structural similarity, several identical adduct masses $[M+NH_4]^+$ showing the same transitions would have been formed. This abundance of isotopologues would have led to various mass spectral overlappings, e.g. interferences of transitions of sitosteryl fatty acid esters with those of stigmasteryl fatty acid esters. Therefore, the developed APCI-based approach offers a decisive advantage over previously described ESI-based approaches, as the number of potentially interfering isotopologues could significantly be reduced by making use of the $[M+H]^+$ ions that were exclusively formed by phytostanyl esters of unsaturated fatty acids. Furthermore, the addition of salts to the mobile phase has been described in the literature to result in longer retention times and broader peak shapes (Caboni *et al.*, 2005), a drawback that could be avoided by using APCI.

Altogether, the developed methodology is essentially based on a unique and previously not described combination of three elements: (i) the formation of $[M-FA+H]^+$ fragment ions via APCI, (ii) a highly efficient UHPLC-based separation allowing the distinction of individual fatty acid esters sharing the same sterol/stanol nucleus and of isotope peaks of phytosteryl fatty acid esters and corresponding phytostanyl fatty acid esters based on these $[M-FA+H]^+$ fragment ions, and (iii) the adjustment of the APCI conditions allowing the differential APCI-MS-SIM detection of phytostanyl esters of linoleic and linolenic acid based on their distinct formation of a $[M+H]^+$ ion. Only this combination allowed for the first time to accomplish the quantitative analysis of both, individual phytosteryl and phytostanyl fatty acid esters in complex mixtures.

The suitability of the novel approach was demonstrated by the investigation of a commercially available enriched margarine containing 12.5 g/100 g plant steryl esters as declared on the label. The enriched margarine was subjected to the previously described aminopropyl-based solid phase extraction, allowing a selective pre-separation of phytosteryl/phytostanyl fatty acid esters from potentially interfering constituents of the lipid matrix. The subsequent UHPLC-APCI-MS analysis of phytosteryl and phytostanyl fatty acid esters according to their $[M-FA+H]^+$ fragment ions, and of phytostanyl esters of linoleic acid corresponding to their $[M+H]^+$ ions, enabled the detection of 35 individual fatty acid esters of sitosterol, campesterol, brassicasterol, stigmasterol, sitostanol, and campestanol (Table 14). Peak assignment was performed using synthesized reference compounds; therefore, this approach is restricted to the identification of these target analytes, and potential co-elutions of other unknown minor isobaric compounds could remain undetected.

Table 14. Relative retention times (RRT) and selected ions of individual phytosteryl and phytostanyl fatty acid esters.

no.	steryl / stanyl fatty acid ester	RRT[a,b]	APCI-fragment	*m/z*[b]
1	brassicasteryl-18:2	0.942	$[M\text{-}FA+H]^+$	381.2
2	brassicasteryl-16:0	0.964	$[M\text{-}FA+H]^+$	381.2
3	brassicasteryl-18:1	1.048	$[M\text{-}FA+H]^+$	381.2
4	brassicasteryl-18:0	1.149	$[M\text{-}FA+H]^+$	381.2
5	campesteryl-16:1	0.974	$[M\text{-}FA+H]^+$	383.2
6	campesteryl-18:2	1.009	$[M\text{-}FA+H]^+$	383.2
7	campesteryl-16:0	1.072	$[M\text{-}FA+H]^+$	383.2
8	campesteryl-18:1	1.122	$[M\text{-}FA+H]^+$	383.2
9	campesteryl-18:0	1.228	$[M\text{-}FA+H]^+$	383.2
10	campesteryl-20:1	1.276	$[M\text{-}FA+H]^+$	383.2
11	campesteryl-20:0	1.384	$[M\text{-}FA+H]^+$	383.2
12	campesteryl-22:0	1.538	$[M\text{-}FA+H]^+$	383.2
13	stigmasteryl-18:2	1.028	$[M\text{-}FA+H]^+$	395.3
14	stigmasteryl-18:1	1.139	$[M\text{-}FA+H]^+$	395.3
15	stigmasteryl-18:0	1.245	$[M\text{-}FA+H]^+$	395.3
16	sitosteryl-18:3	0.984	$[M\text{-}FA+H]^+$	397.2
17	sitosteryl-16:1	1.049	$[M\text{-}FA+H]^+$	397.2
18	sitosteryl-18:2	1.083	$[M\text{-}FA+H]^+$	397.2
19	sitosteryl-16:0	1.152	$[M\text{-}FA+H]^+$	397.2
20	sitosteryl-18:1	1.198	$[M\text{-}FA+H]^+$	397.2
21	sitosteryl-18:0	1.308	$[M\text{-}FA+H]^+$	397.2
22	sitosteryl-20:1	1.359	$[M\text{-}FA+H]^+$	397.2
23	sitosteryl-20:0	1.466	$[M\text{-}FA+H]^+$	397.2
24	sitosteryl-22:0	1.617	$[M\text{-}FA+H]^+$	397.2
25	campestanyl-18:2	1.080	$[M+H]^+$	665.4
26	campestanyl-16:0	1.148	$[M\text{-}FA+H]^+$	385.2
27	campestanyl-18:1	1.196	$[M\text{-}FA+H]^+$	667.3
28	campestanyl-18:0	1.305	$[M\text{-}FA+H]^+$	385.2
29	campestanyl-22:0	1.614	$[M\text{-}FA+H]^+$	385.2
30	sitostanyl-18:2	1.164	$[M+H]^+$	679.5
31	sitostanyl-16:0	1.230	$[M\text{-}FA+H]^+$	399.2
32	sitostanyl-18:1	1.282	$[M\text{-}FA+H]^+$	681.5
33	sitostanyl-18:0	1.389	$[M\text{-}FA+H]^+$	399.2
34	sitostanyl-20:0	1.543	$[M\text{-}FA+H]^+$	399.2
35	sitostanyl-22:0	1.693	$[M\text{-}FA+H]^+$	399.2

[a] Retention times relative to cholesteryl palmitate (Kinetex C8, 1.7 μm).
[b] Relative retention times and mass spectra were determined using synthesized reference compounds.

Figure 21 comprises the SIM chromatograms corresponding to the respective $[M\text{-}FA+H]^+$ fragment ions of the individual phytosteryl fatty acid esters detected in the enriched margarine. Among the phytosteryl fatty acid esters, additional methyl groups in the side chain were reflected in longer retention times. Figure 22 shows of the SIM chromatograms of sitostanyl and campestanyl fatty acid esters.

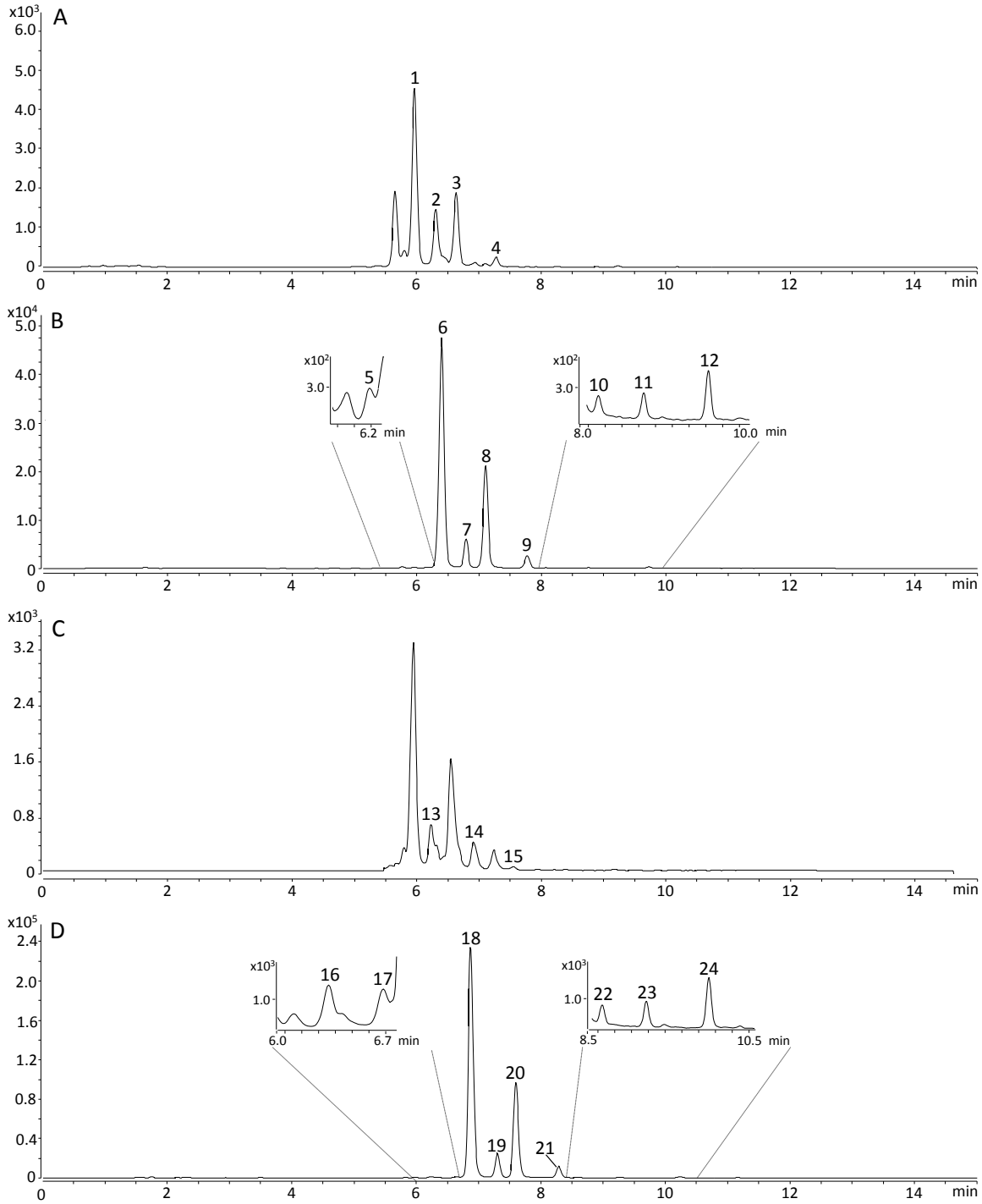

Figure 21. UHPLC-APCI-MS analysis of phytosteryl fatty acid esters in phytosteryl/phytostanyl ester-enriched margarine. SIM chromatogram of brassicasteryl fatty acid esters at *m/z* 381.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**A**). SIM chromatogram of campesteryl fatty acid esters at *m/z* 383.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**B**). SIM chromatogram of stigmasteryl fatty acid esters at *m/z* 395.3, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**C**). SIM chromatogram of sitosteryl fatty acid esters at *m/z* 397.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**D**). The peak numbering is in accordance with that in Table 14. For conditions, cf. 3.2.1.4.

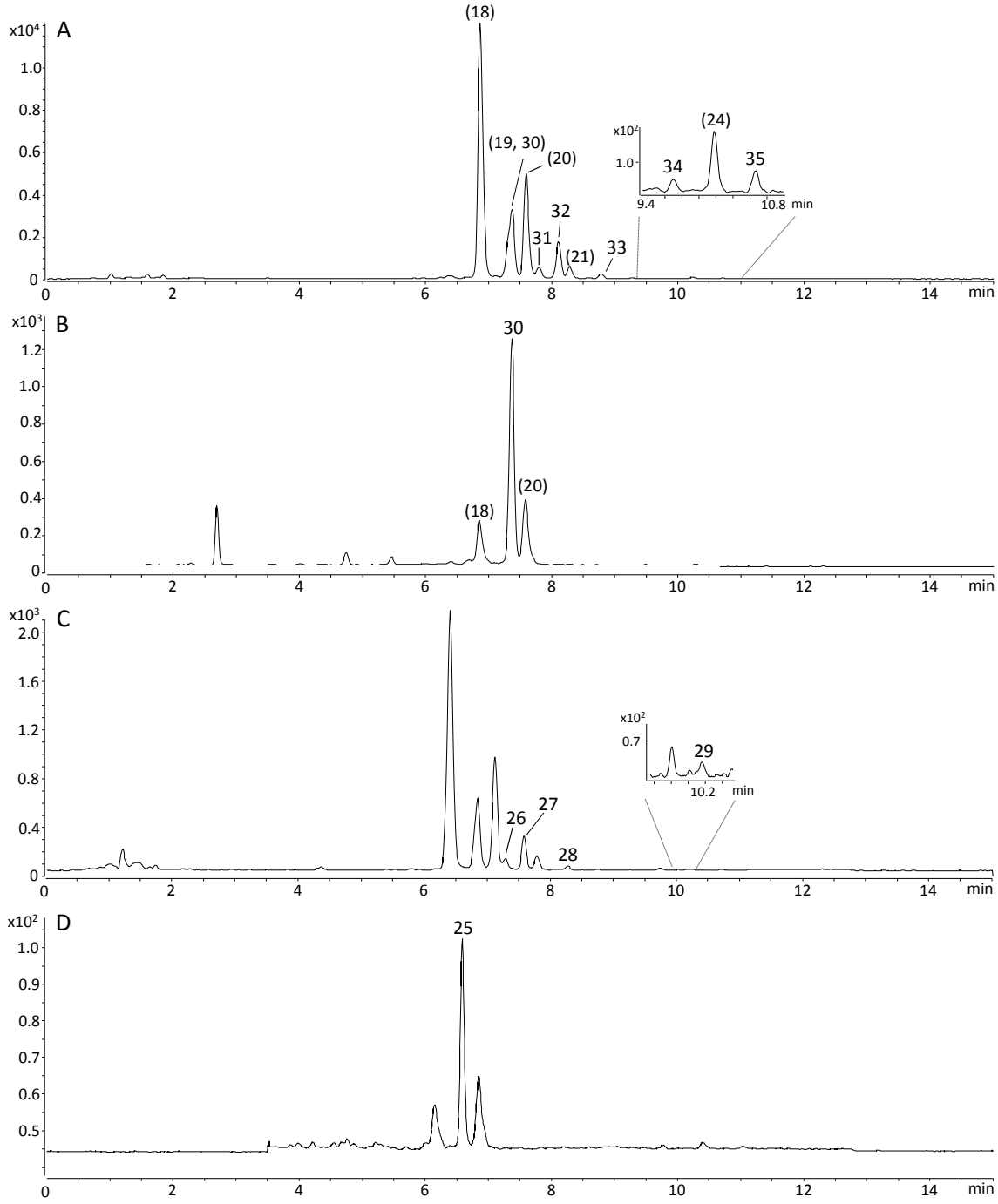

Figure 22. UHPLC-APCI-MS analysis of phytostanyl fatty acid esters in phytosteryl/phytostanyl ester-enriched margarine. SIM chromatogram of sitostanyl fatty acid esters at *m/z* 399.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**A**). SIM chromatogram of sitostanyl-18:2 at *m/z* 679.5, corresponding to the $[M+H]^+$ ion (**B**). SIM chromatogram of campestanyl fatty acid esters at *m/z* 385.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**C**). SIM chromatogram of campestanyl-18:2 at *m/z* 665.4, corresponding to the $[M+H]^+$ ion (**D**). The peaks assigned by numbers in parentheses have not been considered for quantitation in these chromatograms. The peak numbering is in accordance with that in Table 14. For conditions, cf. 3.2.1.4.

Figure 22 A shows the SIM chromatogram of sitostanyl fatty acid esters corresponding to their $[M-FA+H]^+$ fragment ion. The peaks of sitostanyl fatty acid esters that were used for quantitation are assigned by their respective numbers (peaks 31-35). The peaks that were not considered for quantitation in this chromatogram are assigned by their respective numbers in parentheses. These peaks are caused by isotopic mass fragments from sitosteryl fatty acid esters (e.g. sitosteryl-18:2, peak 18); in addition, the non-resolved peaks of sitosteryl-16:0 (peak 19) and sitostanyl-18:2 (peak 30) are assigned. The SIM chromatogram corresponding to the $[M+H]^+$ ion of sitostanyl linoleic acid ester (peak 30) is shown in Figure 22 B. This chromatogram also shows additional peaks from isotopic stearic (peak 18) and oleic (peak 20) acid sitosteryl esters, due to the formation of molecular ions of steryl esters to a minor extent; however, because of their chromatographic separation they did not hamper the quantitation of the sitostanyl linoleic acid ester. Sitostanyl linolenic acid ester was not present in the enriched margarine, the linoleic acid ester of sitostanol was the only sitostanyl ester detected via its $[M+H]^+$ ion. Analogously, Figure 22 C depicts the SIM chromatogram of campestanyl fatty acid esters corresponding to their $[M-FA+H]^+$ fragment ion; in Figure 22, the SIM chromatogram of campestanyl-18:2 detected via its $[M+H]^+$ ion is shown.

4.1.2.2 UHPLC-APCI-MS Quantitation

Cholesteryl palmitate was selected as internal standard and analyzed in the SIM mode corresponding to the $[M-FA+H]^+$ fragment ion; its chromatographic usefulness was confirmed via selected ion monitoring at those masses used for determination of the phytosteryl and phytostanyl fatty acid esters. In order to compensate for varying detector responses of the different phytosteryl and phytostanyl fatty acid esters, external calibration functions were generated, calibrating the response ratio of each individual phytosteryl and phytostanyl fatty acid ester and the internal standard. Due to a higher sensitivity of the Agilent MS compared to the previously used Dionex instrumentation, five-point calibration functions could be generated for all esters.

Preliminary experiments (data not shown) demonstrated that a simultaneous scanning of nine different masses as required for a complete analysis of all esters present in the enriched margarine including the internal standard did not allow an exact quantitation of the esters as the total ester content declared on the label was underestimated by approximately 3 %. A comparative scanning and quantitation of sitosteryl and campesteryl fatty acid esters alone revealed that in particular the quantitation of the dominating sitosteryl and campesteryl fatty acid esters was affected. Therefore, in order to reduce the number of simultaneously scanned masses,

each analysis was divided into two chromatographic runs, with the first run scanning for sitosteryl and campesteryl fatty acid esters and the second run scanning for the remaining brassicasteryl, stigmasteryl, sitostanyl, and campestanyl fatty acid esters.

4.1.2.3 Method Validation

The developed UHPLC-APCI-MS based method was validated in terms of LOD and LOQ, linearity, repeatability and reproducibility, and recovery rates.

The instrumental limits of detection and limits of quantitation determined for the individual phytosteryl and phytostanyl fatty acid esters are compiled in Table 15. For the phytosteryl fatty acid esters, the LOD and LOQ were in the same order of magnitude. Slightly higher values were obtained for phytostanyl fatty acid esters, probably because of a reduced overall ionizability of the stanyl esters due to the lack of the double bond in the B-ring. For oleic and in particular linoleic acid esters of campestanyl and sitostanyl, even higher values were obtained. This is reflecting the relatively intense formation of both, the $[M\text{-}FA+H]^+$ fragment ion and the $[M+H]^+$ peak of these esters and, considering the linoleic acid stanyl esters, the use of the less intensively formed $[M+H]^+$ ion for quantitation. With regard to the phytostanyl fatty acid esters, the improved sensitivity of the Agilent mass spectrometer is reflected in the significantly lower LOD and LOQ (LOD ranging from 4.3 to 30.1 ng/mL) compared to those values previously determined using the Dionex MS instrumentation, with LOD being between 30 and 110 ng/mL (cf. Table 7).

The linearity of all individual esters was determined within their respective calibration range. Coefficients of correlation (r^2) were higher than 0.99 for all compounds, indicating good linearity in the calibration ranges. The determined slopes, intercepts, and coefficients of correlation are given in Table 15.

Table 15. LOD, LOQ, and characteristics of calibration curves of individual phytosteryl and phytostanyl fatty acid esters.

steryl / stanyl fatty acid ester	LOD [ng/mL][a]	LOQ [ng/mL][a]	slope	intercept	r^2
brassicasteryl-18:2	2.90	8.69	0.4097	-0.0005	0.9985
brassicasteryl-16:0	2.83	8.43	1.0763	0.0011	0.9994
brassicasteryl-18:1	3.00	8.99	0.3873	-0.0002	0.9968
brassicasteryl-18:0	3.82	11.42	0.4263	$8x10^{-7}$	0.9967
campesteryl-16:1	0.63	1.88	2.7579	$-2x10^{-5}$	0.9988
campesteryl-18:2	1.57	4.71	0.9110	-0.0058	0.9991
campesteryl-16:0	2.97	8.88	1.1087	-0.0014	0.9979
campesteryl-18:1	2.70	8.09	0.9171	-0.0106	0.9985
campesteryl-18:0	2.31	6.90	0.9070	-0.0007	0.9987
campesteryl-20:1	1.24	3.71	1.7722	$2x10^{-5}$	0.9934
campesteryl-20:0	2.07	6.18	0.9412	$-8x10^{-6}$	0.9964
campesteryl-22:0	1.55	4.62	0.6672	$5x10^{-5}$	0.9921
stigmasteryl-18:2	2.28	6.82	0.4733	0.0001	0.9996
stigmasteryl-18:1	4.22	12.57	0.4737	-0.0002	0.9985
stigmasteryl-18:0	4.85	14.48	0.5647	$-6x10^{-6}$	0.9911
sitosteryl-18:3	1.11	3.31	2.3441	-0.0003	0.9986
sitosteryl-16:1	1.01	3.00	2.9978	$-8x10^{-5}$	0.9983
sitosteryl-18:2	1.63	4.88	0.9806	-0.0577	0.9998
sitosteryl-16:0	2.45	7.31	1.1290	-0.0097	0.9990
sitosteryl-18:1	2.26	7.27	1.0192	-0.1161	0.9979
sitosteryl-18:0	1.10	3.30	0.9526	-0.0054	0.9980
sitosteryl-20:1	1.38	4.12	1.7209	0.0005	0.9995
sitosteryl-20:0	1.97	5.88	0.9455	$-2x10^{-5}$	0.9973
sitosteryl-22:0	1.81	5.40	0.6959	$6x10^{-5}$	0.9981
campestanyl-18:2	23.26	69.54	0.0204	$-2x10^{-6}$	0.9991
campestanyl-16:0	4.30	12.85	0.1836	$-5x10^{-5}$	0.9983
campestanyl-18:1	15.10	45.21	0.1036	$-6x10^{-5}$	0.9998
campestanyl-18:0	8.37	25.00	0.1188	$2x10^{-5}$	0.9919
campestanyl-22:0	5.66	16.19	0.3444	$-5x10^{-5}$	0.9914
sitostanyl-18:2	30.56	75.88	0.0331	-0.0001	0.9992
sitostanyl-16:0	6.94	20.76	0.1683	$-8x10^{-5}$	0.9940
sitostanyl-18:1	11.29	33.78	0.1097	-0.0003	0.9999
sitostanyl-18:0	9.83	29.37	0.1266	$5x10^{-5}$	0.9974
sitostanyl-20:0	8.81	26.03	0.1547	$5x10^{-5}$	0.9906
sitostanyl-22:0	6.66	41.3	0.1148	$5x10^{-5}$	0.9910

[a] LOD and LOQ were determined using the plant steryl/stanyl fatty acid ester mixture "Vegapure 95E" and are expressed as ng/mL of injection volume, determined on the basis of 0.8 μL injection volume.

Recovery rates of the individual esters were determined via the addition of three different amounts of the phytosteryl/phytostanyl fatty acid ester mixture "Vegapure 95E" to a non-enriched margarine; the added amounts corresponded to enrichments of 10 %, 12.3 %, and 15 % phytosteryl/phytostanyl fatty acid esters. Good recovery rates between 95 % and 106 % were determined for the individual compounds at all three concentration levels (Table 16), demonstrating the applicability of the method to a broad concentration range.

Table 16. Recovery rates of individual phytosteryl and phytostanyl fatty acid esters spiked to a non-enriched margarine.

steryl / stanyl fatty acid ester	recovery [%]		
	level 1[a]	level 2[b]	level 3[c]
brassicasteryl-18:2	98.4 ± 6.9[d]	102.6 ± 1.4	102.5 ± 0.1
brassicasteryl-16:0	103.4 ± 4.8	98.3 ± 5.3	102.8 ± 1.3
brassicasteryl-18:1	99.2 ± 1.8	101.2 ± 1.2	95.2 ± 0.4
brassicasteryl-18:0	98.8 ± 0.5	99.0 ± 1.5	99.7 ± 2.7
campesteryl-16:1	99.8 ± 5.0	104.5 ± 3.0	100.0 ± 1.4
campesteryl-18:2	100.4 ± 5.0	100.8 ± 1.8	102.7 ± 2.5
campesteryl-16:0	99.9 ± 4.8	99.6 ± 1.7	100.8 ± 0.8
campesteryl-18:1	100.8 ± 4.9	103.1 ± 6.1	100.4 ± 1.5
campesteryl-18:0	98.7 ± 4.3	101.9 ± 3.8	98.2 ± 2.9
campesteryl-20:1	99.2 ± 0.6	100.7 ± 4.8	101.4 ± 3.4
campesteryl-20:0	100.0 ± 2.4	97.5 ± 4.2	100.2 ± 2.0
campesteryl-22:0	101.1 ± 2.2	97.5 ± 2.2	97.6 ± 3.6
stigmasteryl-18:2	103.5 ± 3.6	102.2 ± 6.7	101.1 ± 2.4
stigmasteryl-18:1	221.5 ± 3.4	225.1 ± 6.1	195.4 ± 3.2
stigmasteryl-18:0	99.5 ± 4.1	104.8 ± 3.3	100.7 ± 1.2
sitosteryl-18:3	108.8 ± 2.8	104.4 ± 8.6	106.5 ± 5.1
sitosteryl-16:1	102.5 ± 3.1	104.1 ± 4.4	102.4 ± 2.2
sitosteryl-18:2	101.8 ± 6.9	104.2 ± 4.1	102.4 ± 3.1
sitosteryl-16:0	101.0 ± 5.1	102.0 ± 3.6	100.3 ± 3.7
sitosteryl-18:1	101.8 ± 5.3	104.7 ± 4.8	104.2 ± 2.9
sitosteryl-18:0	98.6 ± 3.7	101.9 ± 3.6	99.7 ± 3.3
sitosteryl-20:1	97.9 ± 1.0	99.5 ± 3.8	97.9 ± 0.5
sitosteryl-20:0	95.2 ± 2.8	99.7 ± 2.4	103.4 ± 1.5
sitosteryl-22:0	98.6 ± 3.3	104.3 ± 0.6	96.7 ± 2.7
campestanyl-18:2	104.1 ± 1.1	103.5 ± 1.3	101.2 ± 0.2
campestanyl-16:0	99.0 ± 3.5	103.5 ± 5.3	102.2 ± 0.9
campestanyl-18:1	101.9 ± 0.5	105.9 ± 2.6	105.6 ± 1.8
campestanyl-18:0	99.8 ± 3.8	99.4 ± 3.4	101.0 ± 0.0
campestanyl-22:0	99.2 ± 4.4	102.6 ± 3.3	99.2 ± 2.2
sitostanyl-18:2	100.0 ± 3.3	98.8 ± 1.4	102.4 ± 0.6
sitostanyl-16:0	100.3 ± 3.4	106.0 ± 4.1	103.4 ± 1.6
sitostanyl-18:1	100.2 ± 1.8	102.6 ± 5.2	105.1 ± 0.8
sitostanyl-18:0	101.4 ± 3.1	99.7 ± 4.8	100.0 ± 5.6
sitostanyl-20:0	102.5 ± 0.2	101.8 ± 3.7	100.8 ± 1.4
sitostanyl-22:0	102.0 ± 2.5	101.6 ± 4.3	99.8 ± 2.5
total esters	101.6 ± 4.7	104.1 ± 2.9	103.4 ± 0.4

[a] Blank samples were spiked with 3.0 mg of a phytosteryl/phytostanyl fatty acid ester mixture.
[b] Blank samples were spiked with 3.7 mg of a phytosteryl/phytostanyl fatty acid ester mixture.
[c] Blank samples were spiked with 4.5 mg of a phytosteryl/phytostanyl fatty acid ester mixture.
[d] Values represent the mean ± standard deviation (n=3).

Only for stigmasteryl-18:1, consistently high recoveries between 195 % (level 3) and 225 % (level 2) were determined. When subjecting the pure phytosteryl/phytostanyl fatty acid ester mixture to the sample preparation procedure and subsequent UHPLC-MS analysis, the recovery rates determined for stigmasteryl-18:1 amounted to approximately 103 %. Thus, the high recovery

rates in the presence of the margarine matrix must be due to a co-elution with a compound originating from the margarine that is not pre-separated by the SPE procedure; the identity of the compound could not be elucidated. Considering that the amount of the phytosteryl/phytostanyl fatty acid ester mixture added at level 2 (12.3 %) is qualitatively and quantitatively very close to the actual enrichment of the commercial margarine (12.5 %), the recovery rate of 225 % obtained for this amount was used for the calculation of contents of stigmasteryl-18:1 in enriched spreads. In this context, it was tried to improve the specificity of the detection of the stigmasteryl fatty acid esters. As previously described by Mo *et al.* (2013) for free stigmasterol, the unsaturated side chain may be protonated and form characteristic fragments in APCI product ion tandem mass spectra that could be used for a selective MS/MS detection. In the present study, stigmasteryl fatty acid esters formed a specific fragment ion corresponding to the protonated side chain after cleavage from the sterol nucleus at *m/z* 140.9 upon collision-induced dissociation of the $[M\text{-}FA+H]^+$ fragment ion at high collision energies (100 V). Further, lower collision energies (20 V) led to the formation of an even more abundant fragment ion at *m/z* 297.2, deriving from partial cleavage of the side chain (Mo *et al.*, 2013). Exemplarily, the product ion spectrum of the $[M\text{-}FA+H]^+$ fragment ion of stigmasteryl-18:1 at a collision energy of 20 V and the MRM chromatogram of the transition to the fragment ion at *m/z* 297.2 in enriched margarine are illustrated in Figure 23. Obviously, an analysis of the stigmasteryl esters via these transitions eliminated the above-described over-estimation of stigmasteryl-18:1 (peak no. 14). However, as this would have required an additional chromatographic run of the margarine samples and the calibration solutions, it was decided to keep detecting and quantifying the stigmasteryl esters based on their $[M\text{-}FA+H]^+$ fragment ion, in particular as the stigmasteryl esters were shown to be by far the least abundant esters in the enriched margarine (approximately 0.6 % of the total esters).

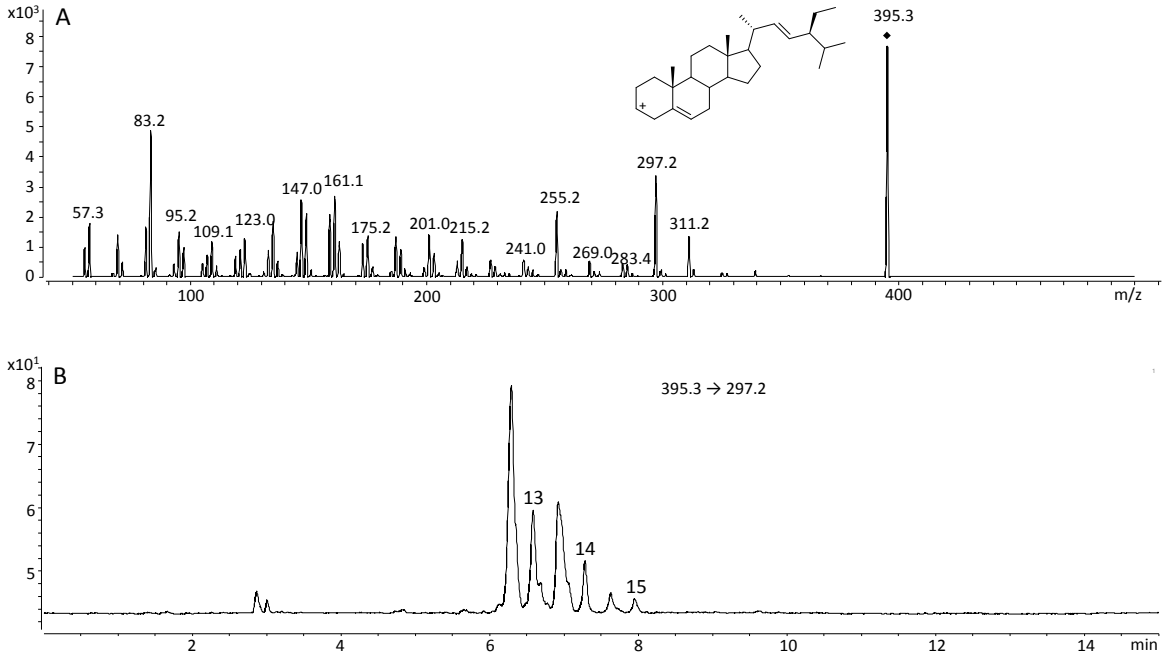

Figure 23. Product ion spectrum of the $[M\text{-}FA+H]^+$ fragment ion of stigmasteryl-18:2 (**A**) and multi reaction monitoring (MRM)-chromatogram of stigmasteryl fatty acid esters in enriched margarine at *m/z* 395.3 → 297.2 (**B**). MS conditions: fragmentor voltage, 70 V; collision energy, 20 V; cell accelerator voltage, 5 V; collision gas, nitrogen. The peak numbering is in accordance with that in Table 14.

To validate the developed method in terms of repeatability and reproducibility, two packages of an enriched margarine were analyzed on three different days by one operator. The reproducibility was confirmed by the additional analysis of each package by a second operator. The results including the coefficients of variation (CV) are summarized in Table 17 and Table 18.

The data indicated very good repeatability and reproducibility of the newly developed approach. The coefficient of variation of the total ester content was 1.6 % for the first package and 2.4 % for the second package, respectively. The coefficients of variation for the individual phytosteryl and phytostanyl fatty acid esters were consistently <5 %, showing that the quantitative reproducibility was maintained also for the low-concentrated analytes. The significant improvement resulting from the use of the Agilent MS instrumentation is reflected by the clearly higher coefficients of variation determined for low-concentrated phytostanyl esters using the Dionex instrumentation (cf. Table 8 - Table 12). Only stigmasteryl-18:1 showed a higher variation between the single analyses of 13 %, which is probably related to the approximated correction of the calculation of the contents of this ester based on its recovery rate. The determined total ester contents of 12.51 % and 12.6 % on average were in very good agreement with the amounts declared on the label (12.5 %).

Table 17. Repeatability and reproducibility data for the UHPLC-APCI-MS analysis of individual phytosteryl and phytostanyl fatty acid esters in a phytosteryl/-stanyl ester-enriched margarine (no. 3).

steryl / stanyl fatty acid ester	operator I			operator II	mean	CV [%]
[g/kg]	day 1	day 2	day 3	day 1		
brassicasteryl-18:2	2.25 ± 0.03[a]	2.28 ± 0.02	2.19 ± 0.09	2.23 ± 0.03	2.24 ± 0.06[b]	2.5
brassicasteryl-16:0	0.27 ± 0.00	0.27 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	0.27 ± 0.01	3.0
brassicasteryl-18:1	0.98 ± 0.01	1.00 ± 0.02	0.98 ± 0.00	1.00 ± 0.06	0.99 ± 0.03	2.7
brassicasteryl-18:0	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	2.5
campesteryl-16:1	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	4.9
campesteryl-18:2	11.47 ± 0.17	11.56 ± 0.30	11.22 ± 0.13	11.41 ± 0.48	11.41 ± 0.29	2.5
campesteryl-16:0	1.10 ± 0.00	1.13 ± 0.02	1.13 ± 0.04	1.12 ± 0.05	1.12 ± 0.03	2.6
campesteryl-18:1	5.20 ± 0.06	5.21 ± 0.16	5.22 ± 0.09	5.23 ± 0.17	5.21 ± 0.11	2.1
campesteryl-18:0	0.64 ± 0.01	0.64 ± 0.02	0.64 ± 0.01	0.64 ± 0.03	0.64 ± 0.02	2.5
campesteryl-20:1	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	4.5
campesteryl-20:0	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	3.3
campesteryl-22:0	0.09 ± 0.01	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	4.2
stigmasteryl-18:2	0.44 ± 0.01	0.44 ± 0.01	0.45 ± 0.01	0.43 ± 0.01	0.44 ± 0.01	2.1
stigmasteryl-18:1	0.29 ± 0.04	0.26 ± 0.04	0.28 ± 0.03	0.30 ± 0.06	0.28 ± 0.04	13.3
stigmasteryl-18:0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	4.2
sitosteryl-18:3	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.00	3.7
sitosteryl-16:1	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	3.6
sitosteryl-18:2	54.60 ± 0.57	54.14 ± 0.92	54.07 ± 1.68	54.60 ± 1.93	54.35 ± 1.21	2.2
sitosteryl-16:0	5.35 ± 0.07	5.37 ± 0.02	5.38 ± 0.16	5.34 ± 0.28	5.36 ± 0.14	2.6
sitosteryl-18:1	25.74 ± 0.11	25.65 ± 0.20	25.70 ± 0.69	25.75 ± 0.85	25.73 ± 0.49	1.9
sitosteryl-18:0	2.96 ± 0.01	3.02 ± 0.08	3.03 ± 0.03	3.03 ± 0.10	3.01 ± 0.06	2.1
sitosteryl-20:1	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	3.2

Table 17. continued.

steryl / stanyl fatty acid ester	operator I			operator II	mean	CV [%]
[g/kg]	day 1	day 2	day 3	day 1		
sitosteryl-20:0	0.17 ± 0.00	0.18 ± 0.00	0.19 ± 0.01	0.17 ± 0.00	0.18 ± 0.01	4.6
sitosteryl-22:0	0.50 ± 0.00	0.48 ± 0.02	0.54 ± 0.01	0.50 ± 0.01	0.50 ± 0.02	4.7
campestanyl-18:2	1.07 ± 0.01	1.11 ± 0.05	1.12 ± 0.05	1.04 ± 0.02	1.09 ± 0.05	4.4
campestanyl-16:0	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	1.6
campestanyl-18:1	0.57 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.57 ± 0.02	0.57 ± 0.01	2.5
campestanyl-18:0	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	3.9
campestanyl-22:0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	4.1
sitostanyl-18:2	6.59 ± 0.21	6.71 ± 0.18	6.61 ± 0.13	6.43 ± 0.09	6.58 ± 0.17	2.6
sitostanyl-16:0	0.67 ± 0.03	0.66 ± 0.01	0.66 ± 0.02	0.67 ± 0.04	0.67 ± 0.02	3.6
sitostanyl-18:1	3.15 ± 0.13	3.14 ± 0.02	3.11 ± 0.14	3.15 ± 0.14	3.14 ± 0.10	3.2
sitostanyl-18:0	0.43 ± 0.01	0.44 ± 0.01	0.44 ± 0.01	0.43 ± 0.01	0.44 ± 0.01	2.1
sitostanyl-20:0	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	4.0
sitostanyl-22:0	0.07 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	3.7
total esters	125.31 ± 0.66	125.13 ± 1.09	124.66 ± 2.53	125.17 ± 3.80	125.07 ± 2.04	1.6

[a] Values represent the mean ± standard deviation (n=3).
[b] Values represent the mean of all analyses ± standard deviation (n=12).

Table 18. Repeatability and reproducibility data for the UHPLC-APCI-MS analysis of individual phytosteryl and phytostanyl fatty acid esters in a phytosteryl/-stanyl ester-enriched margarine (no. 4).

steryl / stanyl fatty acid ester	operator I			operator II	mean	CV [%]
[g/kg]	day 1	day 2	day 3	day 1		
brassicasteryl-18:2	2.37 ± 0.13[a]	2.33 ± 0.12	2.33 ± 0.02	2.37 ± 0.06	2.35 ± 0.08[b]	3.4
brassicasteryl-16:0	0.27 ± 0.01	0.27 ± 0.02	0.25 ± 0.01	0.27 ± 0.01	0.26 ± 0.01[b]	4.7
brassicasteryl-18:1	1.04 ± 0.05	1.06 ± 0.02	1.03 ± 0.02	1.05 ± 0.05	1.05 ± 0.03	3.2
brassicasteryl-18:0	0.11 ± 0.00	0.11 ± 0.00	0.12 ± 0.01	0.12 ± 0.00	0.12 ± 0.00	2.9
campesteryl-16:1	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.016 ± 0.00	2.9
campesteryl-18:2	12.08 ± 0.41	12.22 ± 0.32	12.03 ± 0.14	12.21 ± 0.54	12.13 ± 0.34	2.8
campesteryl-16:0	1.15 ± 0.03	1.21 ± 0.08	1.15 ± 0.02	1.15 ± 0.03	1.16 ± 0.05	4.0
campesteryl-18:1	5.53 ± 0.10	5.57 ± 0.13	5.41 ± 0.02	5.50 ± 0.19	5.50 ± 0.12	2.2
campesteryl-18:0	0.63 ± 0.02	0.66 ± 0.03	0.64 ± 0.00	0.65 ± 0.02	0.65 ± 0.02	3.2
campesteryl-20:1	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	4.6
campesteryl-20:0	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	3.0
campesteryl-22:0	0.08 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	3.1
stigmasteryl-18:2	0.42 ± 0.02	0.44 ± 0.01	0.41 ± 0.00	0.44 ± 0.02	0.43 ± 0.02	4.6
stigmasteryl-18:1	0.31 ± 0.01	0.30 ± 0.01	0.24 ± 0.01	0.25 ± 0.03	0.28 ± 0.04	13.5
stigmasteryl-18:0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	4.3
sitosteryl-18:3	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	2.4
sitosteryl-16:1	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	2.5
sitosteryl-18:2	55.15 ± 0.46	55.11 ± 2.28	54.63 ± 0.14	54.66 ± 3.93	54.89 ± 1.96	3.6
sitosteryl-16:0	5.15 ± 0.10	5.45 ± 0.41	5.29 ± 0.04	5.17 ± 0.13	5.27 ± 0.23	4.3
sitosteryl-18:1	25.58 ± 0.59	25.73 ± 0.68	25.57 ± 0.17	25.45 ± 0.72	25.58 ± 0.51	2.0
sitosteryl-18:0	2.84 ± 0.06	3.01 ± 0.18	2.89 ± 0.03	2.89 ± 0.08	2.91 ± 0.11	3.8
sitosteryl-20:1	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	2.8

Table 18. continued.

steryl / stanyl fatty acid ester	operator I			operator II	mean	CV [%]
[g/kg]	day 1	day 2	day 3	day 1		
sitosteryl-20:0	0.17 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.16 ± 0.00	0.17 ± 0.01	3.7
sitosteryl-22:0	0.45 ± 0.02	0.47 ± 0.01	0.47 ± 0.01	0.47 ± 0.01	0.47 ± 0.01	2.8
campestanyl-18:2	0.97 ± 0.04	1.03 ± 0.02	0.97 ± 0.03	1.04 ± 0.06	1.00 ± 0.05	4.6
campestanyl-16:0	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	2.3
campestanyl-18:1	0.46 ± 0.01	0.45 ± 0.01	0.46 ± 0.03	0.45 ± 0.01	0.46 ± 0.01	3.0
campestanyl-18:0	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	2.0
campestanyl-22:0	0.01 ± 0.000	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	4.8
sitostanyl-18:2	6.68 ± 0.14	6.65 ± 0.20	6.23 ± 0.07	6.68 ± 0.31	6.56 ± 0.26	4.0
sitostanyl-16:0	0.60 ± 0.01	0.61 ± 0.03	0.59 ± 0.01	0.58 ± 0.02	0.60 ± 0.02	3.4
sitostanyl-18:1	3.01 ± 0.08	3.05 ± 0.05	3.06 ± 0.15	3.08 ± 0.12	3.05 ± 0.10	3.2
sitostanyl-18:0	0.36 ± 0.02	0.36 ± 0.01	0.35 ± 0.01	0.35 ± 0.02	0.36 ± 0.02	4.6
sitostanyl-20:0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	3.9
sitostanyl-22:0	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	2.5
total esters	126.02 ± 1.67	126.93 ± 3.27	124.98 ± 0.18	125.66 ± 5.76	125.90 ± 3.01	2.4

[a] Values represent the mean ± standard deviation (n=3).
[b] Values represent the mean of all analyses ± standard deviation (n=12).

Regarding the quantitative and qualitative distribution of the individual esters, sitosteryl and campesteryl esters were dominating, while linoleic acid was the main esterified fatty acid. Sitosteryl esters exhibited the broadest detectable spectrum of esterified fatty acids, as only for sitosteryl esters a linolenic acid ester could be determined. Phytostanyl fatty acid esters accounted for approximately 10 % of the total esters. Also, the profile of the esterified sterols/stanols was in accordance with the specification of the SCF (2003b). Other matrices and/or different quantitative and qualitative compositions of individual esters would possibly require adaptations regarding the working-up procedure as well as instrumental parameters, e.g. the injection volume, and should thus be carefully re-evaluated. In addition, as far as certain naturally occurring plant steryl ester mixtures are concerned, the presented methodology would possibly require extension to another spectrum of analytes than those presently examined.

4.1.3 Summary

In conclusion, the established LC-APCI-MS-based approaches provided novel tools for a simple, robust, and fast detection of plant steryl and/or stanyl fatty acid esters.

For the analysis of foods containing solely phytostanyl fatty acid esters, the crucial step was the excellent resolution of phytostanyl fatty acid esters consisting of the same stanol moiety on a C8 1.7 μm column and their subsequent analysis in SIM mode. This allowed a highly sensitive detection and a differentiation of the esters depending on the stanol moiety based on the $[M-FA+H]^+$ fragment ion.

As far as complex mixtures containing both, phytosteryl and phytostanyl fatty acid esters, are concerned, the previously developed concept for the analysis of phytostanyl fatty acid esters could not be adopted. Due to the presence of isotopologues, phytosteryl fatty acid esters interfere with the detection of phytostanyl fatty acid esters in the SIM mode according to the $[M-F+H]^+$ fragment ion. Therefore, in addition to the excellent chromatographic separation, phytostanyl esters of unsaturated fatty acids had to be detected via an exclusively formed $[M+H]^+$ ion to circumvent isotope effects.

Thus, for both types of mixtures commercially being used to enrich foods, an excellent separation in combination with distinct mass spectrometric features was accomplished. This new possibility to individually determine phytosteryl and or phytostanyl fatty acid esters in their intact forms serves not only as basis for authenticity assessments of enriched foods, but is also the essential precondition for analytically determining the fate of the functionality-bearing esters upon oxidation processes of enriched foods.

4.2 Method Development for the Analysis of Phytosterol / Phytostanol Oxidation Products in Enriched Foods

Oxidation processes of phytosteryl/phytostanyl ester-enriched foods are expected to result in the formation of, among others, secondary oxidation products of the sterol and stanol nuclei. In view of the disadvantages of conventionally employed sample preparation techniques for the analysis of the phytosterol oxidation products (cf. 2.4.2.1), the development of an alternative procedure was targeted.

The on-line coupling of HPLC and GC has been proven useful in the past for several analytical purposes aiming at pre-separating target compounds from abundant matrix constituents (cf. 2.4.2.3). Via LC, a highly selective clean-up, pre-concentration and fractionation of the sample can be achieved; the fraction(s) of interest can then be transferred on-line to the GC (Hyötyläinen and Riekkola, 2004). This allows analyses being performed in a closed and fully automated system, minimizing the risks of sample losses, contaminations, and undesired reactions (Grob, 1997; Hyötyläinen and Riekkola, 2003). Due to these distinct and advantageous features, on-line LC-GC was considered to be a suitable novel analytical platform for the analysis of phytosterol oxidation products, circumventing the drawbacks of the conventional sample preparation techniques. The crucial step of enrichment and purification of the phytosterol oxidation products should be accomplished by LC-separation, followed by an automated on-line transfer of the oxides to GC for the final analysis of individual compounds. Therefore, a further aim of the present study was the development of an on-line LC-GC-based analytical technique in order to enable a sensitive, automated and fast analysis of phytosterol oxidation products in enriched foods. However, the on-line coupling of LC and GC is challenging, requiring the establishment of not only suitable LC- and GC-conditions, but also of an interface technique allowing the evaporation of large amounts of solvents prior to the GC column. In the present study, an on-line LC-GC system equipped with a PTV interface was used.

On-Line Coupling via the Temperature Programmable Multimode Inlet

One type of PTV interface modification to perform eluent evaporation is the temperature programmable Multimode Inlet (MMI), allowing the injection of large volumes. Figure 24 shows a scheme of the on-line LC-GC/MS system equipped with a temperature programmable multimode inlet in the PTV solvent vent mode that was used in the present study. In the solvent vent mode, the sample is injected into a cold inlet, the temperature set below the boiling point of the solvent. The analytes deposit in the inlet liner at the packing material while the solvent is evaporated via a gas flow to split vent. After evaporation of the solvent, the inlet may be switched to a split or

splitless mode and the inlet is heated up fast to transfer the remaining analytes to the capillary column. The LC-GC transfer can be performed by either connecting the carrier gas supply line directly via the transfer valve into the Multimode Inlet or by installing a second line enabling pressure controlled transfer (Figure 24). This allows the setting of a ramp, controlling the pressure in the transfer line, thus preventing a pushing back of solvent vapors into the transfer line. For purge and clean-up, the inlet is switched to a purge mode to allow any retaining material in the inlet liner to be removed to waste (Esche *et al.*, 2011).

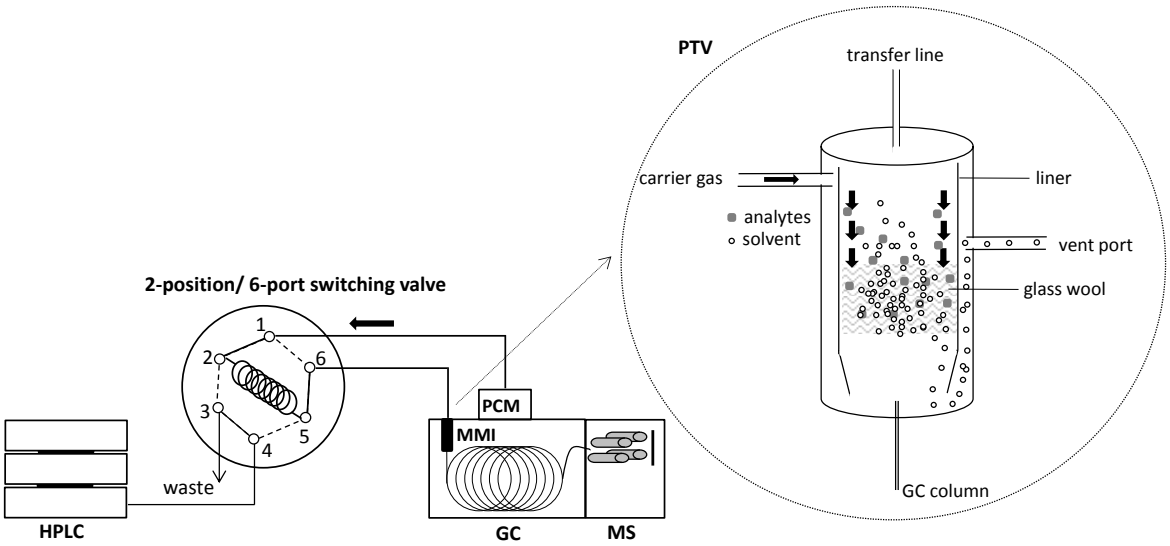

Figure 24. Scheme of the on-line LC-GC/MS system (transfer mode): The analytes are transferred from the HPLC to the GC using a 2-position/6-port switching valve. The evaporation of the solvent is performed via the temperature programmable multimode inlet in the PTV solvent vent mode. The stainless steel transfer line installed between the valve and the inlet is pressure controlled by a second line controlled by the pneumatics control module (PCM).

4.2.1 Preparation of Reference Compounds

While a variety of different cholesterol oxidation products is being commercially available, there are, besides 7-ketostigmasterol, no commercial reference compounds of phytosterol oxidation products. However, the availability of reference compounds is an essential precondition, not only for the development of chromatographic conditions, but also for an unambiguous assignment of the analytes that are to be determined via the developed methodology. The principles of an LC-UV based separation and determination of phytosterol oxidation products (Kemmo *et al.*, 2005) were modified and adapted to a semi-preparative liquid chromatographic system. After unspecific thermo-oxidation of phytosterol standard substances, various oxidation products of phytosterols could be made available in sufficient amounts; as example, the LC/UV chromatogram of thermo-oxidized stigmasterol is shown in Figure 25.

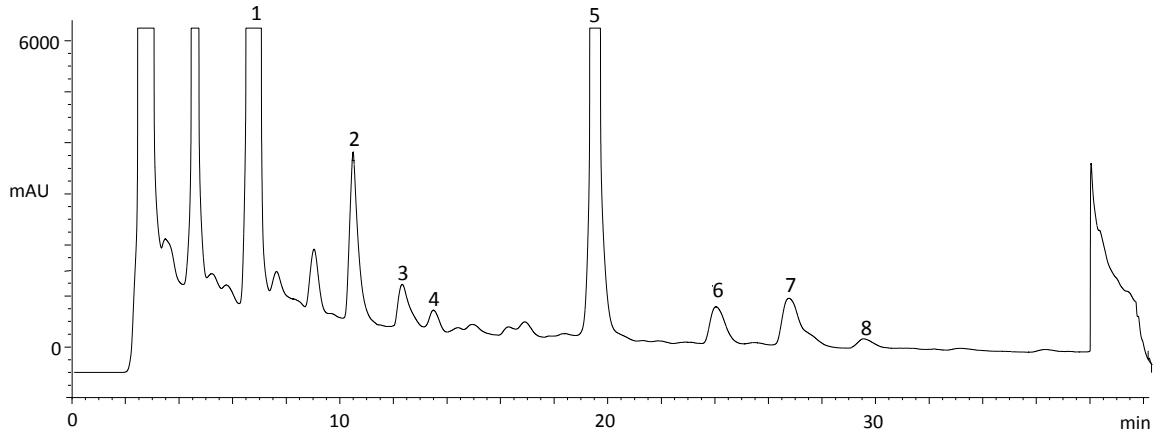

Figure 25. Semi-preparative NP-LC separation of thermo-oxidized stigmasterol, detected at 206 nm: (1) stigmasterol, (2) 6*ß*-hydroxy-3-ketostigmasterol, (3) 6*α*-hydroxy-3-ketostigmasterol, (4) 5,6*α*-epoxystigmasterol, (5) 7-ketostigmasterol, (6) 6*ß*-hydroxystigmasterol, (7) 7*ß*-hydroxystigmasterol, (8) 7*α*-hydroxystigmasterol (for conditions, cf. 3.2.2.1).

As 5,6*ß*-epoxy derivatives could not be detected via semi-preparative HPLC, these compounds were synthesized according to a procedure described in literature (O'Callaghan *et al.*, 2013). The purities and identities of the reference compounds were confirmed via GC/FID and by comparison of the mass spectra of their TMS derivatives to literature data (Conchillo *et al.*, 2005; Apprich and Ulberth, 2004; Dutta and Appelqvist, 1997; Dutta, 1997).

As phytostanols are considered to be rather inert towards oxidative degradation, their oxidation reactions are much less characterized and reference substances are not available. Still, research done by Soupas *et al.* (2004b) elucidated the nature of some secondary oxidation products of sitostanol including their GC-based separation and the mass spectra of the silylated compounds; their formation has subsequently been described upon heating of a sitostanol standard and upon heating of phytostanyl ester-enriched fat-based matrices (Soupas *et al.*, 2004a; 2004b; 2005; 2006; 2007). Therefore, also reference compounds of sitostanol oxidation products should be made available for development of the on-line LC-GC methodology. Due to the reduced reactivity and the scarce absorption of UV-light, thermo-oxidized sitostanol was subjected to a silica-based SPE for isolation of the oxidation products. Via semi-preparative HPLC of thermo-oxidized sitostanol standard, two of the previously described sitostanol oxidation products could be obtained: 6*α*-hydroxysitostanol, being considered to be a marker compound for sitostanol oxidation (Soupas *et al.*, 2004a; 2004b; 2007) was identified. Furthermore, 7-hydroxysitostanol was identified; based on the TMS-mass spectra, the identity of the epimer could, however, not be assigned.

4.2.2 Method Development and Optimization

The primary objective of the initial LC-step was the separation of the non-oxidized sterols from the corresponding oxidation products. In order to establish the appropriate LC conditions, commercially available cholesterol and cholesterol oxidation products were employed, focusing on 7-hydroxy-, 5,6-epoxy-, and 7-ketosterols, as these compounds have consistently been described to be quantitatively dominating among sterol oxidation products (Otaegui-Arrazola *et al.*, 2010). As the subsequent GC analysis would require derivatization in order to protect sterol oxidation products from thermal degradation (van Lier and Smith, 1968; Park and Addis, 1985), the first separation experiments were performed after silylation of the sterol oxidation products, this technique being the most common derivatization approach (Dutta, 2004; Guardiola *et al.*, 2004). However, an LC separation of silylated derivatives of cholesterol and its 7-hydroxy- and 5,6-epoxysterol oxidation products could not be achieved under the tested conditions (Figure 26).

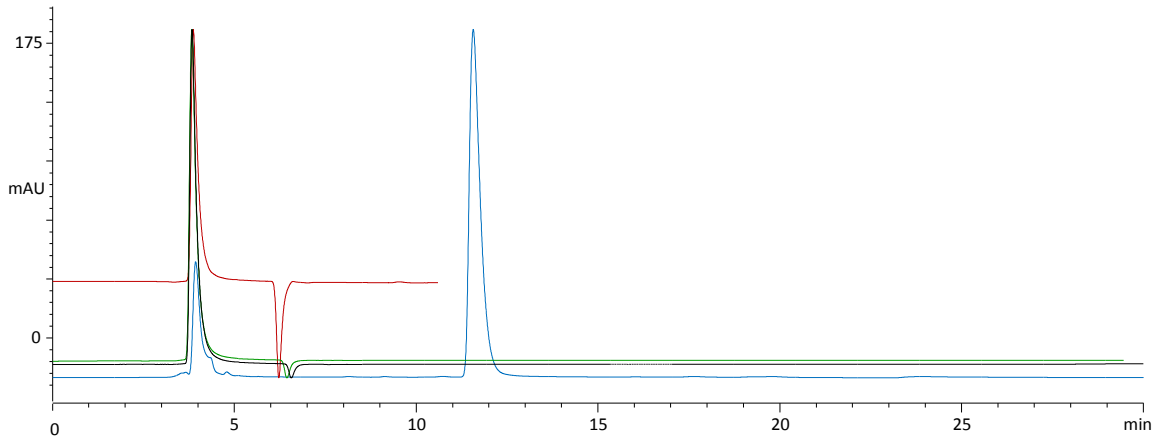

Figure 26. Overlays of LC/UV-chromatograms of silylated cholesterol and of silylated cholesterol oxidation products on a SiOH column, detected at 206 nm (*n*-hexane, 0.2 mL/min).

(——): cholesterol; (——): *7ß*-hydroxycholesterol; (——): 5,*6ß*-epoxycholesterol; (——): 7-ketocholesterol.

Neither the use of different normal phase stationary phases (silica, cyanopropyl, aminopropyl, and diol columns) nor variations of the flow rate and the polarity of the mobile phase resulted in the desired fractionation.

In earlier studies on GC-based analysis of sterols, steroids and also cholesterol oxidation products acetylation has been frequently described as alternative derivatization technique (van Lier and Smith, 1968; Park and Addis, 1985; Nordby and Nagy, 1973; Sax *et al.*, 1964; Aringer and Eneroth, 1973; Meneghetti *et al.*, 1987); therefore, the LC pre-separation of acetylated sterols and sterol oxides was investigated. Ultimately, a derivatization of sterols and the corresponding oxides with acetic anhydride in pyridine enabled the LC separation of (1) non-oxidized sterols, (2) 7-hydroxy-

and 5,6-epoxysterols, and (3) 7-ketosterols on a silica gel column using *n*-hexane/MTBE/*iso*-propanol (80:20:0.3, v/v/v) as eluent; the respective stereoisomers of 7-hydroxy- and 5,6-epoxysterols were eluted at the same time (Figure 27).

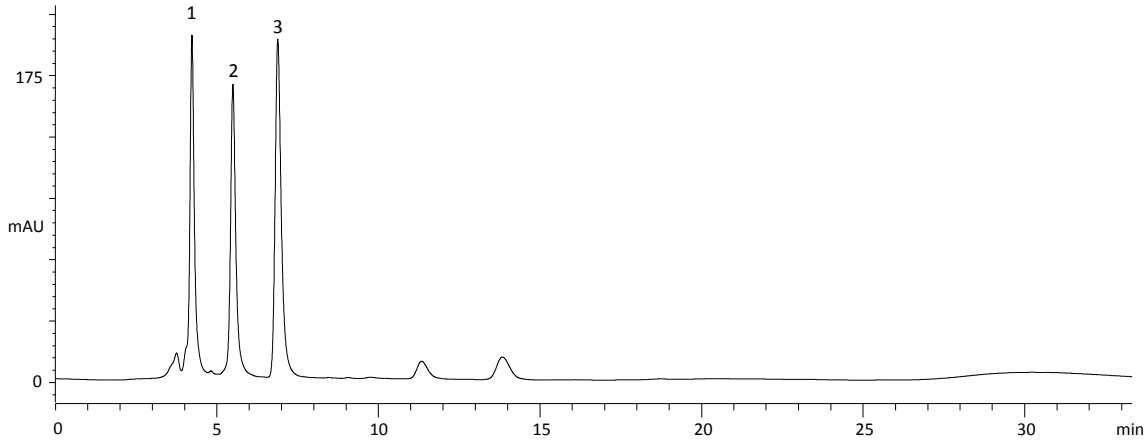

Figure 27. NP-LC separation of a mixture of (1) acetylated cholesterol, (2) acetylated 7*ß*-hydroxycholesterol and 5,6*ß*-epoxycholesterol, and (3) acetylated 7-ketocholesterol on a SiOH-column, detected at 206 nm. For conditions, cf. 3.2.2.5.

LC separations and retentions were shown to be similar for cholesterol and phytosterols and for cholesterol oxidation products and phytosterol/phytostanol oxidation products, respectively, enabling the use of the respective commercially available cholesterol oxidation products as internal standards.

The on-line transfer of the oxysterol fractions to the GC was accomplished by switching the transfer valve; the completeness of the transfer was confirmed by comparing the GC chromatogram of a mixture of acetylated standards that was manually injected into the PTV interface to the on-line LC-GC chromatogram of the same mixture. To vaporize 250 μL of organic eluent transferred from LC, the vent flow was adjusted to 900 mL/min, resulting in an evaporation of the solvent prior to the capillary column through the split vent port. At 0.625 min, the inlet was switched to the purge mode to split vent for clean-up with 2.5 mL/min. The inlet was then heated up with 900 °C/min to 300 °C, inducing a transfer of the analytes to the GC column. Higher vent or purge flows as well as a higher inlet temperature resulted in a decrease of GC response; similarly, reducing vent flow, purge flow, and the maximum inlet temperature also led to a decreased sensitivity due to insufficient solvent elimination and incomplete transfer of the analytes. The use of a completely deactivated ultra-inert liner prevented injection-induced degradations in the PTV interface that were particularly remarkable for 7-ketosterols when conventional liners were employed. Further, the insertion depth of the stainless steel transfer line into the inlet was kept

as low as possible to minimize the active metal surface in the MMI. The low initial GC-temperature of 40 °C allowed a focusing of the analytes and the formation of a sharp band.

A medium-polar trifluoropropylmethyl polysiloxane capillary column was tested for GC analysis of individual constituents as this stationary phase has previously been shown to result in good separations of both free sterols/stanols and intact steryl/stanyl esters (Barnsteiner *et al.*, 2011; Esche *et al.*, 2013e), and in preliminary experiments also of silylated sterol oxidation products. By using a short capillary column (15 m), the retention times of the analytes could be reduced and thus 7-hydroxy steryl acetates could be protected from thermal degradation in the course of GC analysis. This phenomenon had been observed on longer analytical columns (30 m) in preliminary experiments, when up to 30 % of the 7-hydroxylated steryl acetates degraded, as illustrated in Figure 28 at the example of 7*ß*-hydroxycholesterol. A degradation of this compound already in the PTV injector could be excluded by comparison to GC/FID-analysis after cool-on-column injection.

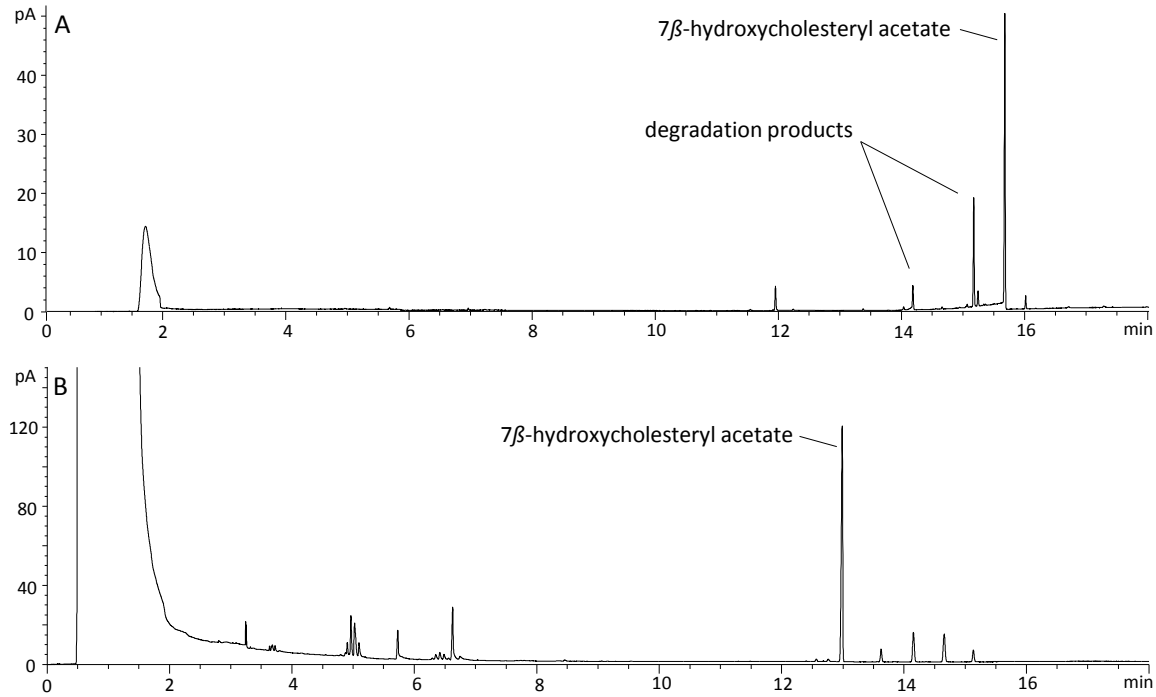

Figure 28. GC/FID analysis of acetylated 7*ß*-hydroxycholesterol after an LC run and subsequent on-line transfer using a 30 m RTX-200MS capillary column (**A**), and a 15 m RTX-200MS capillary column (**B**). The additional peaks in panel (B) are originating from the LC-eluent (cf. 3.2.2.5).

The GC order of elution of acetylated oxidation products deriving from the same sterol was determined by the type of oxidation, as epoxysterols eluted before the corresponding hydroxysterols and ketosterols. Exemplarily, the established on-line LC-GC analysis of a thermo-oxidized and acetylated stigmasterol standard is shown in Figure 29. The stigmasterol oxidation products could be separated from non-oxidized sterols via normal phase LC (Figure 29 A),

enabling a fast subsequent GC analysis of 5,6-epoxy-, 7-hydroxy-, and 7-ketostigmasterol oxidation products after the fully automated on-line transfer of the two oxysterol fractions (Figure 29 B and Figure 29 C). The transferred fraction 1 still comprised a small amount of the non-oxidized sterol; this, however, did not hamper GC analysis, as the non-oxidized sterol was chromatographically separated from the corresponding 7-hydroxy- and 5,6-epoxysterol oxidation products. A determination of phytosterol-triols via the on-line LC-GC methodology was also tested, as triols are also frequently described to be abundantly formed upon oxidation processes; they were, however, not eluted within the two LC-transfer windows and thus could not be determined.

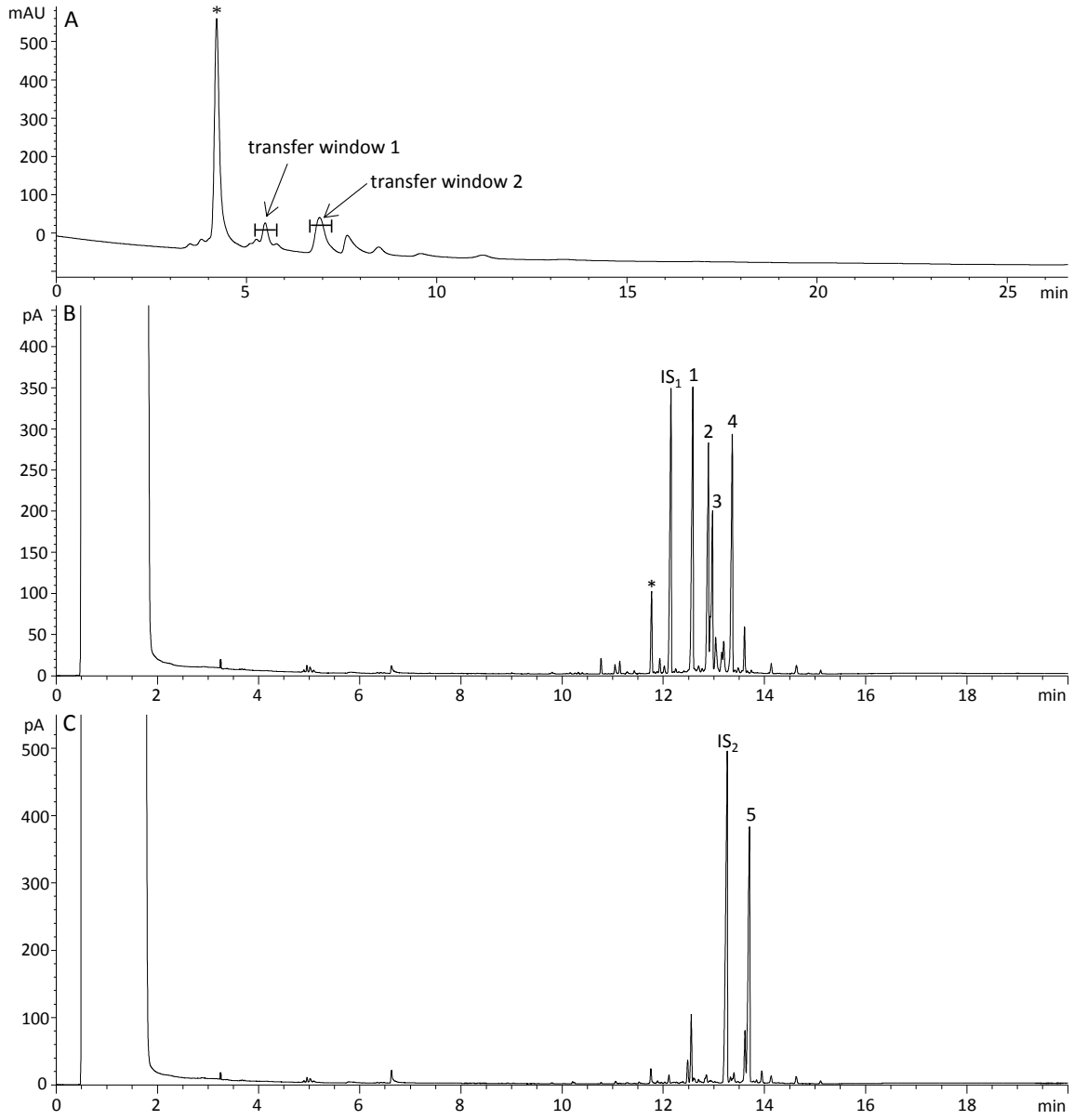

Figure 29. On-line LC-GC/FID analysis of acetylated stigmasterol oxidation products. LC-chromatogram at 206 nm (**A**); GC/FID-chromatogram of the transferred LC-window 1 (**B**): (1) 5,6*ß*-epoxystigmasterol, (2) 5,6*α*-epoxystigmasterol, (3) 7*α*-hydroxystigmasterol, (4) 7*ß*-hydroxystigmasterol, and (IS_1) 5,6*ß*-epoxycholesterol; GC/FID-chromatogram of the transferred LC-window 2 (**C**): (5) 7-ketostigmasterol, and (IS_2) 7-ketocholesterol. The asterisks in panel (A) and (B) indicate non-oxidized acetylated stigmasterol (for conditions, cf. 3.2.2.5).

4.2.3 On-Line LC-GC/MS Identification

In contrast to electron impact mass spectra of silylated phytosterol and cholesterol oxidation products, the fragmentation patterns of acetylated sterol oxidation products have, to the author's knowledge, thus far only been reported in one study, using selected oxidation products of cholesterol acetate as examples (Pelillo *et al.*, 2000). The reference compounds generated via semi-preparative HPLC and via synthesis were used to record the on-line LC-GC/MS spectra of

their acetylated derivatives. However, the 5,6*ß*-epoxy derivative of stigmasterol could not be made available; thus, its identity was deduced from the order of elution and the mass spectra of the 5,6*ß*-epoxy compounds of the other sterols. As examples, the on-line LC-GC mass spectra of representative acetylated sitosterol oxidation products are shown in Figure 30.

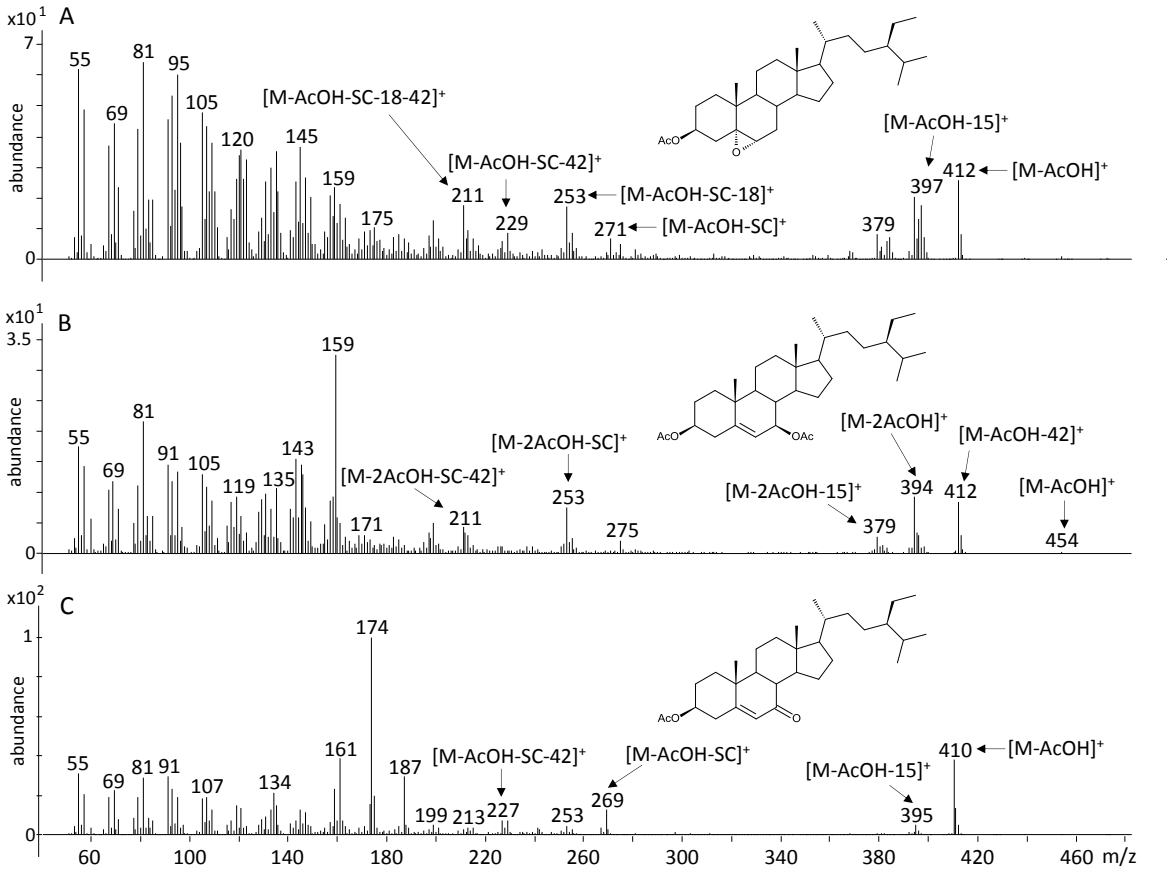

Figure 30. Representative on-line LC-GC/MS (EI) spectra of acetylated sitosterol oxidation products 5,6*α*-epoxysitosteryl acetate (**A**), 7*ß*-hydroxysitosteryl acetate (**B**), 7-ketositosteryl acetate (**C**) (for conditions, cf. 3.2.2.5).

Characteristic fragment ions of acetylated cholesterol and phytosterol/-stanol oxidation products together with the respective relative retention times are summarized in Table 19 - Table 21.

Table 19. Relative retention times (RRT) and characteristic fragment ions of reference compounds of acetylated 5,6-epoxysterols (on-line LC-GC/(EI)MS; 70 eV).

		characteristic fragment ions *m/z* (rel. abundance)							
sterol oxidation product	RRT[a]	M-AcOH	M-AcOH-15	M-AcOH-18	M-AcOH-SC	M-AcOH-SC-18	M-AcOH-SC-42	M-AcOH-SC-18-42	others
5,6*ß*-epoxycholesterol	1.0	384(24)	369(18)	366(13)	271(6)	253(11)	229(10)	211(21)	95(100), 120(28), 247(14)
5,6*α*-epoxycholesterol	1.026	384(18)	369(19)	366(21)	271(6)	253(19)	229(10)	211(22)	81(100), 120(53), 247(10)
5,6*ß*-epoxysitosterol	1.054	412(42)	397(28)	394(19)	271(13)	253(16)	229(17)	211(29)	95(100), 120(29)
5,6*α*-epoxysitosterol	1.079	412(33)	397(25)	394(29)	271(9)	253(26)	229(13)	211(28)	81(100), 120(56)
5,6*ß*-epoxycampesterol	1.030	398(23)	383(17)	380(13)	271(7)	253(12)	229(11)	211(23)	95(100), 120(25)
5,6*α*-epoxycampesterol	1.057	398(15)	383(14)	380(22)	271(7)	253(20)	229(9)	211(23)	55(100), 81(99), 120(47)
5,6*ß*-epoxystigmasterol[b]	1.036	410(4)	395(3)	392(4)	271(4)	253(13)	229(5)	211(7)	55(100), 81(84), 120(12)
5,6*α*-epoxystigmasterol	1.067	410(3)	395(5)	392(6)	271(4)	253(20)	229(4)	211(11)	55(100), 81(90), 120(16)

[a] RRT time compared to the internal standard 5,6*ß*-epoxycholesterol (LC-GC/FID, RTX-200MS, 15 m).

[b] The spectrum was derived from the peak tentatively identified as 5,6*ß*-epoxystigmasterol after thermo-oxidation of stigmasterol, as no authentic reference compound was available.

Table 20. Relative retention times (RRT) and characteristic fragment ions of reference compounds of acetylated 7-hydroxysterols/-stanols (on-line LC-GC/(EI)MS; 70 eV).

		characteristic fragment ions *m/z* (rel. abundance)						
sterol oxidation product	RRT[a]	M-AcOH	M-AcOH-42	M-2AcOH	M-2AcOH-15	M-2AcOH-SC	M-2AcOH-SC-42	others
7*α*-hydroxycholesterol	1.033	426(2)	384(49)	366(48)	351(12)	253(23)	211(17)	159(100), 247(19)
7*β*-hydroxycholesterol	1.068	426(1)	384(26)	366(30)	351(10)	253(21)	211(13)	159(100), 247(12)
7*α*-hydroxysitosterol	1.085	454(1)	412(29)	394(27)	379(7)	253(16)	211(14)	159(100), 275(6)
7*β*-hydroxysitosterol	1.118	454(1)	412(22)	394(19)	379(6)	253(15)	211(11)	159(100), 275(5)
7*α*-hydroxycampesterol	1.064	-	398(18)	380(16)	365(4)	253(10)	211(11)	159(100)
7*β*-hydroxycampesterol	1.098	-	398(24)	380(16)	365(6)	253(10)	211(14)	159(100)
7*α*-hydroxystigmasterol	1.067	452(1)	410(19)	392(19)	377(2)	253(23)	211(9)	83(100), 159(53)
7*β*-hydroxystigmasterol	1.100	452(1)	410(22)	392(22)	377(4)	253(30)	211(10)	55(100), 159(62)
6*α*-hydroxysitostanol	1.103[b]	456(27)	-	396(100)	381(20)	255(47)	213(79)	145(46), 81(53)
7-hydroxysitostanol	1.116[b]	456(44)	-	396(57)	381(25)	255(85)	213(49)	81(67), 57(100)

[a] RRT compared to the internal standard 5,6*β*-epoxycholesterol (LC-GC/FID, RTX-200MS, 15 m).
[b] RRT compared to the internal standard 5,6*β*-epoxycholesterol (LC-GC/FID, RTX-200MS, 14 m).

Table 21. Relative retention times (RRT) and characteristic fragment ions of reference compounds of acetylated 7-ketosterols (on-line LC-GC/(EI)MS; 70 eV).

		characteristic fragment ions *m/z* (rel. abundance)				
sterol oxidation product	RRT[a]	M-AcOH	M-AcOH-15	M-AcOH-SC	M-AcOH-SC-42	others
7-ketocholesterol	1.0	382(30)	367(6)	269(11)	227(7)	174(100), 211(4), 253(6)
7-ketositosterol	1.048	410(38)	395(5)	269(14)	227(8)	174(100), 211(3), 253(5)
7-ketocampesterol	1.025	396(26)	381(5)	269(9)	227(8)	174(100), 211(6), 253(8)
7-ketostigmasterol	1.033	408(15)	393(2)	269(52)	227(11)	81(100), 296(15), 365(13)

[a] RRT compared to the internal standard 7-ketocholesterol (LC-GC/FID, RTX-200MS, 15 m).

For none of the acetylated sterol oxidation products, molecular ions could be observed, being in accordance with the fragmentation described by Pelillo *et al.* (2000). All spectra, except for 7-hydroxycampesterol oxides, showed a characteristic fragment $[M\text{-}AcOH]^+$, corresponding to the loss of acetic acid (AcOH) due to a McLafferty rearrangement (Pelillo *et al.*, 2000). However, regarding the 7-hydroxysteryl diacetates, the intensity of this fragment ion was low; this is probably the reason for the absence of this fragment in the spectra of 7-hydroxycampesterol derivatives due to the low abundance of campesterol (10 %) in the sitosterol/campesterol standard used for preparation of the oxides. Instead, the spectra of the 7-hydroxysterols exhibited a fragment deriving from a double deacetylation, in turn providing evidence for a complete acetylation of the dihydroxy compounds. All acetylated sterol oxidation products showed a fragment correlating to the loss of a methyl group (-15) after complete deacetylation. In addition, for each derivative fragments deriving from a loss of the side chain ($[M\text{-}AcOH\text{-}SC]^+$ for epoxy and keto sterols and $[M\text{-}2AcOH\text{-}SC]^+$ for hydroxysterols) from the deacetylated sterol oxidation product were detected, as well as fragment ions deriving from a subsequent cleavage of the ring D ($[M\text{-}AcOH\text{-}SC\text{-}42]^+$ or $[M\text{-}2AcOH\text{-}SC\text{-}42]^+$, respectively). This fragmentation pattern has previously been described both for oxidation products of cholesterol acetate (Pelillo *et al.*, 2000) and acetates of non-oxidized sterols (Partridge and Djerassi, 1977; Rahier and Benveniste, 1989).

4.2.4 Method Validation

The developed on-line LC-GC-based method was validated in terms of LOD and LOQ, linearity, application to a lipid matrix, matrix effects, recovery rates, repeatability, and reproducibility.

The instrumental LOD and LOQ were exemplarily determined for acetates of cholesterol oxidation products and stigmasterol oxidation products (Table 22). Slightly higher values were obtained for 7-hydroxy compounds in comparison to 5,6-epoxy sterols, reflecting a lower relative response of 7-hydroxy compounds. The linearity was determined for the internal standards 1 and 2, as well as for the representative phytosterol oxidation products 5,6α-epoxysitosterol, 7β-hydroxysitosterol and 7-ketostigmasterol. Coefficients of correlation (r^2) were higher than 0.999 for all of these sterol oxidation products, indicating good linearity in the calibration ranges. The determined slopes, intercepts and coefficients of variation are also given in Table 22.

Table 22. LOD, LOQ, and characteristics of calibration curves of individual cholesterol and phytosterol oxidation products, determined as acetates.

sterol oxidation product	LOD [μg/mL][a]	LOQ [μg/mL][a]	slope	intercept	r^2
cholesterol oxidation products					
5,6β-epoxycholesterol (IS_1)	0.07	0.21	1.8208	0.8857	0.9994
7β-hydroxycholesterol	0.08	0.23	-	-	-
7-ketocholesterol (IS_2)	0.10	0.25	1.8894	1.7286	0.9999
phytosterol oxidation products					
5,6α-epoxystigmasterol	0.08	0.24	-	-	-
5,6α-epoxysitosterol	-	-	1.6772	-1.4873	1
7β-hydroxystigmasterol	0.11	0.33	-	-	-
7β-hydroxysitosterol	-	-	1.2518	-2.594	0.9998
7-ketostigmasterol	0.11	0.31	1.9694	-2.1658	0.9998

[a] LOD and LOQ are expressed as μg/mL of injection volume (on-line LC-GC/FID); determined on the basis of 1 μL injection volume.

As most of the foods enriched with phytosterols or their fatty acid esters are lipid-based products, a phytosteryl ester-enriched margarine was selected as example for further method validation and application. For the release of phytosterol oxidation products from the fatty acid esters, the principles of a recently described fast and mild transesterification method were applied (Rudzińska *et al.*, 2014). The addition of citric acid to the transesterified extract as also described by Schmarr *et al*. (1996) to remove excessive alkali was revealed to be essential in order to accomplish a clear separation of phases upon addition of organic solvent for extraction of the oxides. Further, the use of plastic-based labware was shown to be crucial, as employing glassware led to losses of in particular hydroxy-compounds prior to their derivatization. The transesterification step was followed by the acetylation of the extract and subsequent on-line LC-GC analysis. For achieving a complete acetylation of the oxides, the organic extract had to be washed until a neutral pH was achieved.

The procedure finally employed for the determination of phytosterol/-stanol oxidation products in enriched lipid-based foods is outlined in Figure 31.

For this procedure, matrix effects were determined for each type of oxidation products by analyzing a thermo-oxidized stigmasterol standard, both after direct acetylation and after spiking to non-enriched margarine matrix with subsequent sample preparation. The recoveries of the individual analytes in the non-heated matrix were in the range of 90.5 % ± 4.1 to 102.2 % ± 0.1. Spiking and working up a heated matrix resulted in similar recoveries, ranging from 91.0 % ± 5.5 to 99.3 % ± 1.9. As there were no significant differences in individual or total recoveries between the heated and the non-heated matrix, it was considered reasonable to determine the recovery rates for the complete analytical procedure spiking a non-heated margarine.

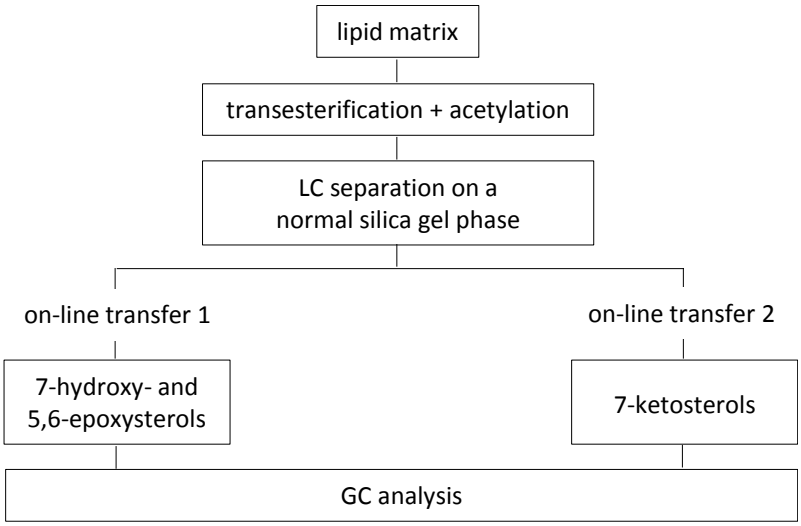

Figure 31. Outline of the on-line LC-GC-based procedure for the analysis of phytosterol and phytostanol oxidation products.

To a non-enriched margarine, three different amounts of stigmasterol oxidation products were added, covering the range of amounts expected to occur in non-heated and heated enriched margarine as derived from preliminary experiments. Good recovery rates between 95 % and 107 % with low standard deviations were obtained for each analyte at all three concentration levels (Table 23), demonstrating the applicability of the methodology to a broad concentration range. Potential carry over effects were excluded by injecting a blank sample after the analysis of the highest concentrated standard mixture.

Table 23. Recovery rates of acetylated stigmasterol oxidation products spiked to a non-enriched margarine.

	recovery [%]		
sterol oxidation product	level 1[a]	level 2[b]	level 3[c]
5,6α-epoxystigmasterol	107.4 ± 3.2	106.7 ± 2.0	106.7 ± 0.5
7β-/7α-hydroxystigmasterol	103.8 ± 1.6	98.5 ± 2.7	99.6 ± 3.6
7-ketostigmasterol	96.9 ± 2.3	95.2 ± 4.2	96.2 ± 0.8

[a] Blank samples were spiked with 0.02 mg 5,6α-epoxystigmasterol, 0.02 mg 7β-hydroxystigmasterol, and 0.02 mg 7-ketostigmasterol.

[b] Blank samples were spiked with 0.135 mg 5,6α-epoxystigmasterol, 0.12 mg 7β-hydroxystigmasterol, and 0.16 mg 7-ketostigmasterol.

[c] Blank samples were spiked with 0.51 mg 5,6α-epoxystigmasterol, 0.51 mg 7α-hydroxystigmasterol, and 0.5 mg 7-ketostigmasterol.

The suitability of the analytical method was further evaluated by investigating the formation of phytosterol oxidation products upon heating of a phytosteryl ester-enriched margarine. For a simulation of cooking conditions, the margarine was thermo-oxidized at 180 °C for different time spans and subjected to analyses. Exemplarily, the on-line LC-GC chromatogram of the margarine heated at 180 °C for 150 min is shown in Figure 32.

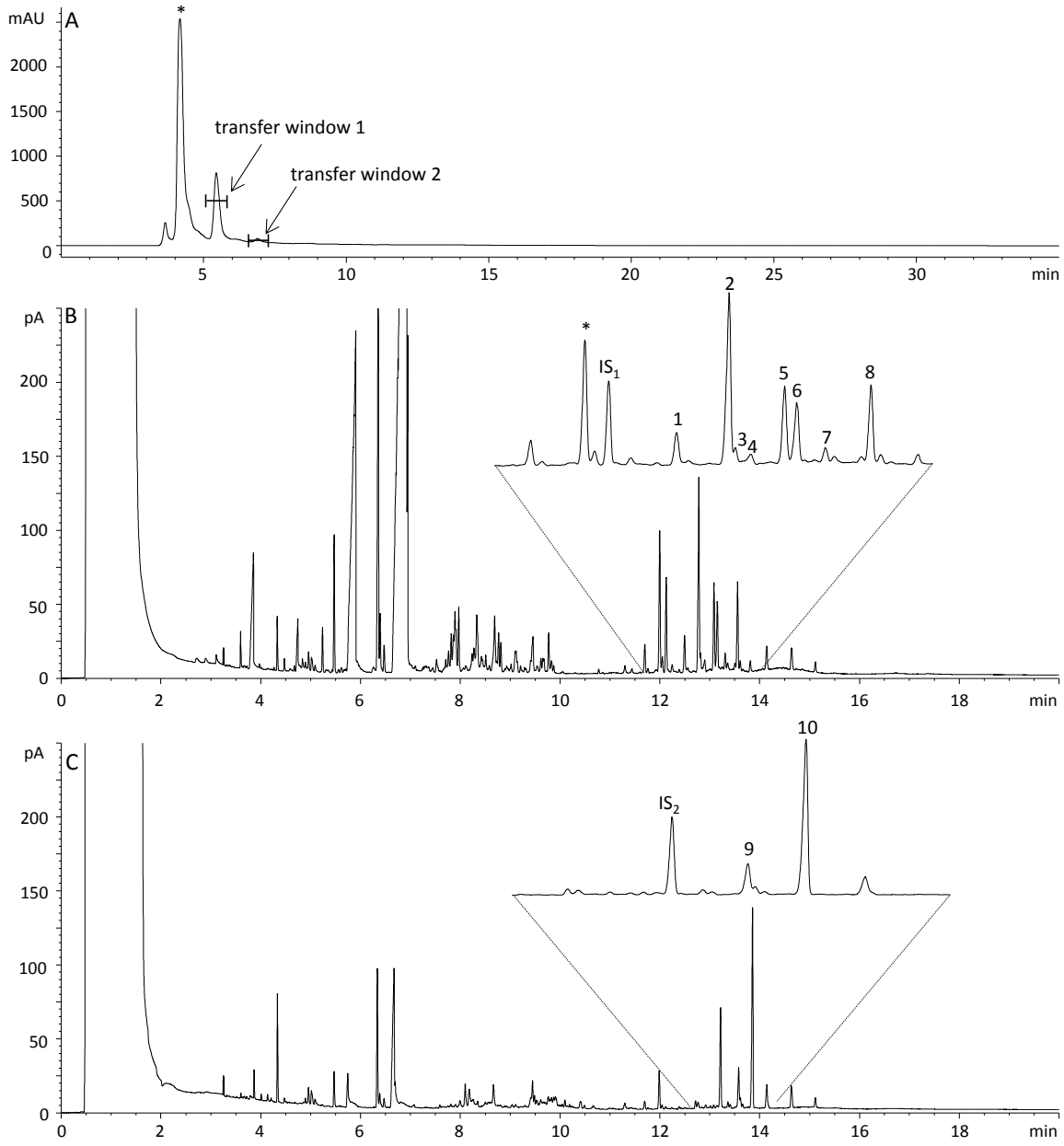

Figure 32. On-line LC-GC/FID analysis of acetylated phytosterol oxidation products in a heated phytosteryl/-stanyl ester-enriched margarine (180 °C, 150 min). LC-chromatogram at 206 nm (**A**); GC/FID-chromatogram of the transferred LC-window 1 (**B**): (1) 5,6*ß*-epoxycampesterol, (2) 5,6*ß*-epoxysitosterol, (3) 5,6*α*-epoxycampesterol, (4) 7*α*-hydroxycampesterol, (5) 5,6*α*-epoxysitosterol, (6) 7*α*-hydroxysitosterol, (7) 7*ß*-hydroxycampesterol, (8) 7*ß*-hydroxysitosterol, and (IS_1) 5,6*ß*-epoxycholesterol; GC/FID-chromatogram of the transferred LC-window 2 (**C**): (9) 7-ketocampesterol, (19) 7-ketositosterol, and (IS_2) 7-ketocholesterol. The asterisks in panel (A) and (B) indicate non-oxidized acetylated phytosterols (for conditions, cf. 3.2.2.5).

The repeatability of the method was determined by analyzing the non-heated margarine, the margarine heated for 90 min and the margarine heated for 150 min on three consecutive days in triplicate analysis, respectively. Reproducibility was confirmed by the additional analysis of each sample by a second operator. The calculated results including the coefficients of variation (CV) are summarized in Table 24 - Table 26.

Table 24. Repeatability and reproducibility of the on-line LC-GC/FID analysis of acetylated phytosterol oxidation products in a phytosteryl/-stanyl ester-enriched margarine (no. 5).

phytosterol oxidation product	operator I			operator II	mean	CV [%]
[mg/kg]	day 1	day 2	day 3	day 1		
5,6*ß*-epoxycampesterol	41.2 ± 1.1[a]	38.5 ± 3.3	38.4 ± 1.1	36.7 ± 3.0	37.9 ± 2.6[c]	6.9
5,6*ß*-epoxysitosterol	195.4 ± 7.6	189.8 ± 21.2	182.6 ± 2.1	186.9 ± 6.6	188.7 ± 11.1	5.9
5,6*α*-epoxycampesterol	16.2 ± 0.9	15.9 ± 1.8	16.5 ± 0.5	15.9 ± 0.6	16.1 ± 1.0	6.1
7*α*-hydroxycampesterol	-[b]	-[b]	-[b]	-[b]	-[b]	-
5,6*α*-epoxysitosterol	82.2 ± 6.3	80.9 ± 7.2	86.5 ± 2.2	79.9 ± 4.9	82.4 ± 5.4	6.5
7*α*-hydroxysitosterol	-[b]	-[b]	-[b]	-[b]	-[b]	-
7*ß*-hydroxycampesterol	-[b]	-[b]	-[b]	-[b]	-[b]	-
7*ß*-hydroxysitosterol	-[b]	-[b]	-[b]	-[b]	-[b]	-
7-ketocampesterol	10.1 ± 0.09	9.6 ± 0.6	9.7 ± 0.4	9.8 ± 0.3	9.8 ± 0.4	4.1
7-ketositosterol	34.9 ± 4.3	36.5 ± 1.5	34.8 ± 0.7	41.4 ± 2.4	36.9 ± 3.6	9.6
total POP	380.0 ± 12.2	371.2 ± 33.7	368.5 ± 3.3	370.5 ± 15.3	372.5 ± 17.3	4.6

[a] Values represent the mean ± standard deviation (n=3).
[b] Content below LOD (cf. Table 22).
[c] Values represent the mean of all analyses ± standard deviation (n=12).

Table 25. Repeatability and reproducibility for the on-line LC-GC/FID analysis of acetylated phytosterol oxidation products in a phytosteryl/-stanyl ester-enriched margarine (no. 5) after heating at 180 °C for 90 min.

phytosterol oxidation product	operator I			operator II	mean	CV [%]
[mg/kg]	day 1	day 2	day 3	day 1		
5,6*ß*-epoxycampesterol	300.2 ± 3.1[a]	301.4 ± 12.1	303.7 ± 4.3	303.3 ± 10.7	302.0 ± 7.6[b]	2.5
5,6*ß*-epoxysitosterol	1399.8 ± 14.0	1411.3 ± 23.6	1413.7 ± 32.7	1375.2 ± 39.4	1398.7 ± 28.7	2.1
5,6*α*-epoxycampesterol	121.5 ± 7.3	131.0 ± 2.7	117.9 ± 8.3	114.5 ± 2.3	121.5 ± 8.0	6.6
7*α*-hydroxycampesterol	177.6 ± 9.2	164.0 ± 6.4	164.1 ± 5.4	170.4 ± 8.3	169.5 ± 8.8	5.2
5,6*α*-epoxysitosterol	646.8 ± 14.8	655.9 ± 16.0	621.8 ± 8.4	608.4 ± 18.7	634.2 ± 24.3	3.8
7*α*-hydroxysitosterol	690.9 ± 10.9	616.9 ± 42.2	605.1 ± 15.8	692.1 ± 29.0	655.5 ± 48.0	7.3
7*ß*-hydroxycampesterol	130.5 ± 4.9	121.5 ± 3.5	127.4 ± 8.0	134.1 ± 0.7	128.5 ± 6.3	4.9
7*ß*-hydroxysitosterol	783.1 ± 36.1	628.4 ± 50.4	594.2 ± 21.1	791.9 ± 3.8	709.0 ± 95.5	13.5
7-ketocampesterol	135.6 ± 3.1	136.7 ± 11.1	121.2 ± 1.2	124.5 ± 6.5	130.3 ± 9.1	7.0
7-ketositosterol	598.3 ± 8.3	596.8 ± 10.0	590.6 ± 12.8	588.1 ± 3.0	593.7 ± 8.5	1.4
total POP	4984.3 ± 59.1	4763.9 ± 126.9	4659.3 ± 14.1	4902.5 ± 39.0	4842.9 ± 118.2	2.6

[a] Values represent the mean ± standard deviation (n=3).
[b] Values represent the mean of all analyses ± standard deviation (n=12).

Table 26. Repeatability and reproducibility for the on-line LC-GC/FID analysis of acetylated phytosterol oxidation products in a phytosteryl/-stanyl ester-enriched margarine (no. 5) after heating at 180 °C for 150 min.

phytosterol oxidation product	operator I			operator II	mean	CV [%]
[mg/kg]	day 1	day 2	day 3	day 1		
5,6*ß*-epoxycampesterol	502.6 ± 28.4[a]	521.4 ± 14.3	498.1 ± 24.9	508.7 ± 6.9	508.6 ± 18.9[b]	3.7
5,6*ß*-epoxysitosterol	2436.8 ± 26.3	2445.5 ± 44.0	2475.7 ± 15.2	2433.4 ± 64.6	2445.3 ± 40.4	1.7
5,6*α*-epoxycampesterol	212.9 ± 11.2	195.1 ± 4.5	214.7 ± 9.8	208.9 ± 5.2	207.3 ± 10.5	5.1
7*α*-hydroxycampesterol	271.0 ± 9.6	266.3 ± 12.1	257.5 ± 4.8	258.1 ± 5.1	263.7 ± 9.5	3.6
5,6*α*-epoxysitosterol	1069.1 ± 21.8	1035.5 ± 4.2	1090.9 ± 42.5	1048.5 ± 16.8	1058.3 ± 27.7	2.6
7*α*-hydroxysitosterol	971.3 ± 65.8	946.1 ± 56.8	980.9 ± 9.7	1013.2 ± 46.7	977.6 ± 51.5	5.3
7*ß*-hydroxycampesterol	192.8 ± 9.9	189.9 ± 8.1	198.8 ± 9.3	206.7 ± 2.6	196.9 ± 9.6	4.9
7*ß*-hydroxysitosterol	1011.0 ± 69.7	980.9 ± 79.3	1086.1 ± 4.9	1052.9 ± 61.2	1027.9 ± 67.8	6.6
7-ketocampesterol	295.0 ± 8.1	285.2 ± 10.1	293.4 ± 10.5	285.9 ± 13.5	289.5 ± 10.1	3.5
7-ketositosterol	1342.1 ± 19.8	1325.0 ± 22.9	1341.0 ± 5.4	1337.0 ± 14.5	1335.9 ± 16.8	1.3
total	8304.8 ± 206.3	8190.9 ± 135.1	8437.1 ± 68.7	8353.2 ± 128.3	8311.0 ± 154.8	1.9

[a] Values represent the mean ± standard deviation (n=3).
[b] Values represent the mean of all analyses ± standard deviation (n=12).

The data indicated very good repeatability and reproducibility regarding all analytes at all concentrations analyzed; the coefficients of variation of the total phytosterol oxidation products were overall <5 %. The CVs for the individual analytes were consistently <10 %, except for 7*ß*-hydroxysitosterol that exhibited a variation of 13.5 % in the margarine heated for 90 min (Table 25). These values comply with the criteria established by Horwitz *et al.* (1980) who defined a CV ≤10 % for within-laboratory repeatability to be acceptable when working at ppm-levels. The instrumental repeatability was determined to be excellent, as after a 10-fold injection of one heated margarine sample, no analyte showed a CV above 6 %, indicating the precision of the LC-transfer. The quantitative development of the individual phytosterol oxidation products in the enriched margarine during the applied heating procedure is illustrated in Figure 33.

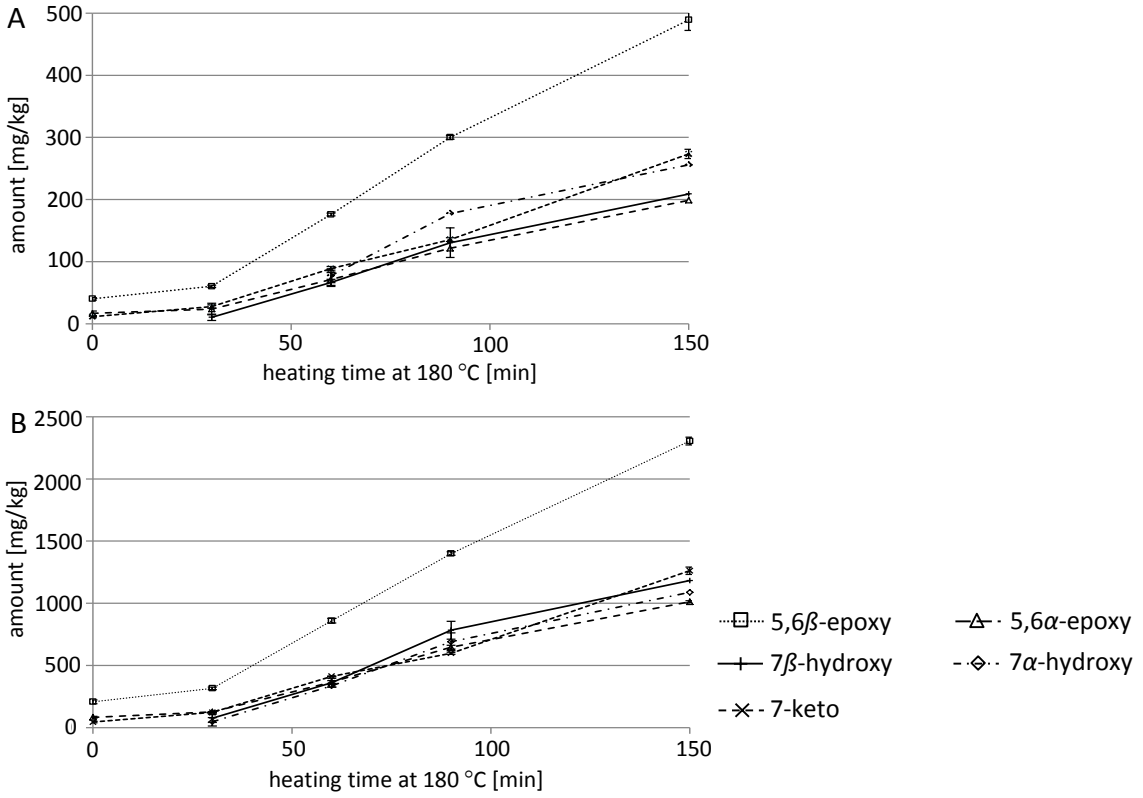

Figure 33. Formation of oxidation products of campesterol (**A**) and sitosterol (**B**) upon heating of a phytosteryl/-stanyl ester-enriched margarine.

Phytosterol oxidation products accumulated during the heating process with a slight increase during the first 30 min, followed by a pronounced formation of phytosterol oxidation products in the next 60 min. Between 90 and 150 min, the increase of the analytes, except for 7-keto compounds, was less pronounced, suggesting an enhanced parallel formation of tertiary oxidation products and/or a degradation of the secondary oxidation products (Otaegui-Arrazola *et al.*, 2010). The quantitative dominance of sitosterol oxidation products over campesterol oxidation

products reflected the phytosterol composition determined in the margarine, as the major esterified phytosterol in the margarine was sitosterol followed by campesterol. Oxidation products of other quantitatively minor phytosterols were not detected. 5,6*ß*-Epoxy compounds were throughout the most abundant compounds, while 7-hydroxysterols could not be determined in the non-heated margarine and were only formed in detectable amounts upon thermal stress. A phytosterol oxidation product concentration of approximately 400 mg/kg determined in the non-heated margarine is in the same order of magnitude as recently reported contents of phytosterol oxidation products in a phytostanyl ester enriched margarine (Rudzińska *et al.*, 2014). The sharp increase of phytosterol oxidation products in the course of the heating procedure is in line with observations reported by Julien-David *et al.* (2014): An analysis of oxidized derivatives of sitosteryl oleate after heating of an enriched spread at various time and temperature conditions showed a similar quantitative development when the margarine was heated at 170 °C and 200 °C for up to 120 minutes; also, 5,6*ß*-epoxygenation of the sterol moiety was the dominating oxidative modification.

4.2.5 Summary

The developed on-line LC-GC/MS methodology represents a novel approach for a highly sensitive and fully automated analysis of phytosterol oxidation products in enriched foods. The acetylation of the oxides permitted a selective LC-based pre-separation using silica as stationary phase and a mixture of *n*–hexane/MTBE/*iso*-propanol as eluent. Two LC-fractions containing (i) 5,6-epoxy- and 7-hydroxyphytosterols, and (ii) 7-ketophytosterols were transferred on-line to the GC for the analysis of their individual compositions on a medium polar trifluoropropylmethyl polysiloxane capillary column. GC/MS spectra of acetylated 5,6-epoxy, 7-hydroxy, and 7-keto derivatives of cholesterol, stigmasterol, sitosterol, campesterol, and of hydroxy derivatives of sitostanol were recorded for the first time using reference compounds. The analytical method was validated using an enriched margarine as example; the formation of phytosterol oxidation products upon heating was followed. Excellent data in terms of sensitivity, recovery, repeatability, and reproducibility could be achieved, illustrating the suitability of the implemented on-line approach as alternative to conventional, laborious off-line techniques that may be accompanied by the risks of sample losses and undesired reactions.

4.3 Determination of Baseline Levels of Phytosterol Oxidation Products in Enriched Margarines

The knowledge on baseline POP contents in enriched margarines not subjected to thermal treatments is only scarce. The contents determined for the thermally untreated margarine used for validation of the on-line LC-GC approach in the present study (cf. 4.2.4) were considerably higher than those described in literature (cf. Table 2); however, a comparative assessment of the existing data is difficult because they were generated via different analytical methodologies. Therefore, employing the established on-line LC-GC methodology, the contents of phytosterol oxidation products of different commercially available phytosteryl and/or phytostanyl ester-enriched margarines should be determined. Four brands of margarines ("Becel pro.activ", "Fruit d'Or pro-activ", "Deli Reform active", "Bellasan activ") enriched with a mixture of phytosteryl/-stanyl fatty acid esters similar to that shown in Table 17 (cf. 4.1.2.3) and purchased at four different supermarkets, were investigated. Further, a phytostanyl ester-enriched margarine ("Benecol") was analyzed, enriched with a mixture of esters similar to that shown in Table 10 (cf. 4.1.1.3). All margarines were suitable for spreading; according to the labelling, the margarines "Fruit d'Or pro-activ" and "Benecol" were additionally suitable to be heat-treated. At purchase, the margarines differed regarding the expiration date and the way they were offered in the supermarkets, as some supermarkets offered them on a cooling shelf while others stored the margarines at room temperature. All margarines were investigated the day after purchase, except for the margarines "Fruit d'Or pro-activ Cuisson & Tartine" from France and "Benecol voi & rypsiöliy" from Finland. Additionally, two of the margarines were stored in a refrigerator at 5 °C, and one week past the expiration date the contents of phytosterol oxidation products were analyzed again.

The on-line LC-GC chromatogram of one of the margarines is exemplarily shown in Figure 34. An overview on the amounts of phytosterol oxidation products determined in the margarine in the previous chapter (cf. 4.2.4, margarine no. 5) and in the additionally investigated margarines is given in Table 27.

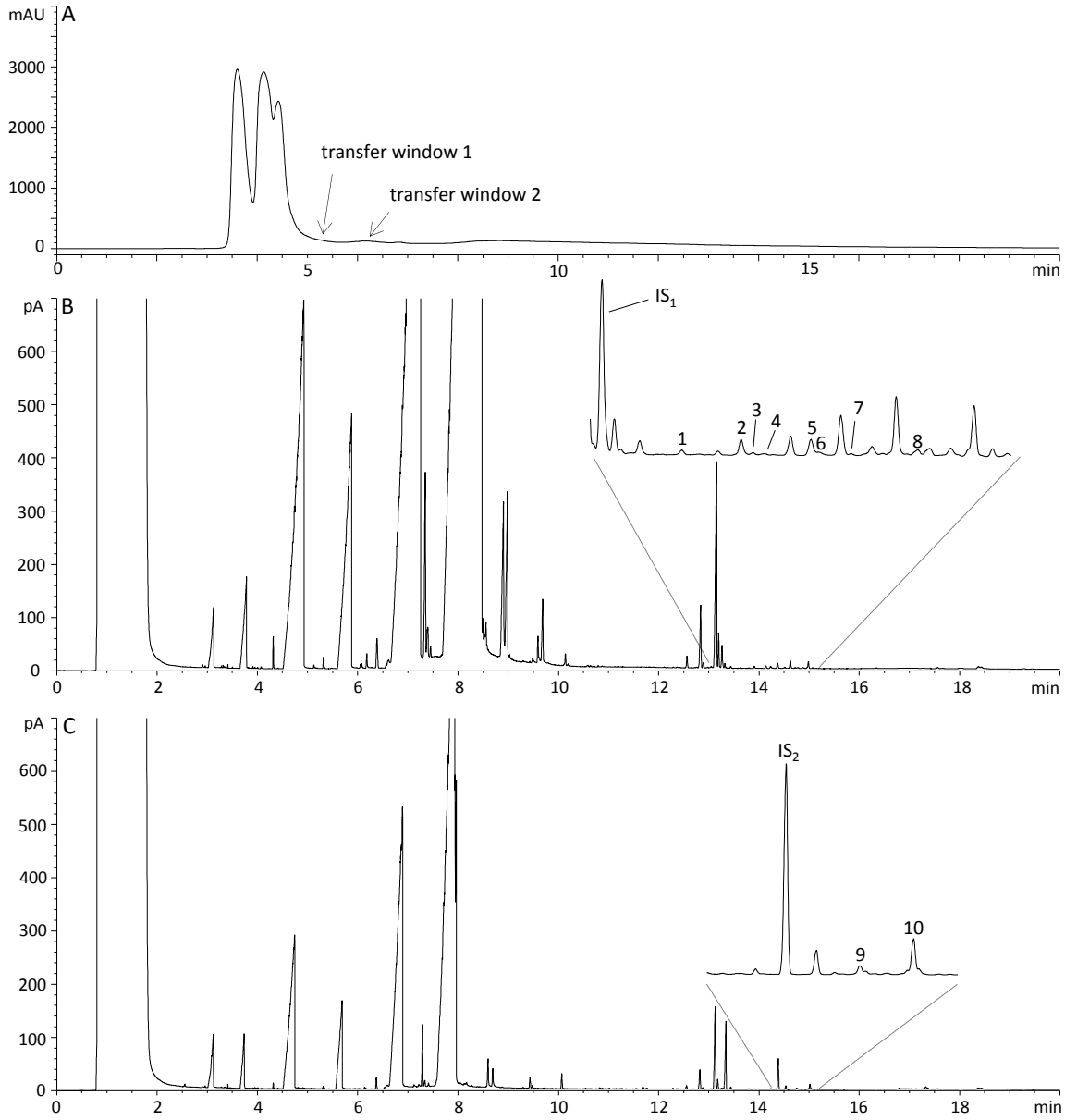

Figure 34. On-line LC-GC/FID analysis of acetylated phytosterol oxidation products in a phytosteryl/ -stanyl ester-enriched margarine ("Bellasan activ", no. 10) at purchase. LC-chromatogram at 206 nm (**A**). GC/FID-chromatogram of the transferred LC-window 1 (**B**): (1) 5,6*ß*-epoxycampesterol, (2) 5,6*ß*-epoxysitosterol, (3) 5,6*α*-epoxycampesterol, (4) 7*α*-hydroxycampesterol, (5) 5,6*α*-epoxysitosterol, (6) 7*α*-hydroxysitosterol, (7) 7*ß*-hydroxycampesterol (8) 7*ß*-hydroxysitosterol, and (IS_1) 5,6*ß*-epoxycholesterol; GC/FID-chromatogram of the transferred LC-window 2 (**C**): (9) 7-ketocampesterol, (10) 7-ketositosterol, and (IS_2) 7-ketocholesterol. For conditions, cf. 3.2.2.5.

Overall, only low amounts of phytosterol oxidation products could be determined in the margarines, and also the differences in total POP contents between the samples were small, ranging between 37.9 ± 1.5 and 92.9 ± 2.2 mg/kg in the phytosteryl/-stanyl ester-enriched margarines, corresponding to oxidation rates between 0.03 – 0.12 % (Table 27). These total POP contents determined for the margarines enriched with mixtures of phytosteryl and phytostanyl fatty acid esters were in good agreement with POP contents in untreated, phytosteryl/-stanyl

ester-enriched margarines described in other studies (Grandgirard *et al.*, 2004c; Conchillo *et al.*, 2005; Johnsson and Dutta, 2006; Husche *et al.*, 2011; Baumgartner *et al.*, 2013b; Lin *et al.*, 2016;) (Table 2, cf. 2.3.2). The described amounts vary between 11 mg/kg (Lin *et al.*, 2016) and 68 mg/kg (Grandgirard *et al.*, 2004c). In the investigated margarines, epoxy-derivatives were the dominating species, followed by keto- and hydroxysterols that were even not detectable in some of the margarines. Individually, except for margarine no. 5, 7-ketositosterol and 5,6*α*-epoxysitosterol were the most abundant phytosterol oxidation products. A preponderance of 7-ketositosterol in similarly enriched margarines has also been described in the literature (Conchillo *et al.*, 2005; Johnsson and Dutta, 2006). However, Husche *et al.* (2011) described a quantitative dominance of 7-hydroxysitosterol for a phytosteryl/-stanyl ester-enriched margarine.

The data obtained for the additionally investigated samples did not confirm the exceptionally high contents determined in the phytosteryl/-stanyl ester-enriched margarine no. 5 (Table 24, cf. 4.2.4), accounting for 372.5 ± 17.3 mg/kg (equivalent to an oxidation rate of 0.5 %), mainly due to the abundance of epoxyphytosterols in this sample.

The phytostanyl ester-enriched margarine exhibited the lowest POP contents. Except for 6*α*-hydroxysitostanol (20.6 mg/kg) and a minor amount of 7-ketositosterol, the concentrations of the other POP were below the limits of detection and the limits of quantitation, respectively. The rate of phytostanol oxidation was calculated to be 0.03 %. In another study, phytosterol oxidation products deriving from the phytosterols present in the analyzed phytostanyl ester-enriched margarines amounted to 5.4 mg/kg (Baumgartner *et al.*, 2013b); this is only slightly above the amount of 1.9 mg/kg 7-ketositosterol determined in the present study. In a further study on a phytostanyl ester-enriched margarine, similarly only phytosterol oxidation products were analyzed (Rudzińska *et al.*, 2014); however, a total of 255 mg/kg phytosterol oxidation products was determined, which is 134-fold higher than the amount of 1.9 mg/kg 7-ketositosterol found in the present study.

Table 27. Phytosterol oxidation products determined in different phytosteryl/-stanyl ester-enriched margarines directly after purchase. The time spans between the date of analysis and the expiration date are given in the square brackets. Margarines no. 8 and no. 2 were not analyzed directly after purchase.

phytosterol oxidation product [mg/kg]	Becel pro.activ				Fruit d'Or pro-activ	Deli Reform active	Bellasan activ	Benecol Voi & Rypsiöliy
	no. 5[a,c]	no. 4 [45 d][c]	no. 6 [45 d][c]	no. 7 [54 d][d]	no. 8 [66 d][c]	no. 9 [58 d][d]	no. 10 [40 d][d]	no. 2[e]
5,6*ß*-epoxycampesterol	37.9 ± 2.6[f]	1.3 ± 0.1[g]	2.1 ± 0.2[g]	2.8 ± 0.2[g]	4.4 ± 0.1[g]	3.5 ± 0.1[g]	2.8 ± 0.2[g]	-[h]
5,6*ß*-epoxysitosterol	188.7 ± 11.1	5.8 ± 0.5	8.9 ± 0.9	10.2 ± 2.3	14.8 ± 0.7	8.7 ± 0.1	10.3 ± 0.6	-[j]
5,6*α*-epoxycampesterol	16.1 ± 1.0	1.8 ± 0.2	2.8 ± 0.5	2.7 ± 0.6	3.8 ± 0.3	1.9 ± 0.2	2.3 ± 0.1	-[h]
7*α*-hydroxycampesterol	-[h]	1.9 ± 0.2	2.4 ± 0.2	1.9 ± 0.2	2.9 ± 0.3	1.8 ± 0.1	2.4 ± 0.2	-[h]
5,6*α*-epoxysitosterol	82.4 ± 5.4	12.2 ± 0.6	9.6 ± 0.7	12.3 ± 0.9	18.7 ± 1.8	12.5 ± 0.0	12.4 ± 0.6	-[h]
7*α*-hydroxysitosterol	-[h]	1.6 ± 0.1	2.8 ± 0.2	2.4 ± 0.2	3.1 ± 0.2	4.3 ± 0.1	3.4 ± 0.3	-[h]
7*ß*-hydroxycampesterol	-[h]	-[h]	-[h]	0.9 ± 0.0	1.4 ± 0.0	0.9 ± 0.1	1.4 ± 0.2	-[h]
6*α*-hydroxysitostanol	-[i]	-[i]	-[i]	-[i]	-[j]	-[i]	-[i]	20.6 ± 1.9[g]
7*ß*-hydroxysitosterol	-[h]	-[h]	-[h]	3.7 ± 0.1	4.7 ± 0.4	4.2 ± 0.1	4.8 ± 0.1	-[h]
7-ketocampesterol	9.8 ± 0.4	2.2 ± 0.1	1.8 ± 0.1	5.2 ± 0.6	10.2 ± 1.0	2.9 ± 0.2	5.5 ± 0.8	-[h]
7-ketositosterol	36.9 ± 3.6	11.1 ± 1.2	10.4 ± 0.9	19.9 ± 2.2	28.9 ± 0.7	12.3 ± 0.3	18.7 ± 3.8	1.9 ± 0.3
total	372.5 ± 17.3	37.9 ± 1.6	40.8 ± 1.4	62.1 ± 6.3	92.9 ± 2.2	52.9 ± 1.0	63.7 ± 5.3	22.6 ± 1.5
oxidation rate[b] [%]	0.50 ± 0.0	0.05 ± 0.0	0.05 ± 0.0	0.08 ± 0.1	0.12 ± 0.0	0.07 ± 0.0	0.08 ± 0.1	0.03 ± 0.0

[a] The numbering is according to that in Table 5.
[b] Calculated as percentage of phytosterol/phytostanol oxidation products with respect to the initial phytosterol/phytostanol content.
[c] Margarines were obtained from supermarkets with cooling shelves.
[d] Margarines were obtained from supermarkets without cooling shelves.
[e] No information on storage condition in supermarket available; the margarine was analyzed 34 days past the expiration date.
[f] Values represent the mean ± standard deviation (n=12).
[g] Values represent the mean ± standard deviation (n=3).
[h] Content below limit of detection (i.e. <0.11 μg/mL of injection volume for 7-hydroxyphytosterols and <0.08 μg/mL of injection volume for 5,6-epoxyphytosterols).
[i] Compound was not analyzed.
[j] Content below limit of quantitation (i.e. <0.33 μg/mL of injection volume for 7-hydroxyphytosterols and <0.24 μg/mL of injection volume for 5,6-epoxyphytosterols).

The POP contents determined in two of the margarines after storage at 5 °C until one week past the expiration date are given in Table 28.

Table 28. Phytosterol oxidation products determined in phytosteryl/-stanyl ester-enriched margarines one week past the expiration date. The days of storage are given in square brackets.

phytosterol oxidation product [mg/kg]	Becel pro.activ no. 4[a], [52 d][c]	Bellasan activ no. 10 [47 d][d]
5,6*ß*-epoxycampesterol	2.1 ± 0.2[e]	3.0 ± 0.3
5,6*ß*-epoxysitosterol	7.8 ± 0.9	9.7 ± 0.9
5,6*α*-epoxycampesterol	1.0 ± 0.0	1.8 ± 0.1
7*α*-hydroxycampesterol	2.1 ± 0.3	1.5 ± 0.1
5,6*α*-epoxysitosterol	8.7 ± 0.8	10.4 ± 0.2
7*α*-hydroxysitosterol	3.5 ± 0.0	2.6 ± 0.2
7*ß*-hydroxycampesterol	1.0 ± 0.1	1.1 ± 0.1
7*ß*-hydroxysitosterol	4.6 ± 0.3	3.9 ± 0.2
7-ketocampesterol	4.3 ± 0.3	7.7 ± 0.2
7-ketositosterol	12.9 ± 1.0	27.3 ± 0.7
total	47.9 ± 2.4	68.9 ± 1.4
oxidation rate[b] [%]	0.06 ± 0.0	0.09 ± 0.0

[a] The numbering is according to that in Table 5.
[b] Calculated as percentage of phytosterol/phytostanol oxidation products with respect to the initial phytosterol/phytostanol content.
[c] Margarine was obtained from a supermarket with cooling shelves.
[d] Margarines was obtained from a supermarket without cooling shelves.
[e] Values represent the mean ± standard deviation (n=3).

Storage led to a slight increase in POP contents in both investigated margarines, reflected by a slight increase in oxidation rates; however, the observed increase was not significant for the "Bellasan activ" margarine (no. 10). Considering the distribution of individual POP species, 7-ketositosterol and 5,6*ß*-epoxysitosterol were still dominating. Though, regarding the changes in the profiles before and after storage, they were observed to vary between the two margarines (Figure 35). In both margarines, the epoxy compounds decreased upon storage, while the keto derivatives increased, however, the extents were differently pronounced. The hydroxy derivatives showed inconsistent changes, as they increased in the "Becel pro.activ" margarine, but decreased in the "Bellasan activ" margarine. The only other study determining the effects of storage on the formation of phytosterol oxidation products in margarine investigated a margarine enriched with phytostanyl esters (Rudzińska *et al.*, 2014). They observed a significantly higher initial POP content and an overall much more pronounced increase of total phytosterol oxidation products from 225 mg/kg to 354 mg/kg margarine after 42 days at 4 °C. However, similar to the observations made in the present study, they also showed that the contents of the individual POP species did not show consistent decreases or increases during a storage time of in total 18 weeks.

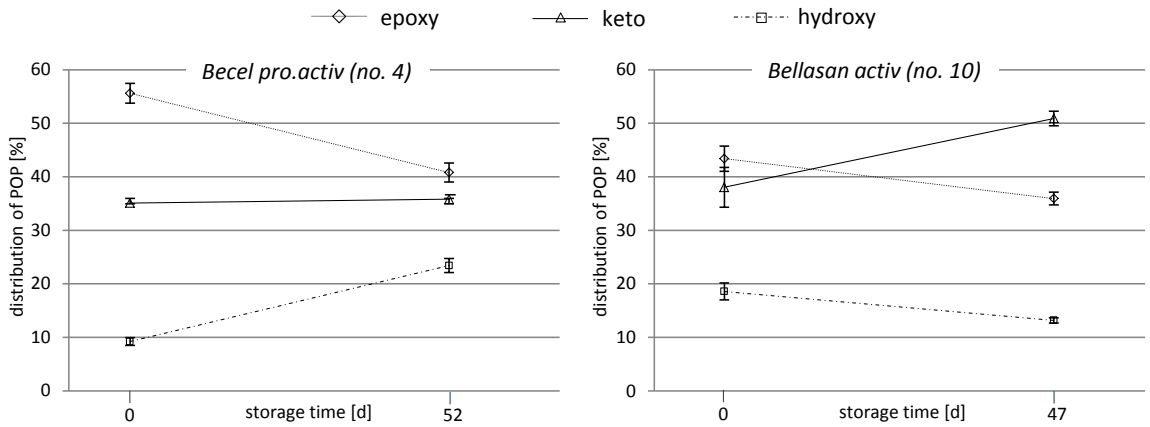

Figure 35. Changes of the distribution of the classes of phytosterol oxidation products upon storage of two phytosteryl/-stanyl ester-enriched margarines.

A comparison of the total POP contents determined in all margarines directly after purchase (except for margarines no. 8 and no. 2) and after storage is depicted in Figure 36. Overall, no difference between samples having been offered on a cooling shelf and those having been stored at ambient temperature could be observed.

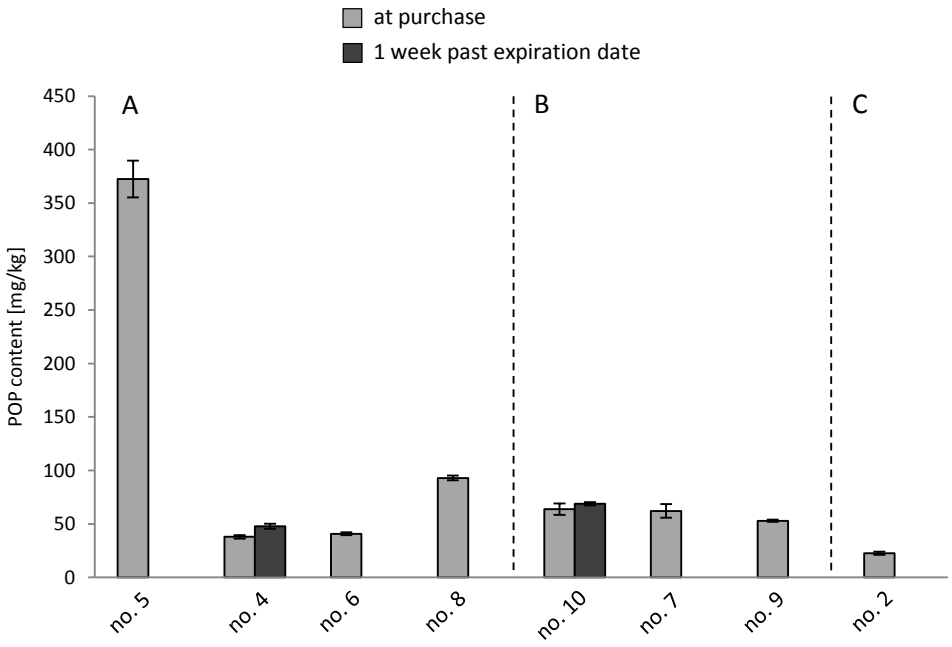

Figure 36. Total contents of phytosterol oxidation products determined via on-line LC-GC in enriched margarines at purchase and upon storage at 5 °C in phytosteryl/-stanyl ester-enriched margarines from supermarkets with (A) and without a cooling shelf (B), and in a phytostanyl ester-enriched margarine (C).

In summary, the analyses of the baseline levels of phytosterol and/or phytostanol oxidation products in untreated enriched margarines revealed overall low levels within a relatively narrow range, in seven of the investigated eight samples. However, for one margarine, exceptionally high

concentrations of phytosterol oxidation products were observed at the time of purchase. Apparently, there may be circumstances leading in single cases to an elevated formation of phytosterol oxidation products, going beyong the normal level. This may be due to conditions of manufacturing, transportation or storage; however, the information available on the “history” of the investigated sample did not allow to explain and to trace back the reasons for this phenomenon.

4.4 Complementary Analysis of Phytosteryl/Phytostanyl Fatty Acid Esters and of Phytosterol/Phytostanol Oxidation Products in Enriched Margarines upon Heating

To increase the understanding of the impact of common heating procedures on phytosteryl/-stanyl fatty acid ester-enriched foods, the investigation of both, the phytosterol oxidation products and the intact esters should be in the focus of analytical efforts. Though, previous approaches were mainly limited to the determination of only the oxidized sterol or stanol moieties (Dutta, 2004; Scholz *et al.*, 2015). Some of the existing studies additionally investigated the decreasing contents of the intact sterol/stanol moieties after cleavage of the ester bonds (Soupas *et al.*, 2007; Menéndez-Carreño *et al.*, 2008a; Rudzińska *et al.*, 2014; Menéndez-Carreño *et al.*, 2016; Nieminen *et al.*, 2016). However, regarding a quantitative determination of the intact esters, only one study is available, following the decrease of one selected ester, sitosteryl oleate, in an enriched margarine upon oven heating (Julien-David *et al.*, 2014). The lack of further data on the changing contents of individual intact phytosteryl and/or phytostanyl fatty acid esters might be due to a long-time unavailability of analytical methodologies allowing their quantitation in complex mixtures. Thus, the developed UHPLC-APCI-MS-based approaches, enabling for the first time the quantitative analysis of mixtures of phytostanyl fatty acid esters and of phytosteryl and phytostanyl fatty acid esters should be used to extend the knowledge on the impact of heating on individual phytosteryl and phytostanyl fatty acid esters in enriched foods. Using the developed on-line LC-GC-based methodology, the analyses should be complemented by the determination of the concurrently formed oxidation products of the phytosteryl/-stanyl moieties. For this purpose, two commercially available margarines explicitly labelled to be used for cooking and baking were subjected to common heating processes such as oven-heating, pan-frying, and microwave-heating. One of the margarines had been enriched with a mixture of phytosteryl and phytostanyl fatty acid esters, while the other margarine contained added phytostanyl fatty acid esters. Data from one set of heating experiments are described in detail in the following chapters. Data from a second set of heating experiments are provided in the Appendix.

4.4.1 Decreases of Intact Phytosteryl and Phytostanyl Fatty Acid Esters

4.4.1.1 Phytosteryl/-stanyl Ester-Enriched Margarine

The contents of individual phytosteryl and phytostanyl fatty acid esters determined via UHPLC-APCI-MS in the untreated margarine as well as in the differently heated samples are compiled in Table 29.

Table 29. Phytosteryl/-stanyl fatty acid esters in phytosteryl/-stanyl ester-enriched margarine (no. 8) before and after heat treatments.[a]

steryl / stanyl fatty acid ester [g/kg]	no treatment	microwave, bottle 800 W, 4 min	pan-frying 200 °C[c], 9 min	oven, bottle 200 °C, 20 min	oven, casserole 200 °C, 20 min
brassicasteryl-18:2***	2.40 ± 0.09[b] (a)	2.19 ± 0.03 (b)	2.13 ± 0.05 (b)	1.92 ± 0.03 (c)	1.39 ± 0.03 (d)
brassicasteryl-16:0***	0.25 ± 0.00 (a)	0.24 ± 0.01 (a)	0.24 ± 0.01 (a)	0.21 ± 0.00 (b)	0.19 ± 0.01 (b)
brassicasteryl-18:1***	1.05 ± 0.02 (a)	0.94 ± 0.01 (b)	0.96 ± 0.03 (b)	0.86 ± 0.05 (c)	0.72 ± 0.02 (d)
brassicasteryl-18:0***	0.11 ± 0.01 (a)	0.10 ± 0.01 (b)	0.11 ± 0.00 (ab)	0.10 ± 0.00 (ab)	0.09 ± 0.01 (c)
campesteryl-16:1***	0.02 ± 0.00 (a)	0.02 ± 0.00 (b)	0.02 ± 0.00 (bc)	0.01 ± 0.00 (c)	0.01 ± 0.00 (d)
campesteryl-18:2***	12.37 ± 0.20 (a)	11.49 ± 0.22 (b)	11.12 ± 0.02 (b)	10.18 ± 0.40 (c)	7.48 ± 0.08 (d)
campesteryl-16:0***	1.08 ± 0.00 (ab)	1.09 ± 0.02 (a)	1.07 ± 0.02 (ab)	1.01 ± 0.04 (c)	0.92 ± 0.04 (d)
campesteryl-18:1***	5.49 ± 0.13 (a)	5.26 ± 0.08 (ab)	5.13 ± 0.05 (bc)	4.78 ± 0.23 (c)	4.10 ± 0.07 (d)
campesteryl-18:0***	0.62 ± 0.01 (a)	0.62 ± 0.02 (a)	0.61 ± 0.00 (a)	0.54 ± 0.01 (b)	0.50 ± 0.03 (b)
campesteryl-20:1***	0.02 ± 0.00 (a)	0.02 ± 0.00 (a)	0.02 ± 0.00 (a)	0.02 ± 0.00 (b)	0.01 ± 0.00 (c)
campesteryl-20:0***	0.03 ± 0.00 (a)	0.03 ± 0.00 (ab)	0.03 ± 0.00 (b)	0.03 ± 0.00 (c)	0.03 ± 0.00 (c)
campesteryl-22:0***	0.10 ± 0.00 (a)	0.09 ± 0.01 (ab)	0.09 ± 0.00 (ab)	0.09 ± 0.00 (b)	0.08 ± 0.00 (c)
stigmasteryl-18:2***	0.39 ± 0.02 (a)	0.30 ± 0.01 (b)	0.29 ± 0.01 (b)	0.27 ± 0.01 (b)	0.21 ± 0.01 (c)
stigmasteryl-18:0***	0.02 ± 0.00 (a)	0.02 ± 0.00 (b)	0.02 ± 0.00 (b)	0.01 ± 0.00 (c)	0.01 ± 0.00 (c)
sitosteryl-18:3***	0.21 ± 0.01 (ab)	0.21 ± 0.01 (a)	0.21 ± 0.01 (a)	0.19 ± 0.01 (b)	0.12 ± 0.00 (c)
sitosteryl-16:1***	0.09 ± 0.00 (a)	0.09 ± 0.00 (a)	0.09 ± 0.00 (ab)	0.08 ± 0.00 (b)	0.05 ± 0.00 (c)
sitosteryl-18:2***	57.59 ± 1.46 (a)	55.35 ± 1.21 (ab)	52.41 ± 0.76 (b)	48.55 ± 1.81 (c)	35.56 ± 1.27 (d)
sitosteryl-16:0**	5.24 ± 0.10 (a)	5.24 ± 0.02 (a)	5.12 ± 0.08 (ab)	4.80 ± 0.19 (ab)	4.71 ± 0.31 (b)
sitosteryl-18:1**	26.04 ± 0.74 (a)	25.87 ± 0.64 (a)	24.93 ± 0.54 (ab)	23.06 ± 1.17 (b)	20.06 ± 0.56 (c)
sitosteryl-18:0***	2.90 ± 0.01 (a)	2.93 ± 0.03 (a)	2.90 ± 0.05 (a)	2.66 ± 0.15 (b)	2.46 ± 0.11 (b)
sitosteryl-20:1***	0.10 ± 0.00 (a)	0.10 ± 0.00 (a)	0.10 ± 0.01 (a)	0.09 ± 0.01 (b)	0.07 ± 0.00 (c)
sitosteryl-20:0***	0.16 ± 0.00 (ab)	0.16 ± 0.00 (a)	0.15 ± 0.00 (ab)	0.15 ± 0.01 (b)	0.13 ± 0.00 (c)
sitosteryl-22:0***	0.47 ± 0.01 (a)	0.47 ± 0.02 (ab)	0.46 ± 0.01 (ab)	0.43 ± 0.02 (b)	0.38 ± 0.01 (c)
campestanyl-18:2***	0.94 ± 0.03 (a)	0.89 ± 0.03 (ab)	0.92 ± 0.04 (ab)	0.81 ± 0.06 (b)	0.63 ± 0.03 (c)

Table 29. continued.

steryl / stanyl fatty acid ester [g/kg]	no treatment	microwave, bottle 800 W, 4 min	pan-frying 200 °C[c], 9 min	oven, bottle 200 °C, 20 min	oven, casserole 200 °C, 20 min
campestanyl-16:0**	0.10 ± 0.00 (a)	0.09 ± 0.01 (ab)	0.10 ± 0.00 (a)	0.09 ± 0.00 (a)	0.08 ± 0.00 (b)
campestanyl-18:1***	0.48 ± 0.01 (a)	0.43 ± 0.03 (b)	0.49 ± 0.00 (a)	0.48 ± 0.01 (a)	0.39 ± 0.00 (c)
campestanyl-18:0*	0.06 ± 0.00 (a)	0.05 ± 0.00 (ab)	0.05 ± 0.00 (a)	0.05 ± 0.00 (b)	0.05 ± 0.00 (ab)
campestanyl-22:0*	0.01 ± 0.00 (a)	0.01 ± 0.00 (ab)	0.01 ± 0.00 (ab)	0.01 ± 0.00 (ab)	0.01 ± 0.00 (ab)
sitostanyl-18:2***	6.50 ± 0.03 (a)	5.85 ± 0.27 (bc)	6.25 ± 0.19 (ab)	5.51 ± 0.30 (c)	4.34 ± 0.14 (d)
sitostanyl-16:0**	0.68 ± 0.03 (a)	0.58 ± 0.02 (b)	0.63 ± 0.02 (ab)	0.60 ± 0.03 (b)	0.57 ± 0.05 (b)
sitostanyl-18:1**	3.30 ± 0.06 (a)	3.22 ± 0.09 (a)	3.23 ± 0.04 (a)	3.10 ± 0.07 (a)	2.67 ± 0.10 (b)
sitostanyl-18:0**	0.36 ± 0.01 (a)	0.33 ± 0.01 (b)	0.35 ± 0.01 (ab)	0.32 ± 0.01 (b)	0.33 ± 0.01 (b)
sitostanyl-20:0***	0.03 ± 0.00 (a)	0.02 ± 0.00 (b)	0.02 ± 0.00 (bc)	0.02 ± 0.00 (c)	0.02 ± 0.00 (bc)
sitostanyl-22:0***	0.06 ± 0.00 (a)	0.04 ± 0.00 (b)	0.04 ± 0.00 (b)	0.05 ± 0.00 (bc)	0.05 ± 0.00 (c)
total esters***	129.28 ± 2.52 (a)	124.36 ± 2.33 (ab)	120.33 ± 1.62 (b)	111.16 ± 4.64 (c)	88.45 ± 2.11 (d)

[a] Analysis of variance was carried out with ANOVA. Levels of significance: $p<0.001$, highly significant (***); $p<0.01$, very significant (**); $p<0.05$, significant (*). Within the same row, different letters indicate significant differences (Tukey's HSD) among treatments ($p<0.05$).

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

[c] Final temperature of the heating process.

In total, 34 individual esters could be identified and quantitated. Stigmasteryl-18:1 was excluded from the quantitation as the coefficients of variation of this compound were unacceptably high in some of the heated samples, probably due to the co-elution of this ester with an unknown compound deriving from the margarine matrix (cf. 4.1.2.3). Sitosteryl esters were dominating followed by campesteryl esters; phytostanyl fatty acid esters made up on average 10 % of the total esters; linoleic acid was the main esterified fatty acid. The profile of the esterified sterols/stanols was in accordance with the specification of the SCF (2003a). The quantitated amount of total esters of 12.9 % was in good agreement with the package labeling (12.5 % plant sterol esters).

As heating procedures, common household ways of preparing foods were chosen: the enriched margarine was heated in a microwave, in a Teflon-coated frying-pan on a hotplate, and in a ventilated oven. Regarding oven heating, two different experiments were conducted in order to investigate the influence of increasing the sample surface exposed to oxygen; in one experiment, the margarine was placed in a bottle, in the other, the margarine was greased in a casserole. The margarines were heated without addition of foods; the applied heating times and temperatures reflected typical household conditions. All heating experiments caused a decrease of the total ester contents. The extent of ester degradation was shown to be impacted by the duration of the heating procedure and by the sample surface exposed to oxygen. Exemplarily, the UHPLC-APCI-MS chromatograms determined in the margarine heated in the casserole are shown in Figure 37 and Figure 38.

Microwave heating at 800 W for 4 min showed the least reduction of ester contents; the remaining total esters still accounted for 96 % of the initially determined content. Menéndez-Carreño *et al.* (2008a) subjected a phytosteryl/-stanyl ester-enriched milk to a similar experiment, heating the milk in a beaker in a microwave at 900 W. After 1.5 min of microwave heating, 71 % of intact phytosterol/-stanol moieties could still be detected, while after 2 min a further reduction to 34 % was described, thus suggesting a stronger impact of microwave heating on the intact sterol/stanol moieties. One explanation for this difference might be the lower energy of 800 W applied in the present study; also different matrices in combination with varying experimental conditions are probably affecting the extent of phytosterol degradation (Otaegui-Arrazola *et al.*, 2010).

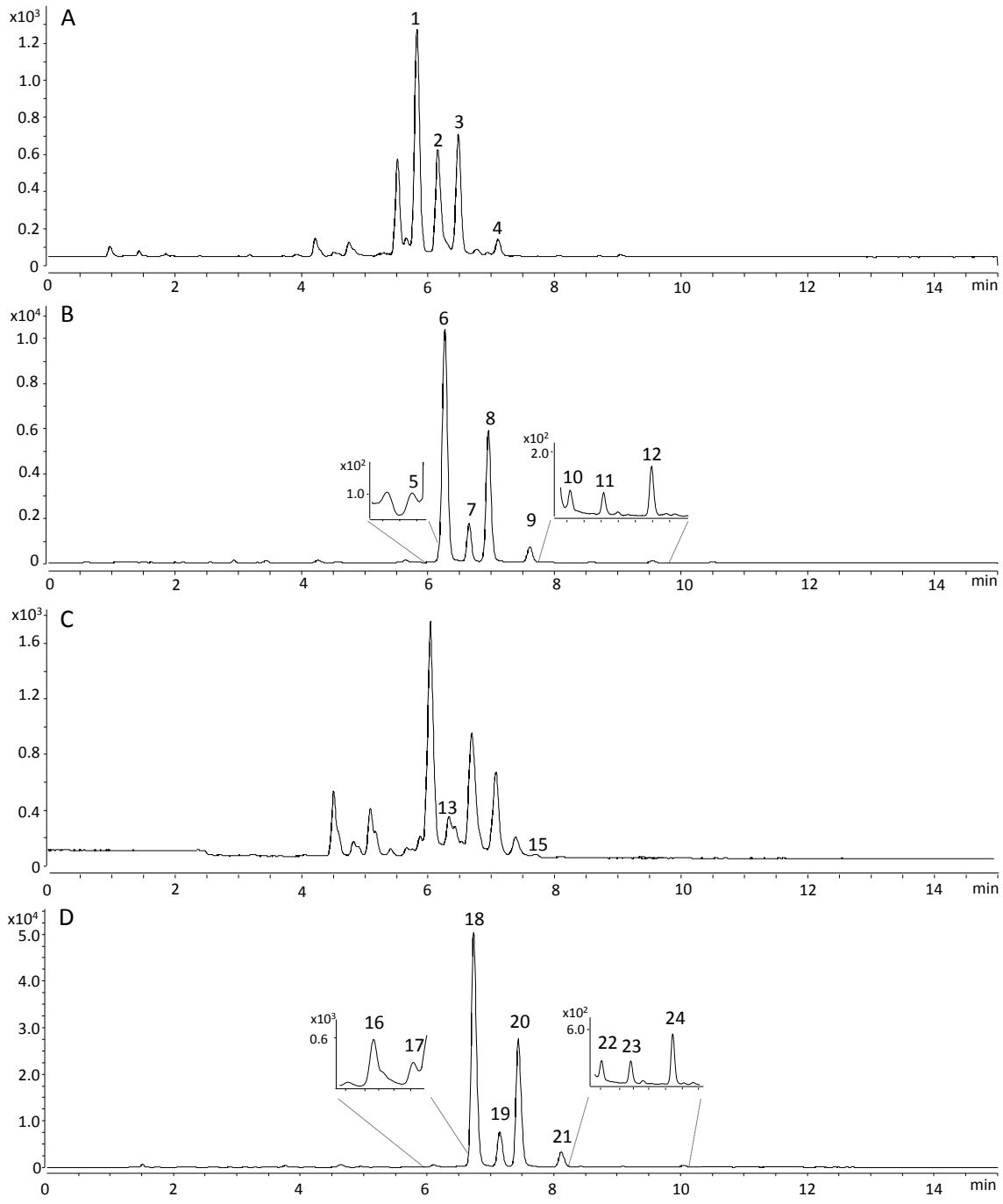

Figure 37. UHPLC-APCI-MS analysis of phytosteryl fatty acid esters in phytosteryl/phytostanyl ester-enriched margarine heated at 200 °C for 20 min in a casserole in an oven. SIM chromatogram of brassicasteryl fatty acid esters at *m/z* 381.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**A**). SIM chromatogram of campesteryl fatty acid esters at *m/z* 383.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**B**). SIM chromatogram of stigmasteryl fatty acid esters at *m/z* 395.3, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**C**). SIM chromatogram of sitosteryl fatty acid esters at *m/z* 397.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**D**). The peak numbering is in accordance with that in Table 14. For conditions, cf. 3.2.3.2.

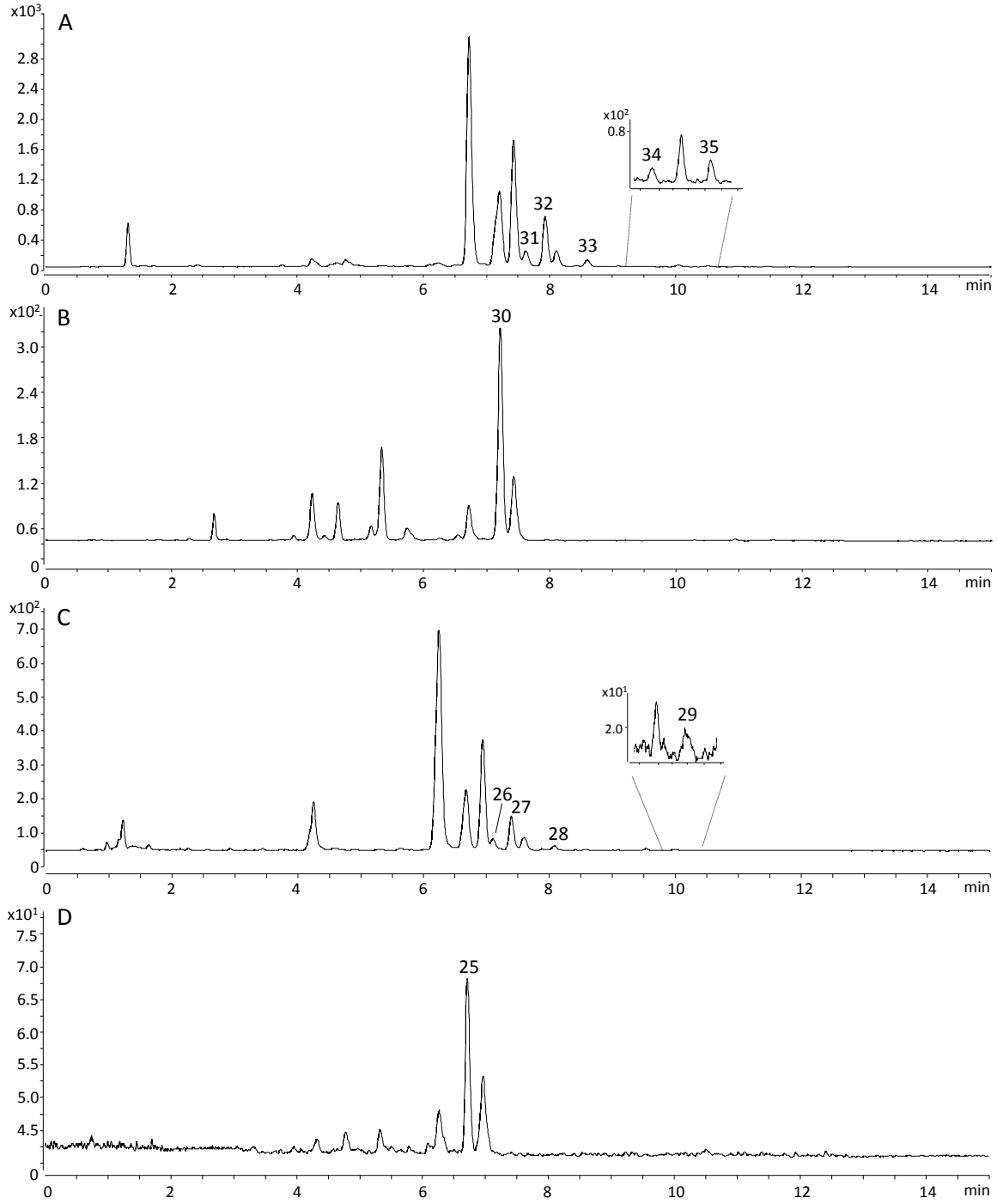

Figure 38. UHPLC-APCI-MS analysis of phytostanyl fatty acid esters in phytosteryl/phytostanyl ester-enriched margarine heated at 200 °C for 20 min in a casserole in an oven. SIM chromatogram of sitostanyl fatty acid esters at *m/z* 399.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**A**). SIM chromatogram of sitostanyl-18:2 at *m/z* 679.5, corresponding to the $[M+H]^+$ ion (**B**). SIM chromatogram of campestanyl fatty acid esters at *m/z* 385.2, corresponding to the $[M\text{-}FA+H]^+$ fragment ion (**C**). SIM chromatogram of campestanyl-18:2 at *m/z* 665.4, corresponding to the $[M+H]^+$ ion (**D**). The peak numbering is in accordance with that in Table 14. For conditions, cf. 3.2.3.2.

After electric heating of the margarine in a pan for 9 min, 120 g/kg fatty acid esters, corresponding to approximately 93 % of the initial ester content, could be detected. Recent results for sunflower oil, where after 30 min of pan-frying at 185 °C approximately 90 % of unaltered phytosteryl/-stanyl moieties were analyzed, are in agreement with these data (Ramadan, 2015). Approximately 94 % remaining unchanged phytosteryl moieties determined after pan-frying of a phytosteryl ester-enriched rapeseed oil for 10 min at 180 °C also indicate a loss in a similar order of magnitude (Soupas *et al.*, 2007). However, when making these comparisons it should be kept in mind that the decreases determined in the present study are based on the actual quantitation of the intact phytosteryl/-stanyl fatty acid esters whereas the decreases reported in other studies are only based on the quantitation of the sterol/stanol moieties after cleavage of the ester bonds.

Oven-heating of the margarine for 20 min at 200 °C led to the most pronounced decreases of the esters. Enlarging the surface to volume ratio via greasing the margarine in the casserole was shown to be of considerable influence, as after oven–heating in the bottle, approximately 86 % of the initial esters could be detected in contrast to only approximately 68 % remaining esters after oven-heating in the casserole. A previous study determined the loss of intact sitosteryl oleate in the same margarine as used in the present study upon heating in open glassware; after 30 min at 200 °C the remaining proportion of intact sitosteryl oleate was calculated to be 89 % (Julien-David *et al.*, 2014). This is in agreement with the decrease of the initially present sitosteryl oleate determined in the present study after oven heating in the bottle.

The employed UHPLC-APCI-MS approach offered the possibility to determine the decreases of esters in mixtures on an individual basis. The determined percentage changes in contents of the individual phytosteryl and phytostanyl fatty acid esters were illustrated by means of a heatmap (Figure 39). Both, the phytosteryl as well as the phytostanyl fatty acid esters decreased upon heat treatment. Still, the decreases of individual compounds upon the applied heating procedures were differently pronounced. Heating in a casserole in the oven led to an obvious decrease of all analyzed esters. In contrast, the heating procedures that were shown to be of a lower impact based on the decreases of total esters, such as microwave heating and pan-frying, seemed to primarily affect esters of brassicasterol, stigmasterol, whereas campesteryl and sitosteryl esters were revealed to be more stable. The higher initial susceptibility of stigmasteryl and brassicasteryl esters towards oxidation might be due to the additional double bond in the side chain. Interestingly, esters of phytostanols also showed more pronounced losses upon the milder heat treatments compared to sitosteryl and campesteryl esters. However, when more drastic heating

conditions such as oven-heating were employed, the decreases of the phytosteryl esters were more pronounced compared to those of the phytostanyl esters.

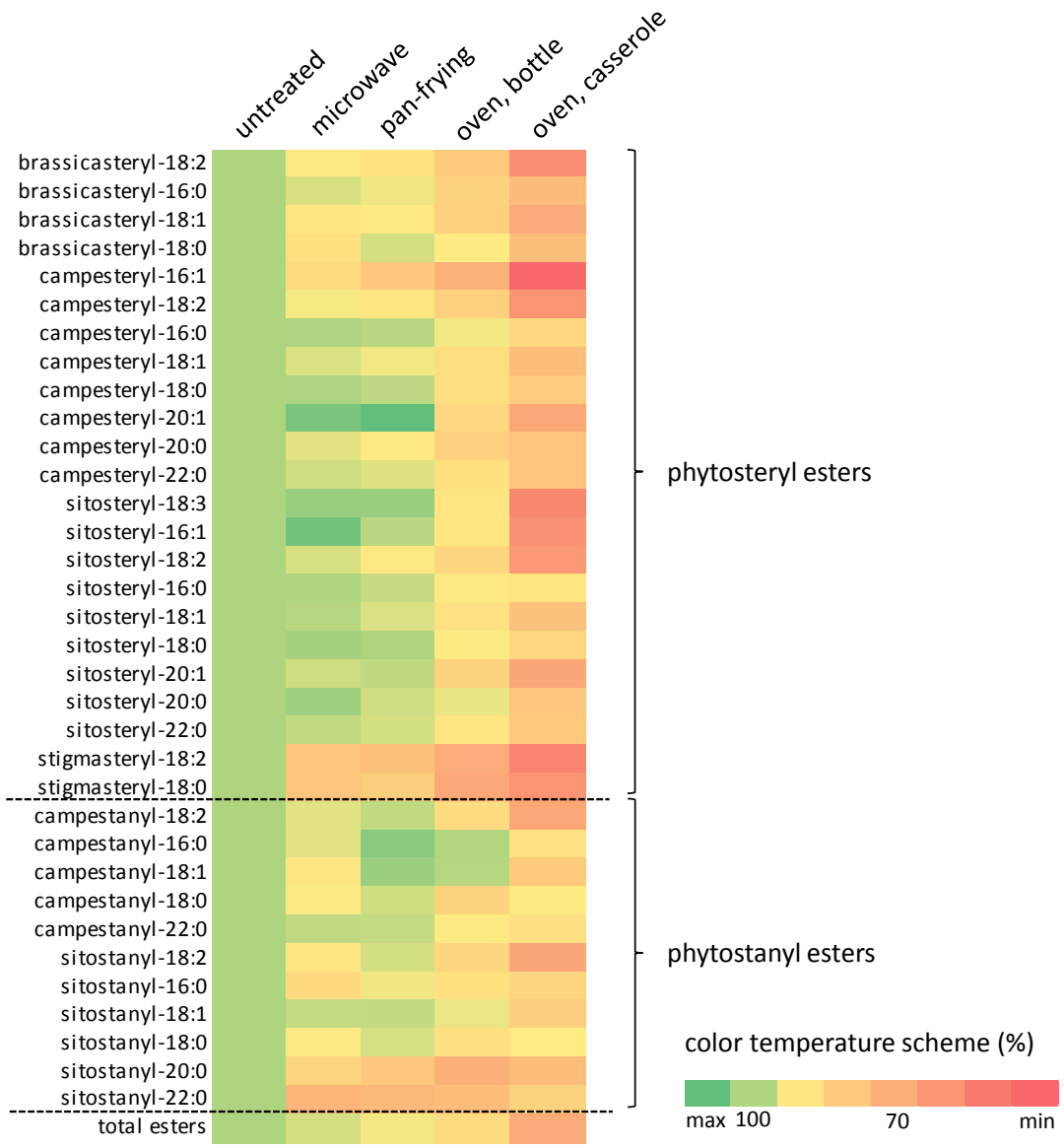

Figure 39. Heatmap of phytosteryl and phytostanyl fatty acid esters in a phytosteryl/-stanyl ester-enriched margarine before and after heat treatments. Analyte levels correspond to the color temperature; the contents at baseline were set to 100 %.

As shown in Figure 40, the extent of percentage decrease was strongly dependent on the degree of saturation of the esterified fatty acids. Regarding the phytosteryl esters, phytosterols esterified to double unsaturated fatty acids were most affected, followed by esters of monounsaturated fatty acids and esters of saturated fatty acids. This pattern was observed for all types of heat treatment. Lehtonen *et al.* (2012) investigated the oxidative susceptibility of differently saturated fatty acid moieties of cholesteryl esters in tripalmitin at the initial stage based on the detection of hydroperoxides. At 100 °C, an increased unsaturation of the acyl moiety was shown to not only

increase the oxidation of the acyl moiety, but also of the cholesterol moiety, probably via intra-molecular promotion of the oxidation reaction. For cholesteryl stearate in turn, only steryl moiety hydroperoxides but no fatty acid moiety hydroperoxides were found. This is in line with another study determining oxidation products of sitosterol and of stearic acid after heating of sitosteryl stearate at 150 °C and subsequent alkaline hydrolysis of the ester bond; no oxidation products of stearic acid could be identified (Yanishlieva-Maslarova *et al.*, 1982).

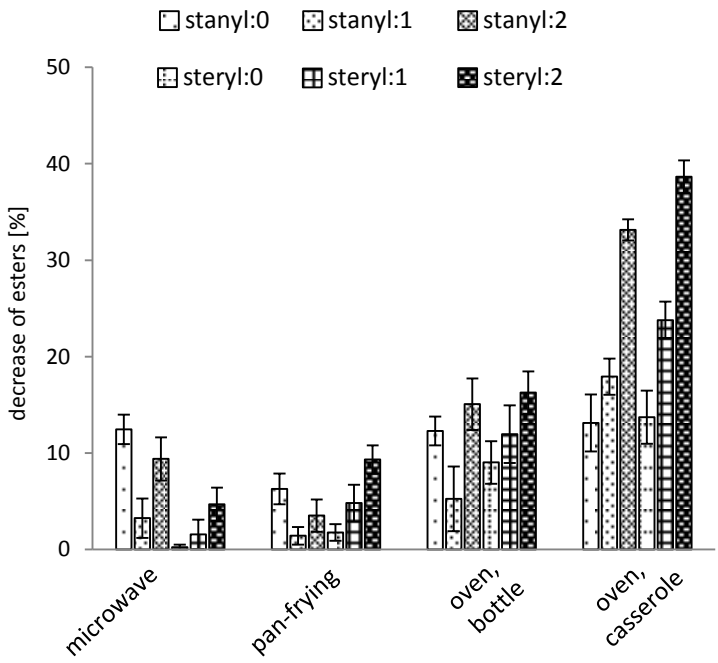

Figure 40. Percentage decreases of individual phytosteryl and phytostanyl fatty acid esters determined in a phytosteryl/-stanyl fatty acid ester-enriched margarine according to the saturation of the fatty acid moiety upon heat treatments

As it can be seen particularly for the margarine heated in the casserole, the degree of unsaturation of the fatty acid impacted the extent of the decrease of the phytostanyl esters in a similar manner, with stanyl esters of double unsaturated fatty acids being most susceptible to degradation. As already mentioned, the percentage decreases of the phytosteryl esters were more pronounced than those of the respective phytostanyl esters and the absolute losses of the phytostanyl esters of saturated fatty acids were small, owing to their already very low initial concentrations in the untreated margarine (Table 29). Nevertheless, it is noteworthy that also phytostanyl esters of saturated fatty acids showed marked percentage losses, thus being in contrast to the general assumption that both, phytostanols and saturated fatty acids are highly stable compounds.

4.4.1.2 Phytostanyl Ester-Enriched Margarine

To study the behavior of phytostanyl fatty acid esters upon heat treatment in more detail, a second margarine enriched with a mixture containing solely phytostanyl fatty acid esters was subjected to the heat treatments. The samples were analyzed based on the previously described approach for mixtures containing solely phytostanyl fatty acid esters. Owing to the use of a more sensitive MS instrumentation as compared to the initial method development, cholesteryl palmitate could be used as internal standard instead of the originally employed cholesterol linolenate, as the peak deriving from a fragment ion exhibiting the same mass as the $[M\text{-}FA+H]^+$ fragment ions of campestanyl esters was now sufficiently resolved. The qualitative and quantitative distributions of the individual esters are shown in Table 30.

Oleic acid esters were the dominating species, followed by esters of linoleic, linolenic, and palmitic acid, whereas C20- and C22-fatty acids were only esterified to a minor extent; this fatty acid pattern indicated rapeseed oil as source of the fatty acids (Codex Alimentarius, 1999). In addition to those phytostanyl fatty acid esters identified using the Dionex MS instrumentation (cf. 4.1.1.3), campestanyl erucic acid ester could be determined with the Agilent mass spectrometer. Sitostanyl esters were quantitatively dominating over campestanyl esters, making up for approximately 86 %. The determined total ester content in the untreated margarine of 138 g/kg, corresponding to approximately 8.5 % phytostanols, was in good accordance with the information on the label.

Table 30. Phytostanyl fatty acid esters in phytostanyl ester-enriched margarine (no. 2) before and after heat treatments.[a]

stanyl fatty acid ester [g/kg]	no treatment	microwave, bottle 800 W, 4 min	pan-frying 205 °C[c], 9 min	oven, bottle 200 °C, 20 min	oven, casserole 200 °C, 20 min
campestanyl-18:3***	1.70 ± 0.04[b] (a)	1.39 ± 0.04 (bc)	1.40 ± 0.07 (bc)	1.51 ± 0.09 (b)	1.26 ± 0.02 (c)
campestanyl-18:2***	4.01 ± 0.08 (a)	3.77 ± 0.14 (b)	3.56 ± 0.04 (b)	3.67 ± 0.02 (b)	3.20 ± 0.04 (c)
campestanyl-16:0**	0.82 ± 0.04 (a)	0.78 ± 0.02 (ab)	0.71 ± 0.04 (b)	0.76 ± 0.02 (ab)	0.74 ± 0.02 (b)
campestanyl-18:1**	11.72 ± 0.26(a)	11.39 ± 0.32 (ab)	10.91 ± 0.23 (bc)	11.11 ± 0.31 (abc)	10.51 ± 0.11 (c)
campestanyl-18:0**	0.32 ± 0.01 (a)	0.30 ± 0.01 (ab)	0.27 ± 0.02 (b)	0.28 ± 0.01 (b)	0.27 ± 0.01 (b)
campestanyl-20:1***	0.28 ± 0.01 (a)	0.25 ± 0.02 (b)	0.21 ± 0.00 (cd)	0.23 ± 0.01 (bc)	0.20 ± 0.01 (d)
campestanyl-20:0***	0.09 ± 0.00 (a)	0.09 ± 0.01 (a)	0.08 ± 0.00 (a)	0.08 ± 0.00 (a)	0.07 ± 0.00 (b)
campestanyl-22:1***	0.09 ± 0.00 (a)	0.08 ± 0.00 (b)	0.07 ± 0.00 (c)	0.07 ± 0.00 (c)	0.07 ± 0.00 (c)
campestanyl-22:0***	0.06 ± 0.00 (a)	0.04 ± 0.00 (bd)	0.04 ± 0.00 (c)	0.05 ± 0.00 (b)	0.04 ± 0.00 (cd)
sitostanyl-18:3***	10.57 ± 0.24 (a)	9.72 ± 0.10 (b)	8.64 ± 0.15 (d)	9.25 ± 0.12 (c)	7.74 ± 0.18 (e)
sitostanyl-16:1**	0.22 ± 0.00 (a)	0.19 ± 0.01 (b)	0.18 ± 0.01 (b)	0.19 ± 0.01 (b)	0.18 ± 001 (b)
sitostanyl-18:2***	25.31 ± 0.27 (a)	23.14 ± 0.97 (b)	21.80 ± 0.18 (b)	22.80 ± 0.71 (b)	19.81 ± 0.23 (c)
sitostanyl-16:0**	5.10 ± 0.18 (a)	5.04 ± 0.03 (ab)	4.67 ± 0.09 (c)	5.06 ± 0.08 (ab)	4.79 ± 0.11 (bc)
sitostanyl-18:1**	73.34 ± 0.87 (a)	70.88 ± 1.78 (ab)	68.24 ± 1.68 (bc)	70.35 ± 2.08 (ab)	64.96 ± 0.67 (c)
sitostanyl-18:0*	1.80 ± 0.05 (a)	1.77 ± 0.03 (ab)	1.72 ± 0.06 (ab)	1.75 ± 0.02 (ab)	1.65 ± 0.06 (b)
sitostanyl-20:1***	1.57 ± 0.04 (a)	1.45 ± 0.05 (b)	1.45 ± 0.02 (b)	1.45 ± 0.02 (b)	1.32 ± 0.03 (c)
sitostanyl-20:0*	0.59 ± 0.03 (a)	0.55 ± 0.02 (ab)	0.53 ± 0.00 (b)	0.55 ± 0.01 (ab)	0.53 ± 0.02 (b)
sitostanyl-22:1***	0.55 ± 0.02 (a)	0.49 ± 0.01 (b)	0.46 ± 0.01 (b)	0.49 ± 0.01 (b)	0.40 ± 0.02 (c)
sitostanyl-22:0*	0.30 ± 0.01 (ab)	0.29 ± 0.01 (ab)	0.29 ± 0.02 (ab)	0.28 ± 0.00 (b)	0.31 ± 0.00 (a)
total	138.44 ± 1.60 (a)	131.61 ± 3.29 (b)	125.24 ± 2.34 (b)	129.93 ± 3.41 (b)	118.03 ± 1.25 (c)

[a] Analysis of variance was carried out with ANOVA. Levels of significance: $p<0.001$, highly significant (***); $p<0.01$, very significant (**); $p<0.05$, significant (*). Within the same row, different letters indicate significant differences (Tukey's HSD) among treatments ($p<0.05$).

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

[c] Final temperature of the heating process.

Similar to the phytosteryl/-stanyl ester-enriched margarine, all heat treatments induced decreases of the total ester contents. As example, the UHPLC-APCI-MS chromatograms of the campestanyl and sitostanyl fatty acid esters as determined in the margarine after oven-heating in the casserole are shown in Figure 41.

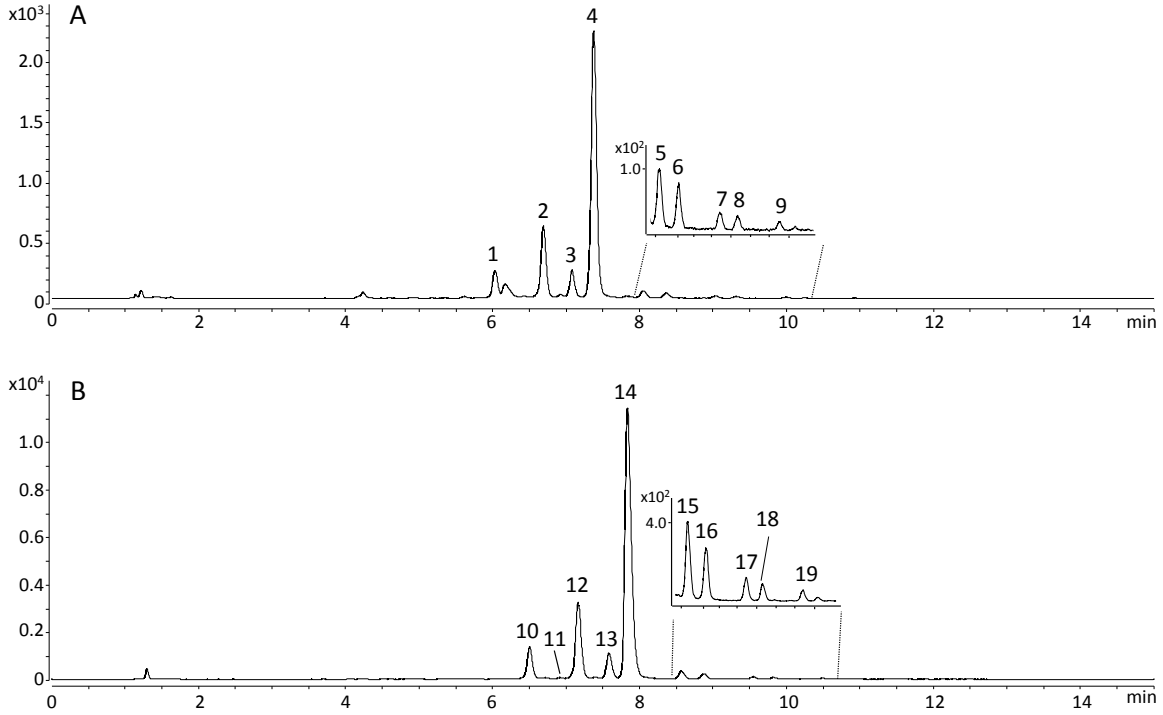

Figure 41. UHPLC-APCI-MS analysis of phytostanyl fatty acid esters in a phytostanyl ester-enriched margarine heated at 200 °C for 20 min in a casserole in an oven. SIM chromatogram of campestanyl fatty acid esters at *m/z* 385.2 (**A**): (1) campestanyl-18:3, (2) campestanyl-18:2, (3) campestanyl-16:0, (4) campestanyl-18:1, (5) campestanyl-18:0, (6) campestanyl-20:1, (7) campestanyl-20:0, (8) campestanyl-22:1, (9) campestanyl-22:0. SIM chromatogram of sitostanyl fatty acid esters at *m/z* 399.2 (**B**): (10) sitostanyl-18:3, (11) sitostanyl-16:1, (12) sitostanyl-18:2, (13) sitostanyl-16:0, (14) sitostanyl-18:1, (15) sitostanyl-18:0, (16) sitostanyl-20:1, (17) sitostanyl-20:0, (18) sitostanyl-22:1, (19) sitostanyl-22:0. For conditions, cf. 3.2.3.2.

Microwave heating showed the least impact on ester degradation, with 95 % of the esters still being detectable, thus resembling the margarine enriched with a mixture of phytosteryl and phytostanyl fatty acid esters. In contrast to the phytosteryl/-stanyl ester-enriched margarine, the ester degradations in the phytostanyl ester-enriched margarine were more pronounced upon electric heating (90 % remaining intact esters) than upon oven heating in a bottle (94 % intact esters). Soupas *et al.* (2007) determined the effect of pan-frying on losses of sitostanyl moieties in phytostanyl ester-enriched rapeseed oil. Pan-frying at 180 °C and at 200 °C for 5 min did not lead to significant decreases of sitostanol. After frying at 180 °C for 10 min, a minor reduction of sitostanol of approximately 3 % was measured. The more pronounced decreases of phytostanyl fatty acid esters in the present pan-frying experiment compared to the study by Soupas *et al*.

(2007) probably mainly reflect the additional oxidative degradations of the fatty acid moieties. The heating of the greased margarine in the casserole in the oven led to the greatest decreases of phytostanyl esters with 85 % of the initial ester content remaining. In comparison to the ester contents determined in the margarine enriched with the mixture of phytosteryl and phytostanyl fatty acid esters after the same experiment, the observed losses were, however, much lower. Apparently, the differences in susceptibility to oxidation reactions between the phytosteryl and the phytostanyl moieties are particularly pronounced under drastic heating conditions, maybe due to an increased intra-molecular propagation of oxidation. This might be further supported by an overall higher oxidative reactivity of the fatty acid moieties of the phytosteryl/-stanyl ester-enriched margarine owing to a lower proportion of esters of saturated and monounsaturated fatty acids of approximately 38 % compared to 70 % in the phytostanyl ester-enriched margarine.

On an individual basis, all esters were shown to decrease upon heat treatments, particularly upon oven-heating in the casserole, as illustrated in Figure 42. Only for sitostanyl-22:0, no loss was observed after this experiment. Campestanyl esters of C20- and C22-fatty acids seemed to decrease most; however, as these are the least concentrated compounds in the mixture, this effect might be due to an overestimation of the percentage changes because of the low initial concentrations.

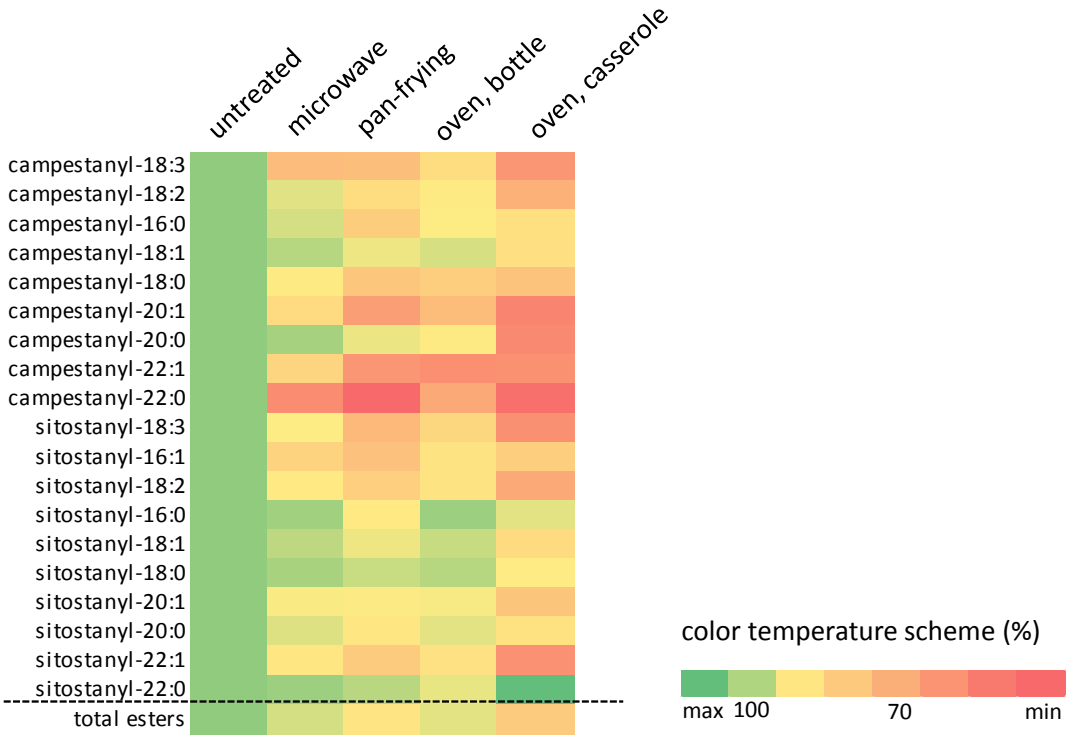

Figure 42. Heatmap of phytostanyl fatty acid esters in a phytostanyl ester-enriched margarine before and after heat treatments. Analyte levels correspond to the color temperature; the contents at baseline were set to 100 %.

Sitostanyl and campestanyl esters of saturated and monounsaturated fatty acids, and of fatty acids with two and three double bonds were present in the margarine. The respective percentage decreases according to the saturation of the fatty acid moiety are illustrated in Figure 43.

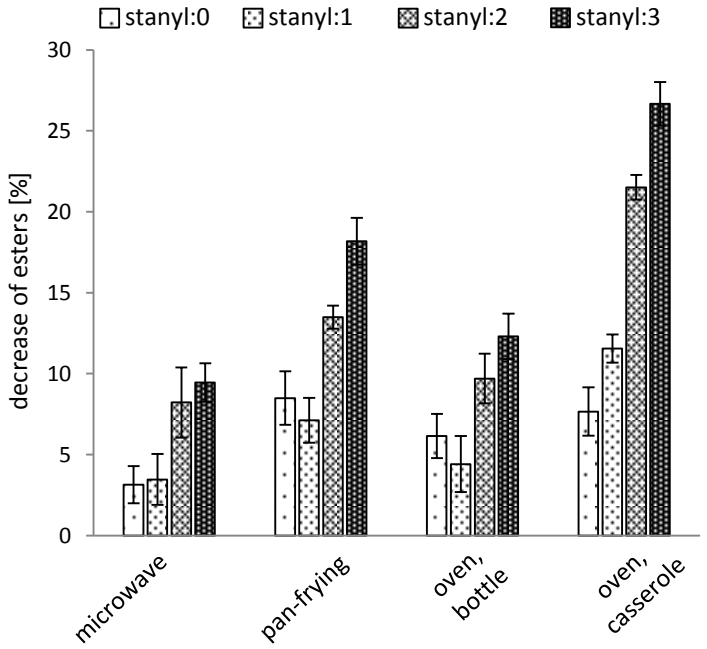

Figure 43. Percentage decreases of individual phytostanyl fatty acid esters determined in a phytostanyl fatty acid ester-enriched margarine according to the saturation of the fatty acid moiety upon heat treatments

Similar to the pattern observed for the margarine enriched with a mixture of phytosteryl and phytostanyl fatty acid esters, the extent of degradation was obviously influenced by the degree of unsaturation of the esterified fatty acid. Phytostanyl esters of linolenic acid were most susceptible to degradation, followed by esters of linoleic acid. The stanyl esters of saturated fatty acids showed a marked percentage decrease, reflecting the observations made for the steryl/stanyl ester-enriched margarine. This decrease is in contrast to the general notion of the stability of saturated compounds. However, it has been described in literature that saturated fatty acids and hydrocarbons might absorb oxygen at 100 °C and higher temperatures, undergoing autoxidation reactions as oxygenated compounds accumulate (Swern *et al.*, 1948).

4.4.2 Formation of Phytosterol and Phytostanol Oxidation Products

4.4.2.1 Phytosteryl/-stanyl Ester-Enriched Margarine

In Table 31, the contents of the individual phytosterol/-stanol oxidation products determined in the margarine enriched with the mixture of phytosteryl and phytostanyl fatty acid esters before and after the different heat treatments are presented.

In the untreated margarine, approximately 93 mg phytosterol oxidation products per kg margarine were quantitated, corresponding to an oxidation rate of 0.12 %. This content was in the median range of contents determined for other margarines, both in the present study and in the literature (cf. 4.3).

The chromatogram of the phytosterol and phytostanol oxidation products determined via on-line LC-GC after heating of the margarine in the casserole is shown in Figure 44.

The different heating procedures led to differently pronounced formations of phytosterol and phytostanol oxidation products, the order of the formed amounts reflecting the observed order of decreases of the amounts of the corresponding intact esters. Once again, the influence of heating time and temperature was apparent. Among the heat treatments, microwave heating resulted in the least amounts of phytosterol oxidation products, yielding 780 mg POP/kg with an oxidation rate of 1 %. Interestingly, in a study investigating the effects of microwave heating on a phytosteryl ester-enriched milk (Menéndez-Carreño *et al.*, 2008a), a much lower oxidation rate of only 0.1 % was determined after 2 minutes of microwave heating at 900 W, along, however, with a remarkable decrease of intact sterol/stanol moieties of 66 %. This is considerably contrasting the above-mentioned reduction of intact esters of 4 % found in the present study for the margarine. These contradictory trends in changes regarding both, phytosterol/-stanol oxidation and degradation might be due to an advanced formation of thermal degradation products of phytosterols/-stanols other than secondary oxidation products, such as transformation of the secondary oxidation products to steradienes and -trienes and/or the formation of tertiary oxidation products in the milk, leading to a reduced rate of secondary oxidation. Moreover, the influence of heating parameters such as time and power on a given food varies between different matrices; for example, different oxidation rates have been described for oil-based matrices and oil-in-water emulsions (Cercaci *et al.*, 2007).

Table 31. Phytosterol and phytostanol oxidation products determined in a phytosteryl/-stanyl ester-enriched margarine (no. 8) before and after heat treatments.[a]

phytosterol oxidation product [mg/kg]	no treatment	microwave, bottle 800 W, 4 min	pan-frying 200 °C[e], 9 min	oven, bottle 200 °C, 20 min	oven, casserole 200 °C, 20 min
5,6*ß*-epoxycampesterol***	4.4 ± 0.1[c] (a)	31.0 ± 1.5 (b)	43.8 ± 0.8 (c)	140.0 ± 1.9 (d)	227.1 ± 6.1 (e)
5,6*ß*-epoxysitosterol***	14.8 ± 0.7 (a)	152.4 ± 5.3 (b)	213.6 ± 9.1 (c)	641.0 ± 11.5 (d)	1030.6 ± 11.5 (e)
5,6*α*-epoxycampesterol***	3.8 ± 0.3 (a)	15.9 ± 0.2 (b)	20.6 ± 0.3 (c)	59.9 ± 1.7 (d)	115.5 ± 2.7 (e)
7*α*-hydroxycampesterol***	2.9 ± 0.3 (a)	26.7 ± 1.0 (b)	42.8 ± 1.7 (c)	97.0 ± 4.5 (d)	207.0 ± 8.1 (e)
5,6*α*-epoxysitosterol***	18.7 ± 1.8 (a)	69.6 ± 3.1 (b)	100.8 ± 5.0 (c)	289.2 ± 15.8 (d)	508.6 ± 4.8 (e)
7*α*-hydroxysitosterol***	3.1 ± 0.2 (a)	90.0 ± 2.5 (b)	155.8 ± 3.6 (c)	338.0 ± 3.5 (d)	704.5 ± 22.5 (e)
7*ß*-hydroxycampesterol***	1.4 ± 0.0 (a)	23.0 ± 1.1 (b)	33.0 ± 1.7 (c)	89.7 ± 4.1 (d)	171.9 ± 3.4 (e)
6*α*-hydroxysitostanol***	-[d]	24.8 ± 2.8 (a)	49.6 ± 3.5 (b)	44.3 ± 3.6 (b)	77.7 ± 2.3 (c)
7*ß*-hydroxysitosterol***	4.7 ± 0.4 (a)	103.5 ± 3.8 (b)	167.6 ± 6.3 (c)	354.6 ± 8.5 (d)	673.5 ± 15.4 (e)
7-ketocampesterol***	10.2 ± 1.0 (a)	40.4 ± 0.8 (b)	46.4 ± 1.2 (b)	157.9 ± 3.3 (c)	257.1 ± 8.9 (d)
7-ketositosterol***	28.9 ± 0.7 (a)	203.0 ± 2.8 (b)	204.9 ± 5.4 (b)	691.0 ± 13.0 (c)	1135.9 ± 40.5 (d)
total***	92.9 ± 2.2 (a)	780.2 ± 13.6 (b)	1078.8 ± 25.2 (c)	2902.6 ± 33.5 (d)	5109.3 ± 28.0 (e)
oxidation rate[b] [%]	0.12 ± 0.0	1.04 ± 0.0	1.44 ± 0.0	3.87 ± 0.0	6.81 ± 0.0

[a] Analysis of variance was carried out with ANOVA. Levels of significance: $p<0.001$, highly significant (***); $p<0.01$, very significant (**); $p<0.05$, significant (*). Within the same row, different letters indicate significant differences (Tukey's HSD) among treatments ($p<0.05$).

[b] Calculated as percentage of phytosterol/phytostanol oxidation products with respect to the initial phytosterol/phytostanol content.

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

[d] Content below limit of quantification (i.e. <0.33 µg/mL of injection volume).

[e] Final temperature of the heating process.

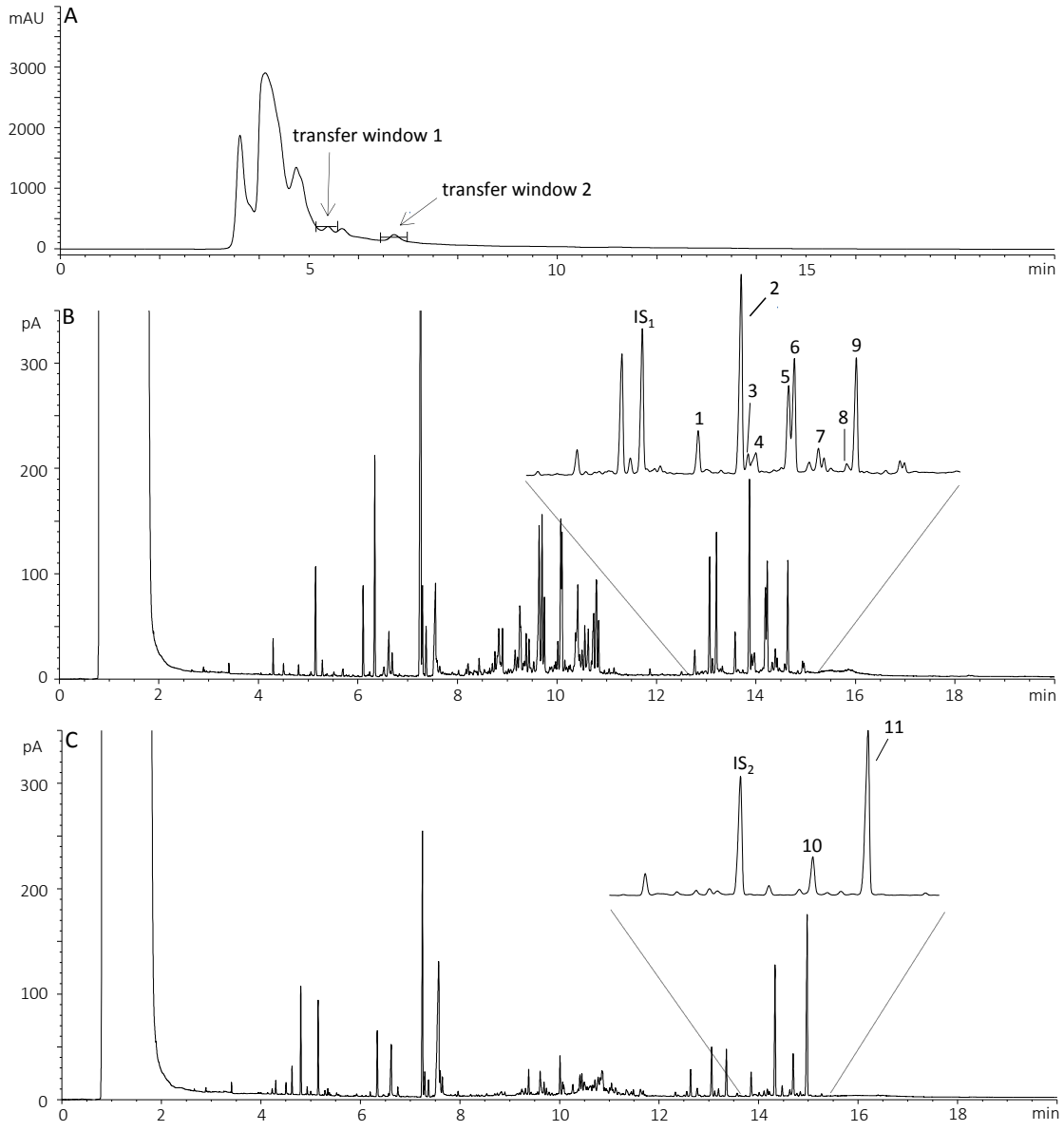

Figure 44. On-line LC-GC/FID analysis of acetylated phytosterol and phytostanol oxidation products in a phytosteryl/-stanyl ester-enriched margarine heated at 200 °C for 20 min in a casserole in an oven. LC-chromatogram at 206 nm (**A**); GC/FID chromatogram of the transferred LC-window 1 (**B**): (1) 5,6*ß*-epoxycampesterol, (2) 5,6*ß*-epoxysitosterol, (3) 5,6*α*-epoxycampesterol, (4) 7*α*-hydroxycampesterol, (5) 5,6*α*-epoxysitosterol, (6) 7*α*-hydroxysitosterol, (7) 7*ß*-hydroxycampesterol, (8) 6*α*-hydroxysitostanol, (9) 7*ß*-hydroxysitosterol, and (IS_1) 5,6*ß*-epoxycholesterol; GC/FID chromatogram of the transferred LC-window 2 (**C**): (10) 7-ketocampesterol, (11) 7-ketositosterol, and (IS_2) 7-ketocholesterol. For conditions, cf. 3.2.3.3.

However, the overall low impact of microwave heating on phytosterol oxidation compared to other heating procedures noticed in the present study is in line with the observations from a recent study, where foods were prepared by several different typical household baking and cooking methods using an enriched margarine; similarly, among all cooking methods, upon microwave heating the least amounts of phytosterol oxidation products were detected (Lin *et al.*, 2016).

Pan-frying of the margarine led to the second least amounts of phytosterol oxidation products (1080 mg/kg, 1.44 % oxidation rate). The oxidation rate is in the same order of magnitude as those determined for sitosterol in a phytosteryl ester-enriched rapeseed oil (1.8 % sitosterol oxidation) and in a phytosteryl ester-enriched liquid margarine (1.3 % sitosterol oxidation) after pan-frying for 10 min at 180 °C (Soupas *et al.*, 2007).

Oven-heating led to the greatest formation of phytosterol oxidation products. Placing the margarine in a bottle resulted in a formation of 2900 mg POP/kg and a corresponding oxidation rate of 3.87 %. These results are different from those described in section 4.2.4, where heating an enriched margarine from the same manufacturer with, however, a fat content of 40 % at 180 °C for 30 min in a similar bottle and analysis via the same analytical method revealed an oxidation rate of only 1.1 % and a formation of total POPs of 809 mg/kg. In a further study, the same margarine as used in the present study was heated in open glassware in an oven at 200 °C for 30 min and oxidized derivatives of sitosteryl oleate were determined via LC-ESI-MS^2; the total amount of 3800 mg/kg oxidized sitosteryl oleate and a resulting oxidation rate of 3 % referring to the total initial ester content are in a similar order of magnitude as in the present study (Julien-David *et al.*, 2014).

Finally, oven-heating in a casserole further increased the formation of secondary oxidation products, as 5110 mg/kg, equivalent to an oxidation rate of 6.81 %, were quantitated. As previously observed for the intact esters, the greater surface in contact to oxygen apparently intensified the oxidation reactions.

In Figure 45, the profiles of the individual phytosterol/-stanol oxidation products determined before and after the different heating experiments are shown. In the untreated margarine, 7-ketophytosterols were the most prominent compounds, while the proportion of 7-hydroxysterols was minor.

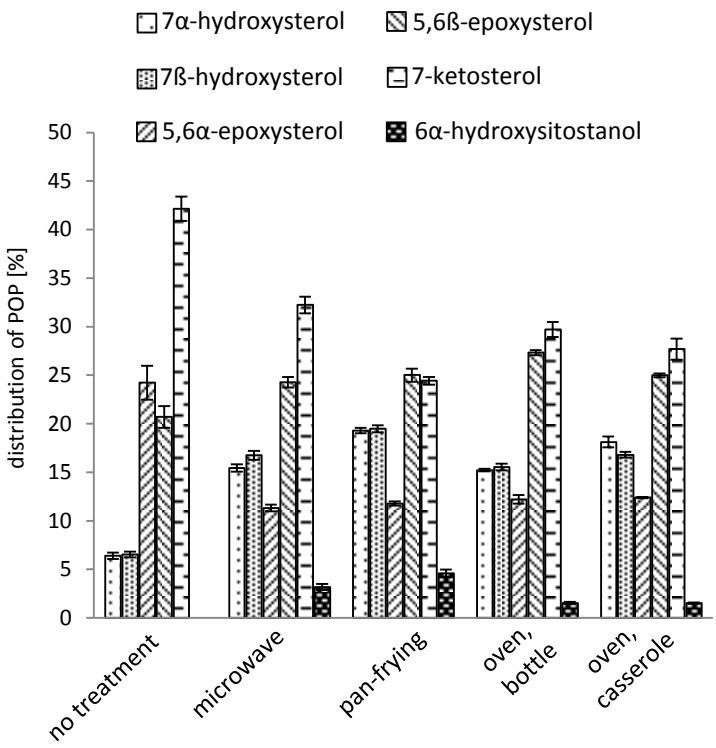

Figure 45. Percentage distributions of individual derivatives of phytosterol and -stanol oxidation products determined in a phytosteryl/-stanyl fatty acid ester-enriched margarine before and after heat treatments.

Upon heating, in addition to the 7-keto-, 7-hydroxy-, and 5,6-epoxyphytosterols, 6*α*-hydroxysitostanol as oxidation product of the sitostanyl esters present in the enriched margarine could be detected. Further, the proportion of 7-ketosterols decreased while a rising contribution of the 7-hydroxysterols was observed. In addition, the ratio of the 5,6-epoxysterol epimers changed, as in the untreated margarine the *α*-epimer was slightly dominating, whereas heating induced a clear dominance of the *ß*-epoxysterols. Soupas *et al.* (2007) also observed a decreasing proportion of 7-ketositosterol along with a relative increase of 7*ß*-hydroxysitosterol and 5,6*ß*-epoxysitosterol upon prolongation of the heating time of a phytosteryl ester-enriched butter oil from 5 min to 10 min. When phytosteryl ester-enriched tripalmitin (1 %) was heated for 30 min at 180 °C in a glass vial, the proportion of 7-ketositosterol once again was shown to decrease compared to the untreated sample; however, the percentage of 7-hydroxysitosterols also declined (Soupas *et al.*, 2005). Both, varying analytical methods and differing sample characteristics such as the degree of enrichment, the fat content or transport and storage conditions are probably contributing to these differences between studies.

4.4.2.2 Phytostanyl Ester-Enriched Margarine

The individual and total contents of the determined oxidation products are compiled in Table 32. LC-APCI-MS-based screening revealed minor amounts of esterified phytosterols in the enriched margarine, probably owing to an incomplete hydrogenation of the sterols used for preparation of the phytostanyl ester mixtures. This is the reason for the detection of several oxidation products of campesterol and mainly sitosterol in the samples besides the expected sitostanol oxidation products.

The on-line LC-GC chromatogram of the phytostanyl ester-enriched margarine heated in the casserole in the oven is shown in Figure 46.

The untreated margarine exhibited rather low POP contents. Except for 6*α*-hydroxysitostanol (20.6 mg/kg) and a minor amount of 7-ketositosterol, the concentrations of the other POP were below the limits of detection and the limits of quantitation, respectively.

Upon heating, the amounts of oxidation products increased; the order of the formed amounts correlated with the order of the determined decreases of the intact phytostanyl fatty acid esters. Microwave heating led only to slightly elevated oxide levels and a rate of phytostanol oxidation of 0.04 %. Pan-frying led to a total POP formation of 137 mg/kg; in addition to 6*α*-hydroxysitostanol, also 7-hydroxysitostanol was detected as phytostanol oxidation product, both oxides making up for approximately 60 mg/kg which corresponds to a phytostanol oxidation rate of 0.08 %. Further, several 5,6-epoxy-, 7-hydroxy- and 7-ketophytosterols were formed. Soupas *et al.* (2007) also determined for both, pan-frying of a phytostanyl ester-enriched rapeseed oil and a phytostanyl ester-enriched liquid margarine sitostanol oxidation rates of 0.1 % after pan-frying at 180 °C for 5 and 10 min, respectively. The contents of sitostanol oxidation products in the liquid margarine, enriched at a similar degree as the margarine used in the present study, were 48.5 mg/kg and 62.9 mg/kg after 5 min and 10 min of pan-frying, respectively, which is in good agreement with the contents found in the present study.

Oven heating in a bottle led to slightly lower amounts of POP compared to pan-frying, while heating in the casserole resulted in the greatest amounts of both, phytostanol and phytosterol oxidation products, accounting for 225 mg POP/kg in total; this amount is, however, 22-fold lower than the total amount of phytosterol and –stanol oxidation products quantitated in the phytosteryl/-stanyl ester-enriched margarine after oven heating in the casserole, underlining the much lower susceptibility of the phytostanol moieties to the formation of secondary oxidation products.

Table 32. Phytosterol and phytostanol oxidation products in phytostanyl ester-enriched margarine (no. 2) before and after heat treatments.[a]

phytosterol oxidation product [mg/kg]	no treatment	microwave, bottle 800 W, 4 min	pan-frying 205 °C[f], 9 min	oven, bottle 200 °C, 20 min	oven, casserole 200 °C, 20 min
6*α*-hydroxysitostanol***	20.6 ± 1.9[c] (a)	30.3 ± 0.8 (b)	51.5 ± 0.5 (d)	44.4 ± 0.7 (c)	69.6 ± 2.5 (e)
7-hydroxysitostanol***	-[d]	-[d]	8.6 ± 0.5 (a)	8.1 ± 0.4 (a)	17.0 ± 0.9 (b)
5,6*ß*-epoxycampesterol***	-[d]	-[d]	8.5 ± 0.5 (b)	7.0 ± 0.0 (a)	15.9 ± 0.4 (c)
5,6*ß*-epoxysitosterol***	-[e]	4.3 ± 0.5 (a)	17.1 ± 0.4 (c)	10.9 ± 0.8 (b)	27.4 ± 1.1 (d)
5,6*α*-epoxysitosterol***	-[d]	5.0 ± 0.4 (a)	15.2 ± 0.3 (c)	10.0 ± 0.6 (b)	27.6 ± 2.5 (d)
7*α*-hydroxysitosterol***	-[d]	-[e]	12.0 ± 0.9 (b)	9.9 ± 0.5 (a)	18.5 ± 0.6 (c)
7*ß*-hydroxysitosterol***	-[d]	-[d]	7.4 ± 0.4 (b)	3.4 ± 0.1 (a)	11.1 ± 0.5 (c)
7-ketocampesterol***	-[d]	-[d]	5.9 ± 0.7 (a)	4.4 ± 0.1 (a)	14.1 ± 1.5 (b)
7-ketositosterol***	1.9 ± 0.3 (a)	3.8 ± 0.2 (b)	10.7 ± 0.9 (d)	6.5 ± 0.1 (c)	24.5 ± 0.6 (e)
total***	22.6 ± 1.5 (a)	43.4 ± 1.5 (b)	137.0 ± 2.1 (d)	104.6 ± 2.1 (c)	225.5 ± 5.5 (e)
oxidation rate[b] [%]	0.03 ± 0.0	0.04 ± 0.0	0.08 ± 0.0	0.07 ± 0.0	0.11 ± 0.0

[a] Analysis of variance was carried out with ANOVA. Levels of significance: $p<0.001$, highly significant (***); $p<0.01$, very significant (**); $p<0.05$, significant (*). Within the same row, different letters indicate significant differences (Tukey's HSD) among treatments ($p<0.05$).

[b] Calculated as percentage of phytostanol oxidation products related to the initial phytostanol content.

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

[d] Content below limit of detection (i.e. <0.11 µg/mL of injection volume for 7-hydroxyphytosterols and <0.08 µg/mL of injection volume for 5,6-epoxyphytosterols).

[e] Content below limit of quantitation (i.e. <0.33 µg/mL of injection volume for 7-hydroxyphytosterols and 0.24 µg/mL of injection volume for 5,6-epoxyphytosterols).

[f] Final temperature of heating process.

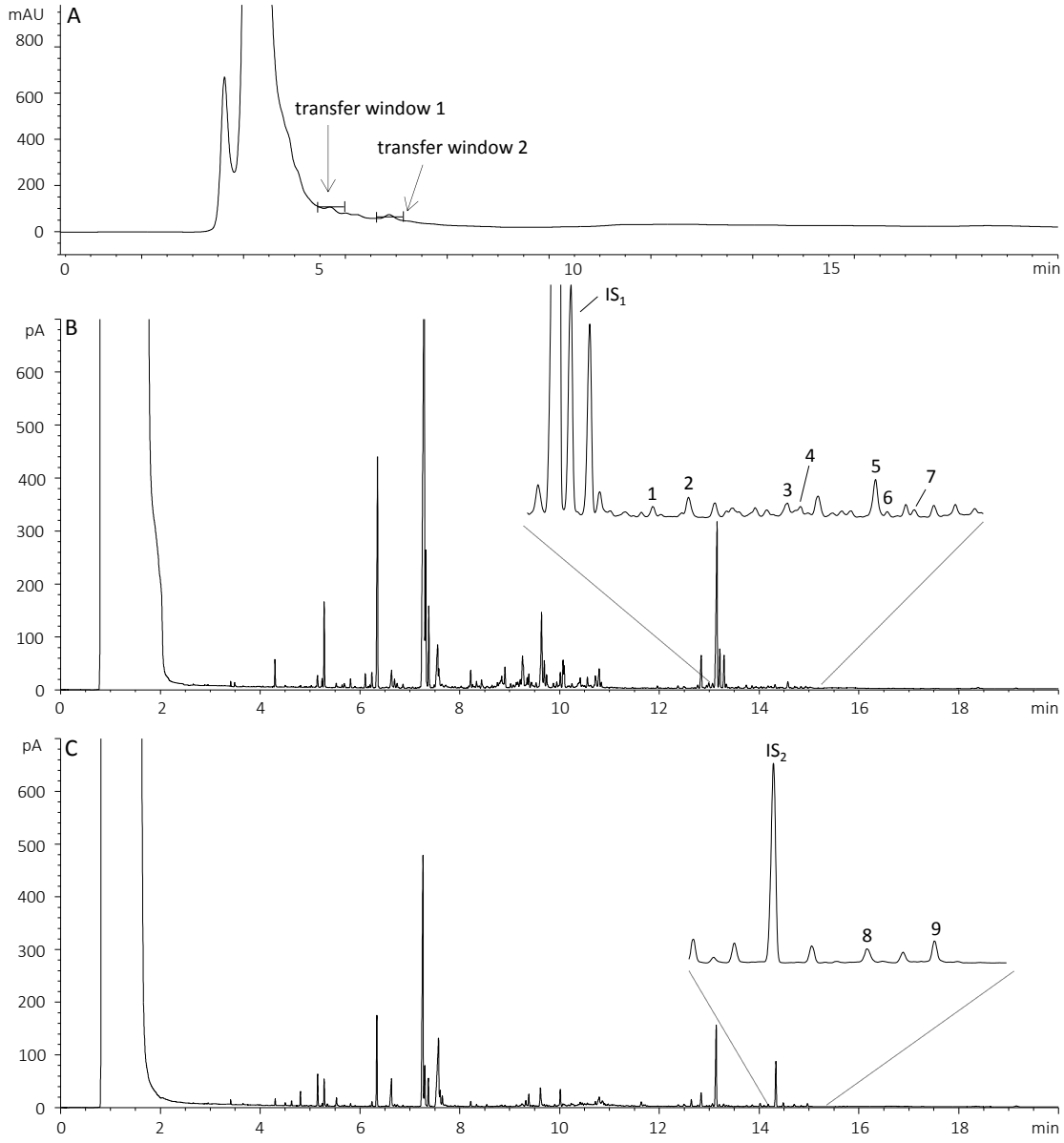

Figure 46. On-line LC-GC/FID analysis of acetylated phytosterol and phytostanol oxidation products in a phytostanyl ester-enriched margarine heated at 200 °C for 20 min in a casserole in an oven. LC-chromatogram at 206 nm (**A**); GC/FID chromatogram of the transferred LC-window 1 (**B**): (1) 5,6*ß*-epoxycampesterol, (2) 5,6*ß*-epoxysitosterol, (3) 5,6*α*-epoxysitosterol, (4) 7*α*-hydroxysitosterol, (5) 6*α*-hydroxysitostanol, (6) 7*ß*-hydroxysitosterol, (7) 7-hydroxysitostanol, and (IS_1) 5,6*ß*-epoxycholesterol; GC/FID chromatogram of the transferred LC-window 2 (**C**): (8) 7-ketocampesterol, (9) 7-ketositosterol, and (IS_2) 7-ketocholesterol. For conditions, cf. 3.2.3.3.

As illustrated in Figure 47, differently changing proportions of phytostanol and phytosterol oxidation products could be observed in the phytostanyl ester-enriched margarine.

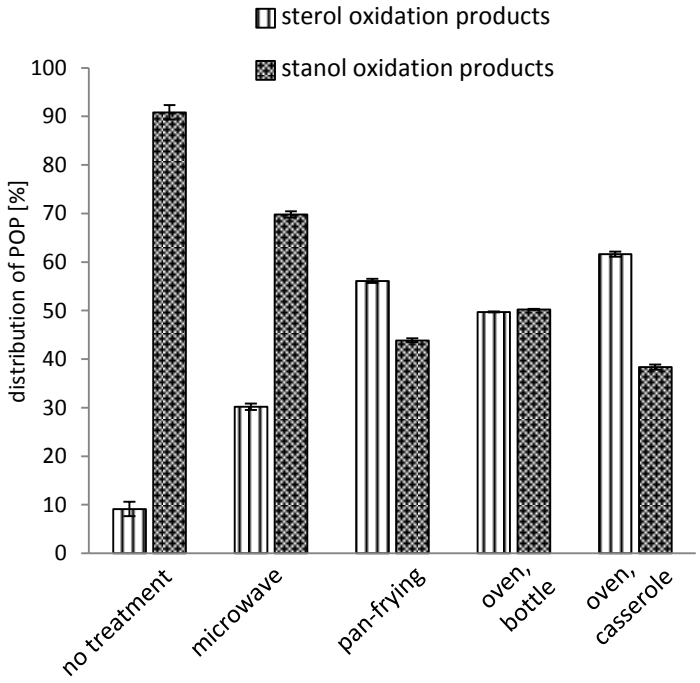

Figure 47. Percentage distributions of phytosterol and -stanol oxidation products determined in a phytostanyl fatty acid ester-enriched margarine before and after heat treatments.

Even though the absolute amounts of the phytostanol oxidation products increased upon heating, their proportions in comparison to the additionally formed phytosterol oxidation products decreased. The stronger the impact of a heating procedure on the overall formation of secondary oxidation products, the more pronounced was the relative decrease of the phytostanol oxidation products; after pan-frying and heating in the casserole, the proportion of phytosterol oxidation products was even higher than that of the phytostanol oxidation products, which is particularly interesting as initially only trace amounts of phytosteryl fatty acid esters could be detected in the margarine. This significantly slower rate of increase of the phytostanol oxidation products is further emphasizing the stability of phytostanols as far as secondary oxidation reactions are concerned.

4.4.3 Calculation of Mass Balances

Previous studies demonstrated contradictions between the oxidation rates calculated on the basis of the formed POP and the actual differences in the amounts of non-oxidized esterified sterols/stanols before and after heat treatment (Soupas *et al.*, 2005; Menéndez-Carreño *et al.*, 2008a). The contents of esterified phytosterols/-stanols were determined after alkaline hydrolysis of the phytosteryl- and phytostanyl fatty acid esters. The LC/MS approaches used in this study allow for the first time to calculate the gap between the losses of phytosteryl/-stanyl esters and the formation of POP by directly determining the decreases of intact esters. In order to assess how much of the losses of the intact phytosteryl and/or phytostanyl fatty acid esters can be explained by the corresponding formation of the secondary phytosterol/-stanol oxidation products, the respective data were compared on a molar basis (Figure 48).

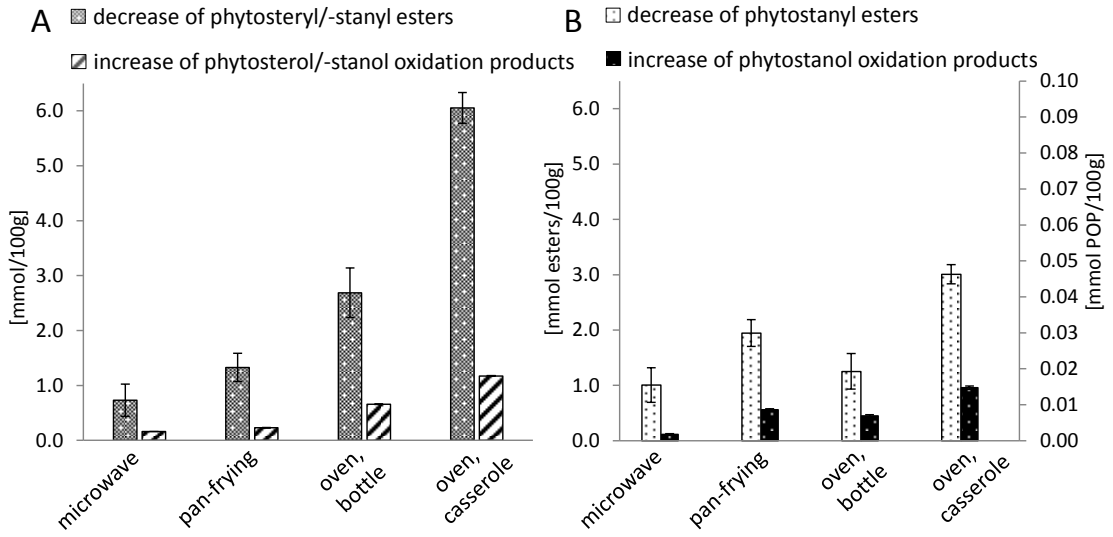

Figure 48. Comparison of decreases of intact phytosteryl and/or phytostanyl fatty acid esters and of increases of phytosterol and/or phytostanol oxidation products in a phytosteryl/-stanyl fatty acid ester-enriched margarine (**A**) and a phytostanyl fatty acid ester-enriched margarine (**B**).

In the margarine enriched with the mixture of phytosteryl and phytostanyl fatty acid esters (Figure 48 A), the observed decreases of intact esters exceeded by far the determined increases of the secondary oxidation products of the phytosterol and -stanol moieties. Approximately 20 % (ranging from 17 % after electric heating to 24 % after oven heating in the bottle) of the ester losses could be explained by the formation of secondary sterol oxidation products. This remaining gap of on average 80 % unexplained losses of the intact esters may partly be due to the formation of further thermal degradation products of phytosterols/-stanols other than the polar secondary oxidation products determined in the present study. However, studies additionally determining

thermal sterol degradation and polymerization products still detected pronounced gaps relating to losses observed for free sterols, ranging from 31 % up to 74 % that could not be explained (Menéndez-Carreño *et al.*, 2010; Derewiaka and Molińska, 2015).

For the phytostanyl ester-enriched margarine, in all heating experiments less than 1 % of the degraded phytostanyl fatty acid esters could be explained via the formation of the corresponding oxidation products, leaving more than 99 % of the losses unaccounted for (Figure 48 B). Despite the common assumption of the particularly high stability of the saturated stanol structure, further degradation of the stanol moiety leading to other products besides the secondary oxidation products cannot be excluded; for example, in a study by Soupas *et al*. (2005) 41 % and 11 % of lost free and esterified sitostanol, respectively, after 6 h of heating at 180 °C could not be explained by the analyzed secondary oxidation products.

A substantial proportion of the gaps observed in the present study is probably to be ascribed to oxidative modifications of the fatty acid moieties. Both, the comparatively low overall contributions of the secondary oxidation products and the observed dependencies of the degradation of the individual esters on the saturation degree of the fatty acid moiety indicate a significant contribution of oxidation reactions of the esterified fatty acids to the observed losses of intact phytosteryl and phytostanyl fatty acid esters.

The observed decreases of intact phytosteryl/-stanyl fatty acid esters in combination with the only minor contributions of formed POP may shed new light on the impact of oxidation reactions on heat-treated enriched foods. Oxidation reactions of sterols and their fatty acid esters are usually raising concerns because of the formation of secondary sterol oxidation products. An inherent health-damaging potential is being discussed for both, dietary consumed cholesterol and phytosterol oxidation products as well as for endogenously formed oxidation products of cholesterol in mammals and of phytosterols in plants (Scholz *et al.*, 2015; Dean and Boyd, 2004). However, as suggested by Dean and Boyd (2004), the adverse potential of physiologically formed sterol oxidation products may not be due to their presence *per se*, but rather to the inevitably related absence of the intact sterols, and thus the absence of certain physiological functionalities. Maybe, this conception should be taken into additional consideration for the assessment of oxidation reactions of phytosteryl and phytostanyl esters in enriched foods. As already mentioned (cf. 2.3.5.6), a previous *in vitro* study by Julien-David *et al.* (2008) showed at the example of sitosteryl oleate, an oxidative modification of the fatty acid moiety leading to sitosteryl-9,10-dihydroxystearate resulted in an almost complete loss of the hydrolytic activity of the cholesterol esterase; in addition, in the presence of sitosteryl-9,10-dihydroxystearate, the hydrolysis of

sitosteryl oleate was significantly reduced. Therefore, as the enzymatic intestinal cleavage of the ester bonds is being recognized as an essential precondition for the eventual exertion of the cholesterol-lowering properties of phytosterols, an absence of the intact esters due to oxidative degradations might be of relevance in the context of quality of enriched foods. Yet, the actual physiological significance of the observed quantitative losses of esters remains to be elucidated.

The obtained results contribute to an extension of the data basis on the contents of secondary phytosterol and phytostanol oxidation products that may be expected. However, they also illustrate the necessity of extending the knowledge on the fate of intact esters upon common household procedures. This may include the generation of further quantitative data on the decreases of intact esters in the course of the preparation of true meals (i.e. including foods), and an advancement of first existing analytical approaches allowing the characterization of oxidized phytosteryl and phytostanyl fatty acid esters formed from the intact esters (Julien-David et al., 2014).

4.5 Estimation of Intakes of Phytosteryl/-stanyl Esters and Phytosterol Oxidation Products

In the course of the risk assessment of novel foods, the estimation of the dietary exposure plays an important role. For foods enriched with phytosteryl/-stanyl fatty acid esters, the intake assessment of both, the intact phytosteryl and phytostanyl esters and the secondary phytosterol oxidation products is crucial.

Phytosterols/-stanols and/or their fatty acid esters are the functionality-bearing ingredients needed to achieve the beneficial effect of lowering LDL-cholesterol levels. However, safety concerns are being raised regarding exceptionally high consumptions of phytosterols, potentially resulting in an accumulation and exertion of pro-atherogenic effects (cf. 2.2.2). Therefore, for the intact esters, it should be ensured that a consumption of 3 g phytosterols/phytostanols per day is not exceeded, and specific consumer information regarding number and size of portions to be consumed of the respective foods has to be provided on the label. On the other hand, as observed in the present study for the heated margarines the ester contents may significantly decrease even under household-type conditions of treatment (cf. 4.4).

In addition, the decrease of the intact esters is accompanied by the concurrent formation of phytosterol oxidation products. A pro-inflammatory and pro-atherogenic potential of POP is being discussed (cf. 2.3.5). In addition, a loss of the anti-atherogenic properties of phytosterols upon oxidation is being considered (cf. 2.3.5). The calculated increases in POP intakes might enhance the risk for adverse health effects and at the same time result in a loss of the anti-atherogenic properties described for the intact phytosterols/-stanols and their esters (cf. 2.3.5).

As a result of these complementary effects, the balance between risk and benefit of foods enriched with phytosteryl/-stanyl esters may change as a result of heat treatment-induced oxidative processes, as illustrated in Figure 49.

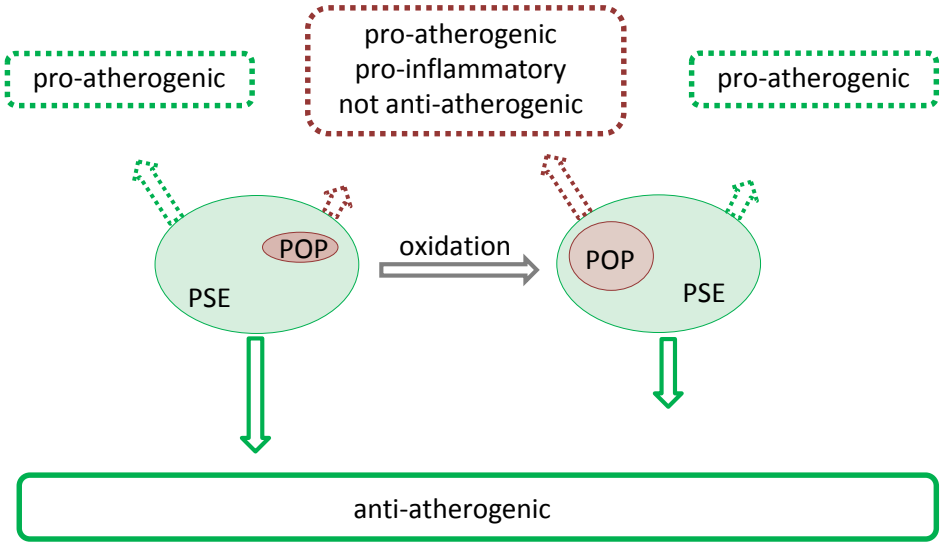

Figure 49. Potential relations of claimed benefits (solid lines) and discussed unintended effects (dashed lines) in phytosteryl/-stanyl fatty acid ester-enriched foods. PSE, phytosteryl/-stanyl fatty acid esters; POP, phytosterol/-stanol oxidation products.

The methodologies developed in the present study allowed for the first time to generate quantitative data on the contents of POP in differently enriched and heat-treated margarines as well as on the contents of the individual intact esters. This novel set of information was used to estimate the intakes of these two groups of constituents (Table 33). The compilation includes data on the baseline contents of different margarines as well as the quantitative data obtained upon household-type thermal treatments of margarines. The approach for estimating the intakes was based on the assumption that the upper daily intake of phytosterols/phytostanols of 3 g is achieved by consuming one of these foods, based on the respective degree of enrichment as indicated on the labels.

As previously discussed, the determined POP contents were strongly dependent on the way the margarines were treated, as well as on the type of enrichment. This is also reflected in the respective intake estimations. For non-heated, phytosteryl/-stanyl ester-enriched margarines, the resulting daily POP intakes ranged from 1.5 to 14.9 mg/d. Upon household heating, the intake was increased to 31.2 - 204.4 mg/d. In particular, oven-heating in a casserole increased the calculated daily POP intake 55-fold. This amount is significantly higher than any other daily POP intake calculated on the basis of existing data in the literature (Scholz *et al.*, 2015; Lin *et al.*, 2015) (cf. 2.3.3); the highest intake of 89 mg POP per day results from the POP content described for two portions of shallow-fried potatoes prepared with a phytosteryl/-stanyl ester enriched margarine (Lin *et al.*, 2016).

Table 33. Contents of intact phytosteryl/phytostanyl fatty acid esters and of phytosterol/phytostanol oxidation products determined in enriched margarines and resulting intakes calculated on the basis of a consumption of enriched foods corresponding to 3 g phytosterols per day.

type of margarine, [no.][a]	treatment	intact esters [g/kg]	POP [mg/kg]	intact esters [g/d][b]	sterols [g/d][b,c]	POP [mg/d][b]
		occurrence		intake		
phytosteryl/-stanyl ester (*Becel pro.activ*), [4]	no treatment	126	38	5.04	3.0	1.5
phytosteryl/-stanyl ester (*Becel pro.activ*), [5]	no treatment	-	373	-	-	14.9
phytosteryl/-stanyl ester (*Bellasan activ*), [10]	no treatment	-	64	-	-	2.5
	storage, 5°C, 47 days	-	69	-	-	2.8
phytosteryl/-stanyl ester (*Fruit d'Or pro-activ*), [8]	no treatment	129	93	5.2	3.1	3.7
	microwave, 800 W, 4min	124	780	5.0	3.0	31.2
	pan-frying, 9 min, 200 °C	120	1079	4.8	2.9	43.2
	oven, casserole, 20 min, 200 °C	88	5109	3.5	2.1	204.4
phytostanyl ester (*Benecol*), [2]	no treatment	138	23	5.2	3.2	0.8
	microwave, 800 W, 4min	132	43	4.9	3.0	1.6
	pan-frying, 9 min, 205 °C	125	137	4.7	2.9	5.1
	oven, casserole. 20 min, 200 °C	118	226	4.4	2.7	8.5

[a] The numbering is according to that in Table 5.
[b] Calculated on the basis of consumptions corresponding to 3 g phytosterols per day.
[c] Calculated on the basis of the intact esters.

Heating the margarine in a casserole led to a reduction of the daily ester consumption from 5.2 g to 3.5 g. This would theoretically correspond to an intake of 2.1 g intact phytosterols/-stanols instead of 3 g per day, which would still be within the range of 2-3 g phytosterols/-stanols per day that has been established to be required for a lowering of LDL cholesterol levels by 9-12 % (Ras *et al.*, 2014). The actual content of intact phytosterol/-stanol moieties is assumed to be even higher because of the unknown proportion of esters with an oxidized fatty acid moiety that were not detected with the employed methodologies.

In contrast, the changes induced by thermal treatment observed for the phytostanyl ester-enriched margarine were much less pronounced. The increase of the daily POP intake from 0.5 to 8.5 mg was comparably small, and also the corresponding decrease of the calculated daily ester consumption from 5.2 to at most 4.4 g after oven-heating in a casserole was less significant as compared to the phytosteryl/-stanyl ester-enriched margarine. Still, enlarging the surface exposed to oxygen during heating seemed to be a decisive parameter for both margarines as the changes in contents and resulting daily intakes were much more pronounced compared to the other heat treatments. Even if the experiments were conducted without the addition of foods, similar scenarios as for example the greasing of meat or vegetables for their preparation in the oven seem to be realistic ways of using enriched margarines in the home.

Risk-benefit evaluations of the effects resulting from the intakes of both, intact esters and their oxidation products deriving from “real” heat-treated foods are still only speculative and limited by various open issues and uncertainties. Definite answers to questions, such as which intake amounts are to be considered as “high” or “low”, or which result in “beneficial” or “adverse” effects, are still lacking. It is hoped that the approaches elaborated in this study will improve the analytical basis to create the quantitative data needed for such assessments.

5 SUMMARY

Phytosterols and -stanols, either in their free forms or esterified to fatty acids are bioactive molecules possessing an LDL-cholesterol-lowering potential. Therefore, they are added as functional ingredients to a variety of foods. However, the fatty acid esters used for enrichment are susceptible to oxidation reactions, particularly in the course of household ways of preparing foods. Such procedures are expected to result in a decrease of the intact esters, initially providing the functionality of the food product, and a concurrent formation of potentially adverse phytosterol oxidation products. To monitor these changes, appropriate analytical methodologies were required.

For the analysis of individual intact phytosteryl and/or phytostanyl fatty acid esters, two RP-UHPLC-APCI-MS-based approaches were established. The first approach enabled the quantitation of individual intact campestanyl and sitostanyl fatty acid esters in foods containing solely phytostanyl esters and was validated for different matrices. The determination of the individual esters was essentially based on an excellent chromatographic separation of those esters sharing the same stanol nucleus using a C8 1.7 μm column and a MeOH/H_2O step gradient. Detection in the SIM-mode enabled the differentiation of those esters exhibiting different stanol nuclei based on the formation of a characteristic $[M\text{-}FA+H]^+$ fragment ion. For foods containing mixtures of both phytosteryl and phytostanyl fatty acid esters, the development of suitable analytical conditions was considerably complicated as isotope peaks of phytosteryl esters interfered with the detection of phytostanyl esters in the SIM mode according to the $[M\text{-}FA+H]^+$ fragment ion. The highly efficient UHPLC-based separation offered a solution to this problem, as the chromatographic resolution of peaks of individual phytostanyl fatty acid esters and isotope peaks of the corresponding phytosteryl esters was possible; only phytostanyl linoleate and phytostanyl linolenate were affected by co-elutions. After adjustment of the APCI conditions, these phytostanyl esters of unsaturated fatty acids could be detected via their exclusive formation of $[M+H]^+$ ions in the corresponding SIM modes. The method was thoroughly validated using an enriched margarine as example and eventually permitted the quantitative analysis of 35 individual intact fatty acid esters of sitosterol, campesterol, brassicasterol, stigmasterol, sitostanol, and campestanol. The developed methodologies offer a novel tool for a comprehensive qualitative and quantitative analysis of phytosteryl and/or phytostanyl fatty acid esters and thus close an analytical gap related to this class of health-relevant food constituents.

For the determination of oxidized phytosterol moieties derived from the esters, on-line LC-GC equipped with a PTV interface was implemented as novel analytical platform. Acetylation of the

phytosterols and the corresponding oxides allowed their selective NP-LC-based pre-separation and the subsequent GC-based analysis of the quantitatively dominating phytosterol oxidation products in enriched foods within two transfers on a medium polar capillary column. On-line LC-GC/MS spectra were recorded for acetylated reference compounds of 5,6-epoxy-, 7-hydroxy-, and 7-keto derivatives of sitosterol, campesterol, stigmasterol, and for hydroxy-derivatives of sitostanol for the first time, and the methodology was extensively validated using an enriched margarine as example. Owing on the on-line sample purification, the drawbacks of conventional off-line procedures could be avoided.
The on-line LC-GC methodology was further applied to the determination of baseline contents of phytosterol oxidation products in various commercially available enriched margarines, revealing overall low contents in thermally untreated margarines.

The developed UHPLC-APCI-MS approaches and the established on-line LC-GC methodology were finally complementarily used to follow for the first time not only the formation of secondary phytosterol/-stanol oxidation products but also the concurrent decreases of the individual intact phytosteryl/-stanyl fatty acid esters being caused by thermo-oxidations. The data were developed by subjecting two differently enriched margarines specifically foreseen for cooking and baking to home food preparation methods. Microwave-heating led to the least decreases of esters of approximately 5 % in both margarines. Oven heating in a casserole caused the greatest decreases, with 68 and 86 % esters remaining; the impact on individual esters was more pronounced with increasing unsaturation of the fatty acid moiety. On the basis of the additional quantitation of concurrently formed oxidation products, a resulting gap of unexplained ester losses could be quantitated for the first time, accounting for approximately 80 % in the phytosteryl/-stanyl ester-enriched and 99 % in the phytostanyl ester-enriched margarine, respectively.

In conclusion, the development and complementary application of the new methodologies allowed the generation of an extended picture of the processes involved in the oxidation of phytosteryl and/or phytostanyl ester-enriched foods. Thereby, the newly developed possibility to determine individual intact esters in complex mixtures allowed for the first time a calculation of mass balances based on the decreases of intact esters. The results provide data not only required for the assessment of food authenticity, but also for the calculation of intakes of both intact phytosteryl/-stanyl fatty acid esters and phytosterol/-stanol oxidation products, eventually allowing an evaluation of potential beneficial and unintended effects.

6 ZUSAMMENFASSUNG

Phytosterole und –stanole, sowohl in freier Form als auch als Fettsäureester, sind bioaktive Verbindungen, die den LDL-Cholesterinspiegel senken können. Daher werden sie als funktionelle Inhaltsstoffe einer Reihe von Lebensmitteln zugesetzt. Die zur Anreicherung eingesetzten Fettsäureester sind jedoch anfällig für Oxidationsreaktionen, insbesondere im Zuge haushaltsüblicher Zubereitungen. Solche Prozeduren führen erwartungsgemäß zu einer Abnahme der intakten, funktionstragenden Ester, sowie zu einer gleichzeitigen Bildung möglicherweise gesundheitsschädlicher Phytosterol-Oxidationsprodukte. Um diese Veränderungen verfolgen zu können, waren zunächst geeignete analytische Methoden notwendig.

Für die Analytik individueller intakter Phytosteryl- und/oder Phytostanylfettsäureester wurden zwei RP-UHPLC-APCI-MS basierte Ansätze etabliert. Der erste Ansatz ermöglichte die quantitative Bestimmung individueller intakter Campestanyl- und Sitostanylfettsäureester in Lebensmitteln, die reine Phytostanylestermischungen enthielten, und wurde für verschiedene Matrizes validiert. Die Erfassung der individuellen Ester basierte wesentlich auf einer exzellenten chromatographischen Trennung von Estern desselben Stanol-Nukleus unter Verwendung einer 1.7 μm C8 Säule und eines MeOH/H_2O Stufen-Gradienten. Die Detektion im SIM-Modus basierend auf der charakteristischen Bildung eines $[M\text{-}FA+H]^+$ Fragmentions erlaubte die Differenzierung der Ester unterschiedlicher Stanolreste. Für mit Mischungen aus Phytosteryl- und Phytostanylestern angereicherte Lebensmittel war die Etablierung geeigneter analytischer Bedingungen ungleich schwerer, da Isotopenpeaks der Phytosterylester mit der Detektion der korrespondierenden Phytostanylester im SIM-Modus entsprechend $[M\text{-}FA+H]^+$ interferierten. Die hocheffiziente UHPLC-Trennung bot die Lösung für dieses Problem, da sie eine Auftrennung individueller Phytostanylester und der Isotopenpeaks der korrespondierenden Phytosterylester ermöglichte. Lediglich Phytostanyllinoleat und –linolenat waren von Co-Elutionen betroffen. Nach einer entsprechenden Adjustierung der APCI-Bedingungen konnten jedoch diese Phytostanylester ungesättigter Fettsäuren aufgrund der nun exklusiven Bildung eines $[M+H]^+$ Molekülions in den entsprechenden SIM-Modi erfasst werden. Die Methode wurde am Beispiel angereicherter Margarine umfassend validiert und ermöglichte letztlich die Erfassung 35 individueller intakter Fettsäureester von Sitosterol, Campesterol, Brassicasterol, Stigmasterol, Sitostanol und Campestanol. Die entwickelten methodischen Ansätze bieten eine neue Möglichkeit der umfassenden qualitativen und quantitativen Analytik von Phytosteryl- und/oder Phytostanylfettsäureestern und schließen eine bis dato existierende analytische Lücke hinsichtlich dieser Gruppe gesundheitlich relevanter Verbindungen.

Für die Bestimmung aus diesen Estern entstehender oxidierter Phytosterol-Reste wurde ein on-line LC-GC System mit PTV-Interface als neue analytische Plattform etabliert. Nach Acetylierung der Phytosterole und ihrer korrespondierenden Oxide war deren selektive NP-LC-basierte Vortrennung und eine anschließende GC-basierte Auftrennung individueller, in angereicherten Lebensmitteln quantitativ dominierender Phytosterol-Oxidationsprodukte auf einer mittelpolaren Kapillarsäule im Rahmen zweier Transfers möglich. On-line LC-GC/MS Spektren wurden erstmalig für acetylierte Referenzen von 5,6-Epoxy-, 7-Hydroxy- und 7-Keto-Derivate von Sitosterol, Campesterol, Stigmasterol sowie von Hydroxy-Derivaten von Sitostanol aufgenommen; die Methode wurde am Beispiel einer angereicherten Margarine umfangreich validiert. Aufgrund der on-line basierten Vortrennung konnten die Nachteile konventioneller off-line Prozeduren umgangen werden.
Die on-line LC-GC Methode wurde anschließend zur Bestimmung der Oxid-Grundgehalte an Phytosterol-Oxidationsprodukten in verschiedenen kommerziell erhältlichen Margarinen eingesetzt, wobei insgesamt niedrige Gehalte quantifiziert wurden.

Die entwickelten UHPLC-APCI-MS-basierten Ansätze sowie die etablierte on-line LC-GC Methode wurden schließlich komplementär eingesetzt, um erstmalig nicht nur die Bildung sekundärer Phytosterol/-stanol-Oxidationsprodukte, sondern auch die gleichzeitigen Abnahmen individueller intakter Phytosteryl-/Phytostanylfettsäureester in Folge thermisch bedingter Oxidationen beobachten zu können. Entsprechende Daten wurden am Beispiel zweier unterschiedlich angereicherter Margarinen, die insbesondere zum Braten und Backen vorgesehen sind, durch verschiedene haushaltsübliche Hitzebehandlungen erzeugt. Dabei führte das Erhitzen in der Mikrowelle in beiden Margarinen zu den geringsten Abnahmen intakter Ester von etwa 5 %. Das Erhitzen in einer Auflaufform im Ofen verursachte mit 68 bzw. 86 % verbleibenden Estern die stärksten Verluste; das Ausmaß der Abnahmen war umso größer, je ungesättigter die veresterte Fettsäure war. Auf der Grundlage der zusätzlichen Quantifizierung der gleichzeitig gebildeten Phytosterol/-stanol-Oxidationsprodukte konnte erstmalig die resultierende Lücke nicht erklärter Verluste der intakten Ester berechnet werden; diese Lücke betrug ca. 80 % in der mit Phytosteryl-/Phytostanylestern angereicherten bzw. 99 % in der mit Phytostanylestern angereicherten Margarine.

Zusammenfassend ermöglichten die Entwicklung und anschließende kombinierte Anwendung neuer analytischer Methoden eine erweiterte Betrachtung von Oxidationsprozessen in mit Phytosteryl-/Phytostanylestern angereicherten Lebensmitteln. Die neue Möglichkeit der Erfassung intakter, individueller Ester in komplexen Mischungen erlaubte dabei erstmalig die Berechnung von Massenbilanzen auf Basis der Abnahmen der intakten Ester. Die Ergebnisse

liefern nicht nur einen weiteren zur Authentizitätsbewertung dieser Lebensmittel notwendigen Beitrag. Sie bieten auch die Grundlagen für Abschätzungen von Aufnahmemengen sowohl der intakten Phytosteryl-/Phytostanylfettsäureester als auch der Phytosterol/-stanol-Oxidationsprodukte und damit letztlich für die Evaluierung daraus möglicherweise resultierender vorteilhafter sowie unerwünschter Effekte.

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8 APPENDIX

The temperature profiles of the phytosteryl/-stanyl ester-enriched (no. 8) and of the phytostanyl ester-enriched (no. 2) margarines during pan-frying are shown in Figure 50.

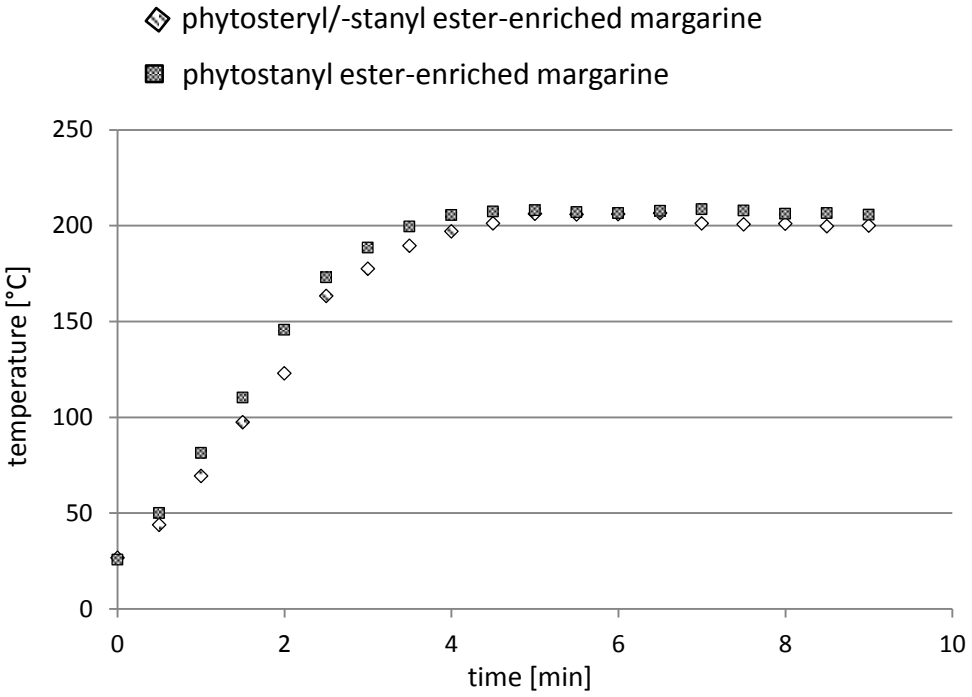

Figure 50. Temperature profiles of the margarines during pan-frying.

The changes of intact phytosteryl/-stanyl fatty acid esters and of intact phytostanyl fatty acid esters in the two margarines determined in a second series of heat treatments are compiled in Table 34 and Table 35. The data for the untreated margarine are those presented in Table 29 and Table 30.

The formations of phytosterol and phytostanol oxidation products in the two margarines determined in a second series of heat treatments are summarized in Table 36 and Table 37. The data for the untreated margarine are those presented in Table 31 and Table 32.

Table 34. Phytosteryl/-stanyl fatty acid esters in in phytosteryl/-stanyl ester-enriched margarine (no. 8) before and after a second series of heat treatments.[a]

steryl / stanyl fatty acid ester [g/kg]	no treatment	microwave, bottle 800 W, 4 min	pan-frying 193 °C[c], 9 min	oven, bottle 200 °C, 20 min	oven, casserole 200 °C, 20 min
brassicasteryl-18:2***	2.40 ± 0.09[b] (a)	2.34 ± 0.10 (a)	1.94 ± 0.14 (b)	1.88 ± 0.05 (b)	1.42 ± 0.03 (c)
brassicasteryl-16:0***	0.25 ± 0.00 (b)	0.31 ± 0.01 (a)	0.27 ± 0.01 (b)	0.27 ± 0.00 (b)	0.24 ± 0.01 (c)
brassicasteryl-18:1***	1.05 ± 0.02 (a)	1.00 ± 0.04 (a)	0.87 ± 0.07 (b)	0.85 ± 0.02 (b)	0.74 ± 0.03 (c)
brassicasteryl-18:0***	0.11 ± 0.01 (a)	0.10 ± 0.00 (ab)	0.09 ± 0.00 (bc)	0.09 ± 0.00 (b)	0.08 ± 0.01 (c)
campesteryl-16:1***	0.02 ± 0.00 (a)	0.02 ± 0.00 (b)	0.02 ± 0.00 (c)	0.01 ± 0.00 (d)	0.01 ± 0.00 (e)
campesteryl-18:2***	12.37 ± 0.20 (a)	11.89 ± 0.17 (b)	10.82 ± 0.15 (c)	9.98 ± 0.23 (d)	7.89 ± 0.09 (e)
campesteryl-16:0***	1.08 ± 0.00 (b)	1.13 ± 0.02 (a)	1.03 ± 0.00 (c)	1.00 ± 0.01 (c)	0.94 ± 0.03 (d)
campesteryl-18:1***	5.49 ± 0.13 (a)	5.42 ± 0.08 (a)	4.97 ± 0.09 (b)	4.66 ± 0.19 (b)	4.14 ± 0.03 (c)
campesteryl-18:0***	0.62 ± 0.01 (a)	0.61 ± 0.01 (a)	0.58 ± 0.01 (b)	0.55 ± 0.01 (c)	0.51 ± 0.01 (d)
campesteryl-20:1***	0.02 ± 0.00 (a)	0.02 ± 0.00 (a)	0.02 ± 0.00 (a)	0.02 ± 0.00 (a)	0.01 ± 0.00 (b)
campesteryl-20:0**	0.03 ± 0.00 (a)	0.03 ± 0.00 (ab)	0.03 ± 0.00 (ab)	0.03 ± 0.00 (bc)	0.03 ± 0.00 (c)
campesteryl-22:0***	0.10 ± 0.00 (a)	0.10 ± 0.01 (a)	0.09 ± 0.00 (ab)	0.09 ± 0.00 (bc)	0.08 ± 0.00 (c)
stigmasteryl-18:2***	0.39 ± 0.02 (a)	0.32 ± 0.02 (b)	0.26 ± 0.02 (c)	0.26 ± 0.01 (c)	0.19 ± 0.00 (d)
stigmasteryl-18:0***	0.02 ± 0.00 (a)	0.02 ± 0.00 (ab)	0.01 ± 0.00 (bc)	0.01 ± 0.00 (bc)	0.01 ± 0.00 (c)
sitosteryl-18:3***	0.21 ± 0.01 (b)	0.23 ± 0.01 (a)	0.20 ± 0.01 (bc)	0.18 ± 0.01 (c)	0.14 ± 0.00 (d)
sitosteryl-16:1***	0.09 ± 0.00 (a)	0.09 ± 0.00 (a)	0.08 ± 0.00 (b)	0.08 ± 0.00 (b)	0.06 ± 0.00 (c)
sitosteryl-18:2***	57.59 ± 1.46 (a)	57.61 ± 0.91 (a)	50.26 ± 0.70 (b)	46.60 ± 2.00 (c)	36.86 ± 0.36 (d)
sitosteryl-16:0***	5.24 ± 0.10 (a)	5.35 ± 0.13 (a)	4.92 ± 0.12 (b)	4.73 ± 0.13 (bc)	4.44 ± 0.11 (c)
sitosteryl-18:1***	26.04 ± 0.74 (a)	26.26 ± 0.39 (a)	23.78 ± 0.59 (b)	22.18 ± 0.97 (b)	20.27 ± 0.23 (c)
sitosteryl-18:0***	2.90 ± 0.01 (a)	3.00 ± 0.07 (a)	2.73 ± 0.07 (b)	2.62 ± 0.08 (bc)	2.47 ± 0.06 (c)
sitosteryl-20:1**	0.10 ± 0.00 (a)	0.10 ± 0.00 (ab)	0.09 ± 0.00 (abc)	0.09 ± 0.01 (bc)	0.08 ± 0.01 (c)
sitosteryl-20:0**	0.16 ± 0.00 (a)	0.15 ± 0.00 (a)	0.15 ± 0.01 (ab)	0.13 ± 0.01 (b)	0.13 ± 0.01 (b)
sitosteryl-22:0***	0.47 ± 0.01 (a)	0.46 ± 0.01 (a)	0.43 ± 0.00 (b)	0.41 ± 0.01 (c)	0.38 ± 0.01 (d)
campestanyl-18:2***	0.94 ± 0.03 (a)	0.91 ± 0.05 (a)	0.81 ± 0.05 (b)	0.94 ± 0.01 (a)	0.67 ± 0.04 (c)

Table 34. continued.

steryl / stanyl fatty acid ester [g/kg]	no treatment	microwave, bottle 800 W, 4 min	pan-frying 193 °C[c], 9 min	oven, bottle 200 °C, 20 min	oven, casserole 200 °C, 20 min
campestanyl-16:0	0.10 ± 0.00	0.09 ± 0.01	0.09 ± 0.02	0.10 ± 0.01	0.08 ± 0.00
campestanyl-18:1***	0.48 ± 0.01 (b)	0.49 ± 0.03 (b)	0.40 ± 0.02 (b)	0.51 ± 0.10 (a)	0.40 ± 0.02 (b)
campestanyl-18:0	0.06 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.00	0.04 ± 0.00
campestanyl-22:0***	0.01 ± 0.00 (a)	0.01 ± 0.00 (b)	0.01 ± 0.00 (bc)	0.01 ± 0.00 (ab)	0.01 ± 0.00 (c)
sitostanyl-18:2***	6.50 ± 0.03 (a)	6.27 ± 0.06 (ab)	5.68 ± 0.23 (b)	6.05 ± 0.08 (ab)	4.54 ± 0.42 (c)
sitostanyl-16:0*	0.68 ± 0.03 (ab)	0.62 ± 0.01 (ab)	0.62 ± 0.08 (ab)	0.72 ± 0.06 (a)	0.55 ± 0.02 (b)
sitostanyl-18:1**	3.30 ± 0.06 (ab)	3.37 ± 0.11 (a)	2.85 ± 0.25 (b)	3.21 ± 0.41 (ab)	2.84 ± 0.03 (b)
sitostanyl-18:0*	0.36 ± 0.01 (a)	0.36 ± 0.01 (a)	0.33 ± 0.02 (ab)	0.37 ± 0.03 (a)	0.31 ± 0.00 (b)
sitostanyl-20:0*	0.03 ± 0.00 (a)	0.02 ± 0.00 (ab)	0.02 ± 0.00 (ab)	0.03 ± 0.01 (ab)	0.02 ± 0.00 (b)
sitostanyl-22:0*	0.06 ± 0.00 (a)	0.06 ± 0.01 (ab)	0.05 ± 0.01 (ab)	0.06 ± 0.00 (ab)	0.04 ± 0.01 (b)
total esters***	129.28 ± 2.52 (a)	128.80 ± 1.65 (a)	114.55 ± 2.38 (b)	110.28 ± 4.35 (b)	92.34 ± 3.21 (c)

[a] Analysis of variance was carried out with ANOVA. Levels of significance: $p<0.001$, highly significant (***); $p<0.01$, very significant (**); $p<0.05$, significant (*). Within the same row, different letters indicate significant differences (Tukey's HSD) among treatments ($p<0.05$).

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

[c] Final temperature of the heating process.

Table 35. Phytostanyl fatty acid esters in in phytostanyl ester-enriched margarine (no. 2) before and after a second series of heat treatments.[a]

stanyl fatty acid ester [g/kg]	no treatment	microwave, bottle 800 W, 4 min	pan-frying 200 °C[c], 9 min	oven, bottle 200 °C, 20 min	oven, casserole 200 °C, 20 min
campestanyl-18:3***	1.70 ± 0.04[b] (a)	1.47 ± 0.05 (b)	1.46 ± 0.06 (b)	1.59 ± 0.06 (ab)	1.12 ± 0.02 (c)
campestanyl-18:2***	4.01 ± 0.08 (a)	3.71 ± 0.04 (b)	3.60 ± 0.02 (b)	3.75 ± 0.11 (b)	3.20 ± 0.03 (c)
campestanyl-16:0***	0.82 ± 0.04 (a)	0.71 ± 0.03 (bc)	0.73 ± 0.01 (b)	0.77 ± 0.02 (ab)	0.65 ± 0.01 (c)
campestanyl-18:1***	11.72 ± 0.26(a)	11.32 ± 0.15 (ab)	11.16 ± 0.08 (b)	11.17 ± 0.13 (b)	10.39 ± 0.06 (c)
campestanyl-18:0***	0.32 ± 0.01 (a)	0.26 ± 0.01 (cd)	0.28 ± 0.01 (c)	0.30 ± 0.01 (b)	0.25 ± 0.00 (d)
campestanyl-20:1***	0.28 ± 0.01 (a)	0.24 ± 0.01 (b)	0.24 ± 0.01 (b)	0.25 ± 0.01 (b)	0.19 ± 0.01 (c)
campestanyl-20:0***	0.09 ± 0.00 (a)	0.08 ± 0.01 (b)	0.08 ± 0.00 (b)	0.08 ± 0.00 (ab)	0.06 ± 0.00 (c)
campestanyl-22:1***	0.09 ± 0.00 (a)	0.07 ± 0.00 (b)	0.06 ± 0.00 (d)	0.07 ± 0.00 (bc)	0.06 ± 0.00 (cd)
campestanyl-22:0***	0.06 ± 0.00 (a)	0.04 ± 0.00 (cd)	0.04 ± 0.00 (c)	0.05 ± 0.00 (b)	0.03 ± 0.00 (d)
sitostanyl-18:3***	10.57 ± 0.24 (a)	9.05 ± 0.28 (b)	8.49 ± 0.06 (b)	9.12 ± 0.33 (b)	7.75 ± 0.23 (c)
sitostanyl-16:1***	0.22 ± 0.00 (a)	0.18 ± 0.01 (b)	0.17 ± 0.01 (c)	0.19 ± 0.00 (b)	0.16 ± 0.00 (c)
sitostanyl-18:2***	25.31 ± 0.27 (a)	22.94 ± 0.62 (b)	21.02 ± 0.62 (cd)	22.39 ± 0.70 (bc)	19.87 ± 0.47 (d)
sitostanyl-16:0**	5.10 ± 0.18 (a)	4.86 ± 0.19 (a)	4.80 ± 0.11 (a)	4.77 ± 0.14 (a)	4.37 ± 0.04 (b)
sitostanyl-18:1***	73.34 ± 0.87 (a)	71.29 ± 0.49 (b)	68.74 ± 0.66 (c)	66.84 ± 0.76 (c)	63.26 ± 0.74 (d)
sitostanyl-18:0**	1.80 ± 0.05 (a)	1.61 ± 0.05 (b)	1.68 ± 0.05 (ab)	1.64 ± 0.07 (b)	1.55 ± 0.07 (b)
sitostanyl-20:1**	1.57 ± 0.04 (a)	1.40 ± 0.05 (b)	1.46 ± 0.04 (ab)	1.43 ± 0.06 (b)	1.34 ± 0.04 (b)
sitostanyl-20:0**	0.59 ± 0.03 (a)	0.53 ± 0.01 (b)	0.53 ± 0.02 (b)	0.52 ± 0.01 (b)	0.50 ± 0.02 (b)
sitostanyl-22:1***	0.55 ± 0.02 (a)	0.47 ± 0.03 (b)	0.48 ± 0.02 (b)	0.47 ± 0.02 (b)	0.41 ± 0.01 (c)
sitostanyl-22:0***	0.30 ± 0.01 (a)	0.29 ± 0.01 (a)	0.28 ± 0.01 (ab)	0.26 ± 0.01 (bc)	0.25 ± 0.01 (c)
total	138.44 ± 1.60 (a)	130.49 ± 1.42 (b)	125.30 ± 1.14 (c)	125.65 ± 1.95 (c)	115.42 ± 1.57 (d)

[a] Analysis of variance was carried out with ANOVA. Levels of significance: $p<0.001$, highly significant (***); $p<0.01$, very significant (**); $p<0.05$, significant (*). Within the same row, different letters indicate significant differences (Tukey's HSD) among treatments ($p<0.05$).

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

[c] Final temperature of the heating process.

Table 36. Phytosterol and phytostanol oxidation products determined in a phytosteryl/-stanyl ester-enriched margarine (no. 8) before and after a second series of heat treatments.[a]

phytosterol oxidation product [mg/kg]	no treatment	microwave, bottle 800 W, 4 min	pan-frying 193 °C[e], 9 min	oven, bottle 200 °C, 20 min	oven, casserole 200 °C, 20 min
5,6*ß*-epoxycampesterol***	4.4 ± 0.1[c] (a)	27.4 ± 0.7 (b)	49.4 ± 2.3 (c)	137.4 ± 2.9 (d)	265.7 ± 2.2 (e)
5,6*ß*-epoxysitosterol***	14.8 ± 0.7 (a)	139.4 ± 7.3 (b)	222.8 ± 6.7 (c)	614.9 ± 6.6 (d)	1162.2 ± 4.8 (e)
5,6*α*-epoxycampesterol***	3.8 ± 0.3 (a)	16.4 ± 0.7 (b)	25.8 ± 0.7 (c)	57.2 ± 0.4 (d)	121.6 ± 2.3 (e)
7*α*-hydroxycampesterol***	2.9 ± 0.3 (a)	27.8 ± 0.9 (b)	43.5 ± 0.3 (c)	98.8 ± 0.6 (d)	238.5 ± 4.8 (e)
5,6*α*-epoxysitosterol***	18.7 ± 1.8 (a)	70.4 ± 2.4 (b)	115.0 ± 0.9 (c)	278.8 ± 9.4 (d)	566.9 ± 4.2 (e)
7*α*-hydroxysitosterol***	3.1 ± 0.2 (a)	71.5 ± 2.8 (b)	144.2 ± 4.1 (c)	328.0 ± 16.3 (d)	812.8 ± 5.3 (e)
7*ß*-hydroxycampesterol***	1.4 ± 0.0 (a)	23.2 ± 1.1 (b)	39.4 ± 1.4 (c)	85.2 ± 2.7 (d)	221.3 ± 2.9 (e)
6*α*-hydroxysitostanol***	-[d]	35.7 ± 2.5 (a)	42.9 ± 3.5 (a)	39.3 ± 3.0 (a)	63.2 ± 3.9 (b)
7*ß*-hydroxysitosterol***	4.7 ± 0.4 (a)	87.9 ± 2.3 (b)	162.4 ± 4.0 (c)	338.0 ± 16.3 (d)	803.9 ± 3.5 (e)
7-ketocampesterol***	10.2 ± 1.0 (a)	37.2 ± 1.7 (b)	41.5 ± 0.3 (b)	146.6 ± 3.3 (c)	264.0 ± 6.5 (d)
7-ketositosterol***	28.9 ± 0.7 (a)	202.8 ± 3.2 (b)	212.9 ± 1.2 (b)	609.7 ± 4.8 (c)	1160.2 ± 5.6 (d)
total***	92.9 ± 2.2 (a)	739.6 ± 10.8 (b)	1099.7 ± 20.3 (c)	2733.7 ± 43.6 (d)	5680.3 ± 7.9 (e)
oxidation rate[b] [%]	0.12 ± 0.0	0.99 ± 0.0	1.47 ± 0.0	3.64 ± 0.1	7.57 ± 0.0

[a] Analysis of variance was carried out with ANOVA. Levels of significance: $p<0.001$, highly significant (***); $p<0.01$, very significant (**); $p<0.05$, significant (*). Within the same row, different letters indicate significant differences (Tukey's HSD) among treatments ($p<0.05$).

[b] Calculated as percentage of phytosterol/phytostanol oxidation products with respect to the initial phytosterol/phytostanol content.

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

[d] Content below limit of quantification (i.e. <0.33 *µ*g/mL of injection volume).

[e] Final temperature of the heating process.

Table 37. Phytosterol and phytostanol oxidation products determined in a phytostanyl ester-enriched margarine (no. 2) before and after a second series of heat treatments.[a]

phytosterol oxidation product [mg/kg]	no treatment	microwave, bottle 800 W, 4 min	pan-frying 200 °C[f], 9 min	oven, bottle 200 °C, 20 min	oven, casserole 200 °C, 20 min
6*α*-hydroxysitostanol***	20.6 ± 1.9[c] (a)	26.7 ± 1.0 (b)	46.6 ± 0.5 (d)	32.9 ± 2.0 (c)	61.8 ± 1.8 (e)
7-hydroxysitostanol***	-[d]	-[d]	8.7 ± 0.7 (b)	3.3 ± 0.2 (a)	11.1 ± 0.6 (c)
5,6*ß*-epoxycampesterol***	-[d]	-[d]	11.4 ± 0.3 (b)	4.5 ± 0.1 (a)	11.3 ± 0.7 (b)
5,6*ß*-epoxysitosterol***	-[e]	-[d]	27.8 ± 0.2 (c)	6.9 ± 0.6 (a)	17.7 ± 0.2 (b)
5,6*α*-epoxysitosterol***	-[d]	-[d]	17.4 ± 1.3 (b)	7.3 ± 0.4 (a)	20.5 ± 0.5 (c)
7*α*-hydroxysitosterol***	-[d]	-[d]	8.6 ± 0.7 (b)	5.9 ± 0.1 (a)	16.8 ± 0.9 (c)
7*ß*-hydroxysitosterol***	-[d]	-[d]	9.5 ± 0.4 (c)	1.7 ± 0.1 (a)	8.1 ± 0.6 (b)
7-ketocampesterol	-[d]	-[d]	9.6 ± 0.6	-[e]	9.6 ± 0.7
7-ketositosterol***	1.9 ± 0.3 (a)	-[e]	36.0 ± 1.1 (d)	6.1 ± 0.6 (b)	16.1 ± 0.4 (c)
total***	22.6 ± 1.5 (a)	26.7 ± 1.0 (a)	175.4 ± 4.3 (c)	68.6 ± 2.1 (b)	173.0 ± 1.2 (c)
oxidation rate[b] [%]	0.03 ± 0.0	0.03 ± 0.0	0.07 ± 0.0	0.05 ± 0.0	0.09 ± 0.0

[a] Analysis of variance was carried out with ANOVA. Levels of significance: $p<0.001$, highly significant (***); $p<0.01$, very significant (**); $p<0.05$, significant (*). Within the same row, different letters indicate significant differences (Tukey's HSD) among treatments ($p<0.05$).

[b] Calculated as percentage of phytostanol oxidation products related to the initial phytostanol content.

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

[d] Content below limit of detection (i.e. <0.11 μg/mL of injection volume for 7-hydroxyphytosterols and <0.08 μg/mL of injection volume for 5,6-epoxyphytosterols).

[e] Content below limit of quantitation (i.e. <0.33 μg/mL of injection volume for 7-hydroxyphytosterols and 0.24 μg/mL of injection volume for 5,6-epoxyphytosterols).

[f] Final temperature of heating process.

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9 PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS (peer reviewed)

Scholz, B.; Menzel, N.; Lander, V.; Engel, K.-H., Heating two types of enriched margarine: Complementary analysis of phytosteryl/phytostanyl fatty acid esters and phytosterol/phytostanol oxidation products. *J. Agric. Food Chem.* **2016**, *64*, 2699-2708.

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POSTER PRESENTATION

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

12/2011 – 03/2016	Research Assistant (PhD Student) Technische Universität München, Research Department of Nutrition and Food Science, Chair of General Food Technology (Univ.-Prof. Dr. Karl-Heinz Engel), Freising-Weihenstephan, Germany
08/2010 – 09/2010	Research Internship VDLUFA-QLA GmbH, Bioanalytik Weihenstephan ZIEL, Freising, Germany
07/2008 – 10/2008	Internship in Product Development ALPENHAIN Käsespezialitäten-Werk GmbH & Co. KG, Lehen, Germany

ACADEMIC STUDIES AND EDUCATION

10/2011	Master Degree (M. Sc.) in Nutritional Science Master's Thesis: "Analysis of Phytosterols/-stanols and Intact Phytosteryl/-stanyl Esters in Corn (Zea mays L.)" Technische Universität München, Research Department of Nutrition and Food Science, Chair of General Food Technology (Univ.-Prof. Dr. Karl-Heinz Engel), Freising-Weihenstephan, Germany
09/2009	Bachelor Degree (B. Sc.) in Nutritional Science Bachelor's Thesis: „Metabolite Profiling von Gerste im Verlauf der Mälzung" Technische Universität München, Research Department of Nutrition and Food Science, Chair of General Food Technology (Univ.-Prof. Dr. Karl-Heinz Engel), Freising-Weihenstephan, Germany
10/2006 – 10/2011	Academic Studies of Nutritional Science Technische Universität München Research Department of Nutrition and Food Science, Study Program Division Nutrition, Freising-Weihenstephan, Germany
10/2005 – 08/2006	Romance Philology and English studies Ludwigs-Maximilians-Universität, München, Germany
09/1996 – 07/2005	Allgemeine Hochschulreife Gymnasium Grafing, Grafing, Germany