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New Methodologies for the Analysis of Phytosterols and Their Intact Esters: Applications to Cereals, Nuts and Edible Plant Oils

Rebecca Esche

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ABBREVIATIONS

ABC	adenosine triphosphate-binding cassette
ACAT	acyl CoA cholesteryl acyltransferase
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
CC	column chromatography
CoA	coenzym A
dm	dry matter
DMAPP	3,3-dimethylallylpyrophosphate
ELSD	evaporative light scattering detector
FAME	fatty acid methyl ester
FID	flame ionization detector
FPP	farnesyl pyrophosphate
GC	gas chromatography/gas chromatographic
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography
IPP	isopentyl pyrophosphate
IS	internal standard
i.v.	injection volume
i.d.	inner diameter
IUPAC-IUB	International Union of Pure and Applied Chemistry and International Union of Biochemistry
LC	liquid chromatography/liquid chromatographic
LDL	low-density lipoprotein
LOD	limit of detection
LOQ	limit of quantification
LXR	liver X receptor
MS	mass spectrometry/mass spectrometric
MTBE	methyl *tert*-butyl ether
MTP	microsomal transport protein
NPC1L1	Nieman-Pick C1-Like 1
NP	normal phase
PCA	principal component analysis
PTV	programmable temperature vaporizer
Rf	response factor
RP	reversed phase
SMT	sterol methyl transferase
SPE	solid-phase extraction

TG	triglycerides
TLC	thin layer chromatography
TMCS	trimethylchlorosilan
TMS	trimethyl silyl
UV	ultraviolet

1 INTRODUCTION AND OBJECTIVES

Phytosterols/-stanols are cholesterol-like molecules which play important roles in the regulation of plant cell membrane fluidity and permeability (Piironen *et al.*, 2000). They occur in free form, esterified with fatty acids or phenolic acids, and as glycosides or acylated glycosides (Piironen *et al.*, 2000; Moreau *et al.*, 2002). The nutritional interest in these compounds mainly arises from their potency to decrease blood serum levels of low-density lipoprotein (LDL) cholesterol, a potential risk factor for the formation of cardiovascular diseases (Plat and Mensink, 2005; Brufau *et al.*, 2008; Calpe-Berdiel *et al.*, 2009). A daily intake of about 2 g phytosterols/-stanols can reduce the levels of LDL-cholesterol of hypercholesterolemic patients by 10 % (Katan *et al.*, 2003). For this purpose, phytosterols/-stanols are currently incorporated, particularly in form of their fatty acid esters, into a broad spectrum of foods such as spread, margarine, yogurt, or milk. In addition to the cholesterol-lowering effect, phytosterols/-stanols and their derivatives were also suggested to possess other health benefits such as anti-oxidative, anti-inflammatory, or anti-carcinogenic properties (Wang *et al.*, 2002; Woyengo *et al.*, 2009; Othman and Moghadasian, 2011). In this context, there is growing interest in increasing the intake of these compounds also via natural diets. Cereals, nuts, seeds, and edible plant oils are rich sources of free sterols/stanols and steryl/stanyl esters (Piironen *et al.*, 2000). Even though the natural intake of total phytosterols/-stanols is low compared to the amounts ingested by the consumption of fortified foods, there is clear evidence that also moderately low intakes of phytosterols/-stanols significantly influence the cholesterol absorption and serum LDL-cholesterol concentrations (Ostlund *et al.*, 2002a; Ostlund *et al.*, 2003; Andersson *et al.*, 2004; Sanclemente *et al.*, 2012). Further, it is suggested that the gastrointestinal hydrolysis of steryl/stanyl esters by digestive enzymes depends on the type of ester. *In vitro* and *in vivo* studies reported an impact of the sterol/stanol and fatty acid moiety on the hydrolysis rate, which, in turn, could influence the cholesterol-lowering effect of dietary phytosterols (Brown *et al.*, 2010; Lubinus *et al.*, 2013).

Thus, it is of great interest to establish analytical methods which provide qualitative and quantitative data on the contents and compositions of the naturally occurring phytosterol classes. In particular, steryl/stanyl fatty acid esters have been less studied to date, also because of the lack of appropriate analytical methods. Previous investigations have mainly been focused on the analysis of total phytosterols/-stanols, commonly determined after alkaline or after a combination of alkaline and acidic hydrolysis. The liberated sterols and stanols can then be extracted as part of the unsaponifiable matter, followed by a purification step, and the qualitative and quantitative analysis via gas chromatography (GC) or high

performance liquid chromatography (Abidi, 2001; Lagarda *et al.*, 2006). However, due to the hydrolysis steps, information on the distributions and contents of the individual steryl/stanyl esters is lost.

The aim of the present study was the development of analytical approaches, which enable the comprehensive analysis of naturally occurring individual free sterols/stanols, intact steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters. The methods should be based on high temperature capillary GC, which was demonstrated to be suitable for the analysis of several mixtures of intact steryl/stanyl esters (Miller *et al.*, 2003; Barnsteiner *et al.*, 2011; Barnsteiner *et al.*, 2012). However, as some lipid constituents, mainly triglycerides, hamper the direct chromatographic analysis of intact steryl/stanyl esters, a pre-separation of the extracted plant lipids is essential. On the one hand, an approach based on solid-phase extraction (SPE) for the fractionation of the lipids into free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters should be established and validated. The individual compositions of the obtained sterol fractions should then be analyzed via GC. On the other hand, a method based on on-line liquid chromatography-gas chromatography should be developed and validated as an efficient, automated, and rapid alternative to the SPE-based approach. The developed methodologies should be applied to demonstrate the natural variability in compositions and contents of individual free sterols/stanols and steryl/stanyl esters in different types of cereals, nuts, and edible plant oils.

2 BACKGROUND

2.1 Phytosterols/-stanols and Their Conjugates

2.1.1 Structural Properties, Biosynthesis, and Biological Function in Plants

Phytosterols are steroidal alcohols belonging to the group of triterpenoids. The basic sterol structure with standard carbon numbering according to the IUPAC-IUB recommendations is shown in Figure 1 (IUPAC-IUB, 1989). Phytosterols are characterized by a tetracyclic cyclopenta[*a*]phenantrene ring, two methyl groups at C10 and C13, a flexible side chain on C17, and a hydroxyl group on C3 in *ß*-stereochemistry (Piironen *et al.*, 2000).

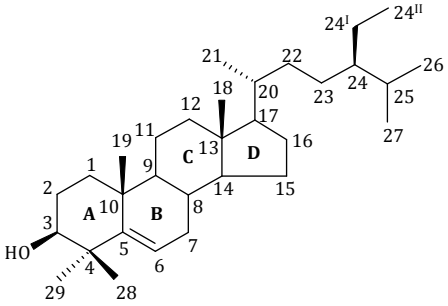

Figure 1. Basic structure of sterols according to official recommendations (IUPAC-IUB, 1989).

Depending on the number of methyl groups on C4, sterols are categorized as 4-desmethylsterols, 4-monomethylsterols, or 4,4'-dimethylsterols (Figure 2).

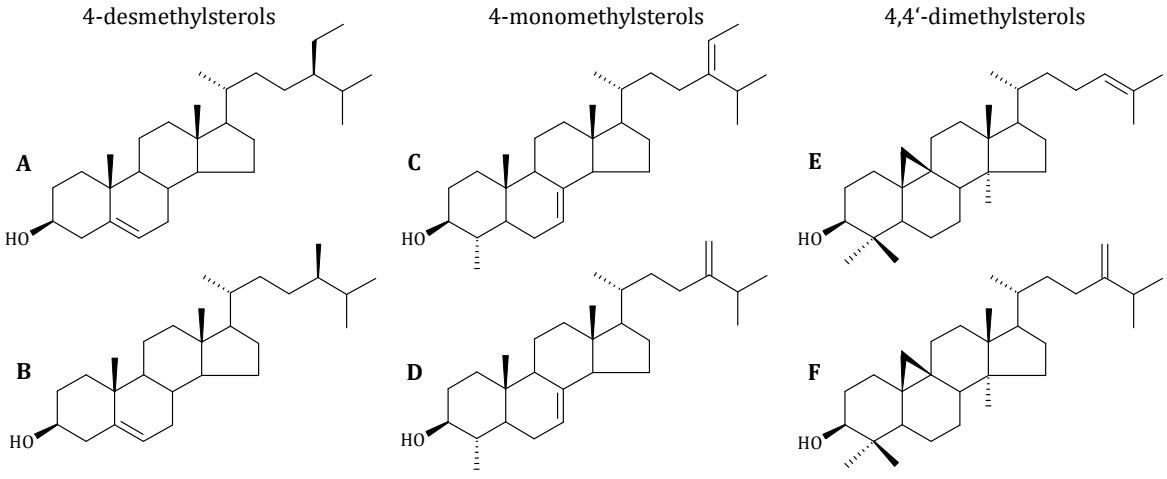

Figure 2. Representative structures of 4-desmethylsterols, 4-monomethylsterols, and 4,4'-dimethylsterols: (**A**) sitosterol, (**B**) campesterol, (**C**) citrostadienol, (**D**) gramisterol, (**E**) cycloartenol, and (**F**) 24-methylene cycloartanol.

4-Desmethylsterols are predominant and can further be classified according to the position of the double bond(s) in the B-ring and the side chain into Δ^5 sterols, Δ^7 sterols, $\Delta^{5,7}$ sterols, or $\Delta^{5,22}$ sterols (Nes, 1987). Stanols, i.e. sitostanol and campestanol, are the fully saturated forms of the sterols.

The biosynthesis of phytosterols, which occurs exclusively in the cytoplasm, involves a series of at least 30 different enzyme-catalyzed reactions (Piironen *et al.*, 2000). A schematic and simplified pathway of the biosynthesis of sterols in plants is shown in Figure 3.

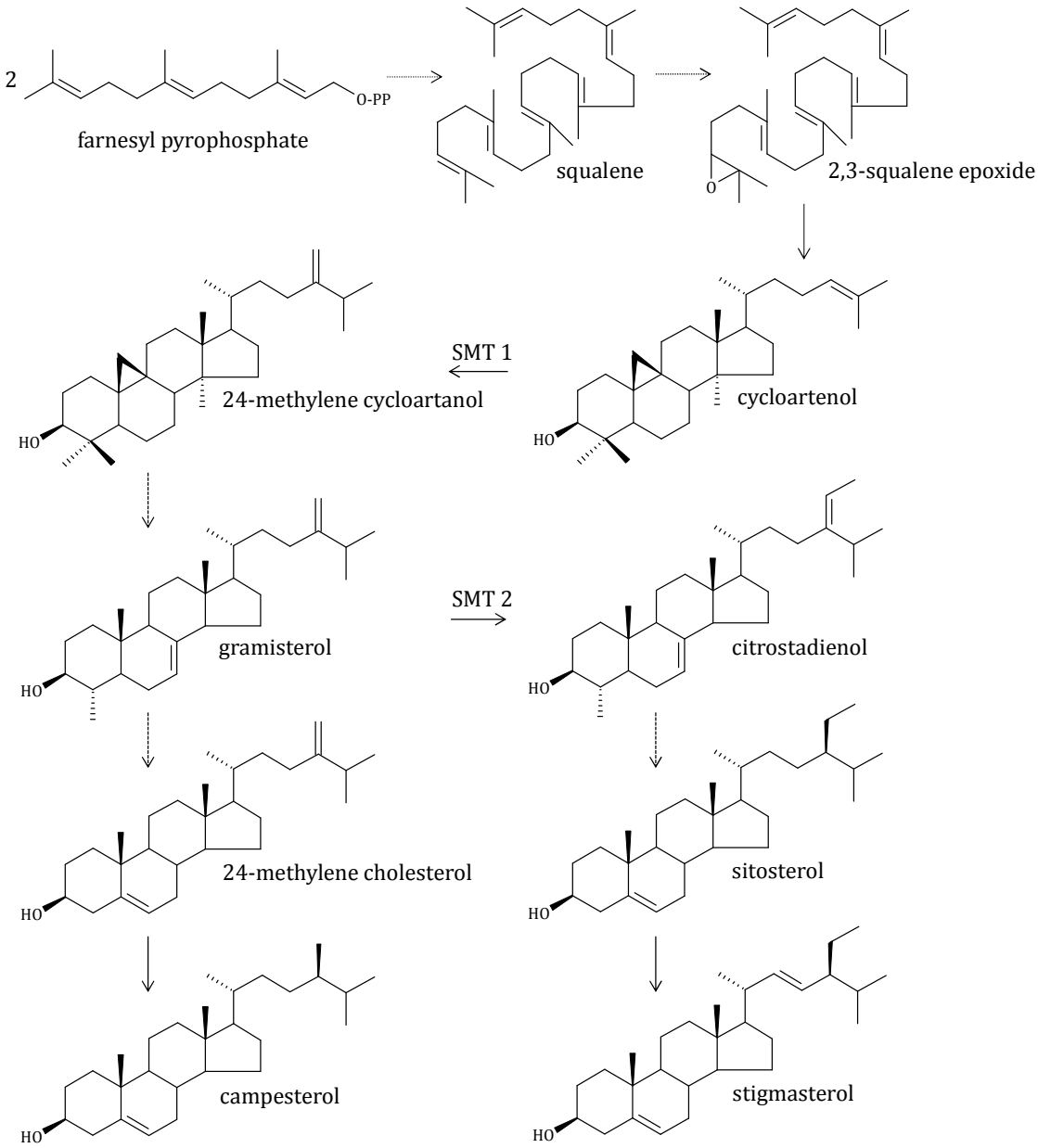

Figure 3. Simplified schematic representation of the biosynthetic pathway of phytosterols in plants. SMT: sterol methyl transferase

The first basic building block isopentyl pyrophosphate (IPP) is derived from acetyl-CoA via the cytosolic mevalonate pathway. IPP is then partially isomerized to 3,3-dimethylallyl pyrophosphate (DMAPP). The condensation of IPP and DMAPP leads to the formation of

geranyl pyrophosphate, the addition of a second IPP unit to the formation of farnesyl pyrophosphate (FPP). The enzyme squalene synthase catalyzes the reaction of two molecules FPP to squalene. Squalene is further oxidized to squalene 2,3-epoxide, which, in turn, undergoes a cyclization to form the first steroidal intermediate cycloartenol (Piironen *et al.*, 2000; Augustin *et al.*, 2011; Nes, 2011). The following pathway involves a series of methylation, desmethylation, desaturation, and isomerization reactions. The alkylation of the side chain at C24 for the synthesis of 24-methyl and 24-ethyl sterols is catalyzed by two families of sterol methyltransferases (SMT 1 and SMT 2) and is one of the critical rate-limiting steps in the regulation of the phytosterol biosynthesis (Bouvier-Navé *et al.*, 1998; Piironen *et al.*, 2000).

Free sterols/stanols are structural components of plant cell membranes where they serve to regulate the fluidity and permeability of the intercellular phospholipid bilayers (Hartmann and Benveniste, 1987; Hartmann, 1998; Piironen *et al.*, 2000; Tjellström *et al.*, 2010). Sterols are also suggested to be involved in the control of membrane-associated metabolic processes, such as the modulation of the activity of membrane-bound H^+-ATPase (Grandmougin-Ferjani *et al.*, 1997; Piironen *et al.*, 2000). There is evidence that phytosterols also play a role in cellular proliferation and differentiation as well as in plant versus pathogen interactions (Hartmann, 1998; Griebel and Zeier, 2010). In addition, phytosterols act as precursors for the biosynthesis of plant steroid hormones and serve as substrates for a wide variety of secondary metabolites such as glycoalkaloids, cardenolides, and saponins (Piironen *et al.*, 2000; Moreau *et al.*, 2002).

In plants, sterols/stanols occur not only as free alcohols but also as esters with fatty acids or phenolic acids, as steryl glycosides, or acylated steryl glycosides (Piironen *et al.*, 2000; Moreau *et al.*, 2002).

Steryl/Stanyl Fatty Acid Esters

Two representative structures of steryl fatty acid esters are shown in Figure 4. Commonly, fatty acids with 12-22 carbon atoms, mainly C16 and C18, are the predominant esterified fatty acid moieties (Dyas and Goad, 1993).

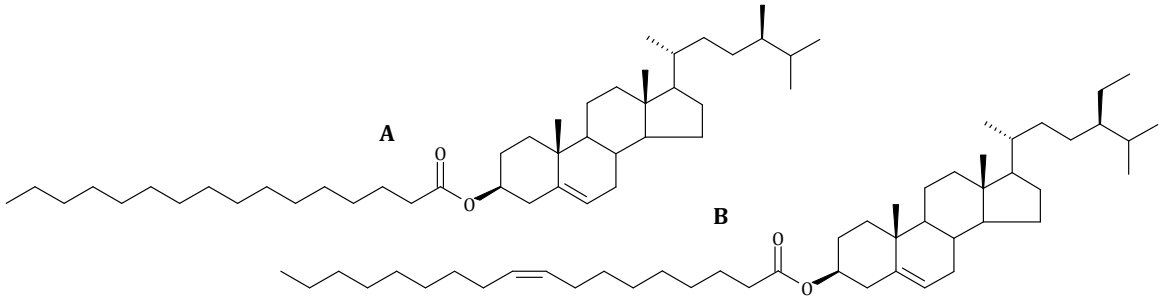

Figure 4. Representative structures of steryl fatty acid esters: (**A**) campesteryl palmitate and (**B**) sitosteryl oleate.

Unlike free sterols/stanols, steryl/stanyl fatty acid esters are largely excluded from membranes, but are mainly localized in lipid bodies in the cytoplasm (Gondet *et al.*, 1994; Moreau *et al.*, 2002). The acylation of sterols via sterol acyl transferases is believed to maintain the free sterol/stanol contents of cell membranes at their physiological levels (Schaller, 2004). Steryl/stanyl fatty acid esters are also presumed to be of storage and transport function (Piironen *et al.*, 2000). Triglycerides, diglycerides, and phospholipids are suggested as the predominant acyl donors (Dyas and Goad, 1993; Chen *et al.*, 2007). However, varying compositions of esterified sterols and fatty acids were observed in different plant tissues, indicating a selective biosynthesis of steryl/stanyl fatty acid esters dependent on the affinity of the acyl transferase as well as on the substrate availability (Kemp *et al.*, 1967; Kemp and Mercer, 1968; Dyas and Goad, 1993).

Steryl/Stanyl Phenolic Acid Esters

Steryl/stanyl phenolic acid esters (Figure 5) seem to be unique constituents of cereal grains (Piironen *et al.*, 2000; Moreau *et al.*, 2002). The occurrence of these esters was reported for the first time in the 1950s, when a mixture of steryl/stanyl ferulic acid esters (called *γ*-oryzanol) was extracted from rice bran oil (Kaneko and Tsuchiya, 1954). Besides steryl/stanyl ferulic acid esters, which are the predominant ester forms, steryl/stanyl *p*-coumaric acid esters have additionally been detected as minor lipid constituents in corn (Seitz, 1989; Norton, 1994; Norton, 1995).

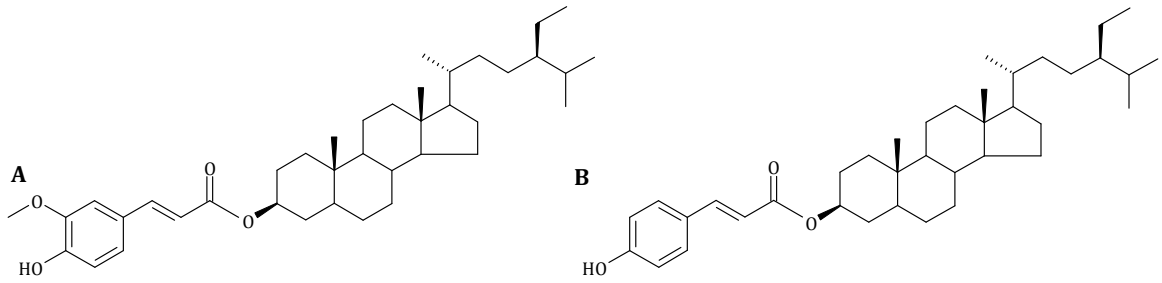

Figure 5. Representative structures of stanyl phenolic acid esters: (**A**) *trans*-sitostanyl ferulate and (**B**) *trans*-sitostanyl *p*-coumarate.

Tyrosine and phenylalanine, amino acids derived of the shikimate pathway, are precursors for the synthesis of ferulic acid and *p*-coumaric acid (Dewick, 2002). However, pathway, regulation, and location of the biosynthesis of steryl/stanyl phenolic acid esters are not known. Steryl/stanyl phenolic acid esters occur in *cis*- and *trans*-form, but the *trans* configuration is the naturally synthesized form (Shahidi and Chandrasekara, 2010). Isomerization can occur when the plants and sample material are exposed to ultraviolet (UV)-light or daylight (Hartley and Jones, 1975; Fenton *et al.*, 1978).

The biological functions of steryl/stanyl phenolic acid esters are still not well known. In plant cells, steryl/stanyl phenolic acid esters may have a protective role as they were suggested to

be involved in the resistance against fungal or other pathogenic infections. However, studies regarding this issue reported contradictory results (Seitz, 1989; Norton, 1994; Norton and Dowd, 1996). The role of steryl/stanyl phenolic acid esters in plants may also be linked to their antioxidant properties, which have been demonstrated in several *in vitro* studies (Juliano *et al.*, 2005; Nyström *et al.*, 2005; Tan and Shahidi, 2013b).

Steryl Glycosides and Acylated Steryl Glycosides

Like free sterols, steryl glycosides and acylated steryl glycosides (Figure 6) are structural components of plant cell membranes (Grille *et al.*, 2010). The glycosylation of the 3ß-hydroxyl group of sterols, usually with the pyranose form of D-glucose, is catalyzed by uridine diphosphate (UDP)-glucose-sterol glucosyltransferases (Grunwald, 1978; Grille *et al.*, 2010). The acylation of the steryl glycosides with fatty acids via acyl transferases may occur at the C6 position of the sugar moiety with palmitic and oleic acid as predominant fatty acid moieties; stearic, linoleic, and linolenic acid are less common (Grunwald, 1978; Grille *et al.*, 2010). Glycosylated sterols have been proposed to be involved in biosynthetic processes and to be also a transport and storage form of sterols (Moreau *et al.*, 2002; Grille *et al.*, 2010). Moreover, one study reported that sitosteryl glucoside serves as primer for the synthesis of cellulose in plants (Peng *et al.*, 2002).

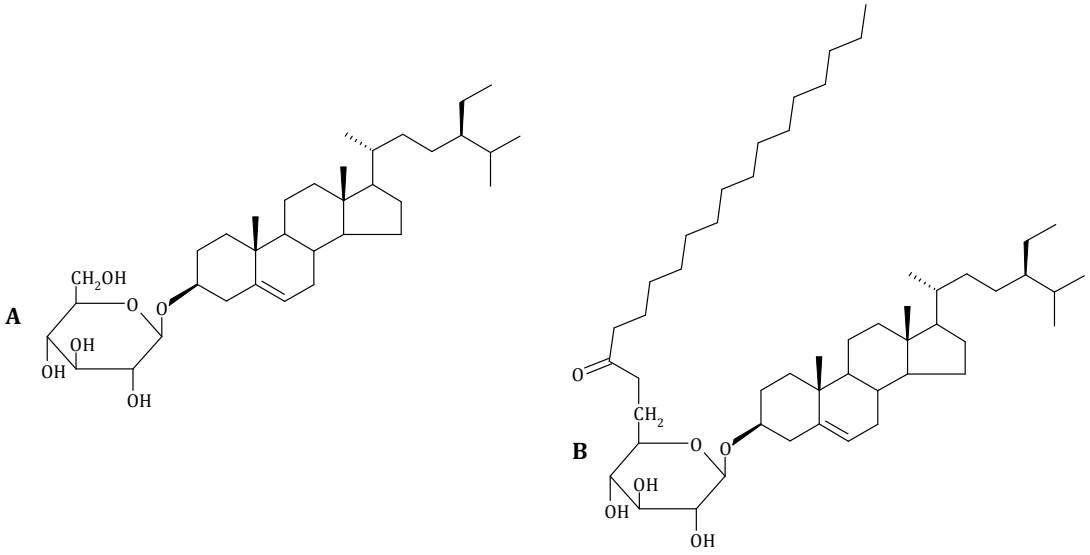

Figure 6. Representative structures of glycosylated sterols: (**A**) sitosteryl-*β*-D-glucoside and (**B**) sitosteryl (6'-*O*-palmitoyl)-*β*-D-glucoside.

2.1.2 Metabolism

In contrast to cholesterol, phytosterols are not synthesized by humans, but are exclusively derived from the intake of plant foods (Salen *et al.*, 1970). The metabolism of phytosterols differs in some issues from that of cholesterol, but the exact molecular mechanisms regarding absorption and excretion are still not completely understood (Betters and Yu, 2010).

A schematic representation of the major pathways of the metabolism of sterols in the human body is shown in Figure 7.

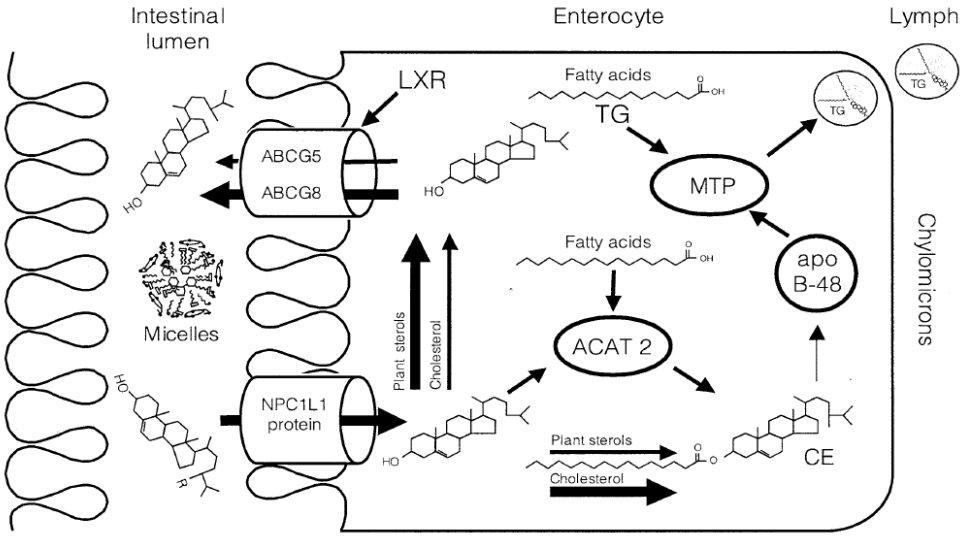

Figure 7. Major pathways of the metabolism of cholesterol and phytosterols in humans (von Bergmann *et al.*, 2005).
ABCG5/G8: adenosine triphosphate-binding cassette G5/G8, NPC1L1: Nieman-Pick C1-Like 1 transport protein; LXR: liver X receptor; TG: triglycerides; ACAT: acyl CoA cholesteryl acyltransferase; MTP: microsomal transport protein; apoB48: apoprotein B48

In the intestinal lumen, sterols are incorporated into mixed micelles, which then are absorbed mainly via the Nieman-Pick C1-Like1 (NPC1L1) transport protein localized in the apical brush boarder membrane of enterocytes (Altmann *et al.*, 2004; Davis *et al.*, 2004; Sané *et al.*, 2006). Also other transport proteins such as the scavenger receptor B type I or the cluster determinant 36 have been suggested to be involved in the intestinal absorption of sterols (Werder *et al.*, 2001; Altmann *et al.*, 2002; Hui *et al.*, 2008; Nguyen *et al.*, 2009). Within the enterocytes, sterols are esterified by acyl CoA cholesteryl acyltransferase 2 (ACAT 2). However, phytosterols are poor substrates for that enzyme and are esterified to a lower degree than cholesterol (Lin *et al.*, 2010a). Non-esterified sterols are excreted back into the intestinal lumen via two adenosine triphosphate-binding cassette (ABC) co-transporters, ABCG5 and ABCG8, which are controlled by the liver X receptor (LXR) (Berge *et al.*, 2000; Lu *et al.*, 2001; Yu *et al.*, 2002). Non-absorbed and re-excreted sterols may be esterified with fatty acids during their intestinal passage or may undergo bacterial transformation by the intestinal colon flora (Wilkins and Hackman, 1974; McNamara *et al.*, 1981; Nissinen *et al.*, 2007). The esterified sterols in the enterocyctes, in turn, are incorporated by microsomal transport protein (MTP) into chylomicrons along with triglycerides and apoprotein B48 (apoB48) (Jia *et al.*, 2011). The chylomicrons are then secreted across the basolateral membrane of the enterocyte into the lymph and are transported to the blood stream (Jia *et al.*, 2011). The triglycerides in the core of the circulating chylomicrons are hydrolyzed by lipoprotein lipase and the remnants including the sterols are adsorbed by the liver via apoB

and apoE receptors (Lecerf and de Lorgeril, 2011). However, in contrast to the metabolism of cholesterol, there is no evidence that phytosterols are converted into bile salts (Boberg *et al.*, 1990). They are either esterified by ACAT 2, followed by their incorporation into very low density lipoprotein and secretion or they are directly excreted into the bile via ABCG5 and ABCG8 (Yu *et al.*, 2002; Jia *et al.*, 2011)

The intestinal absorption of phytosterols/-stanols is much lower than that of cholesterol, although there is a large inter-individual variation reported for the absorption rates of sterols (Lecerf and de Lorgeril, 2011). In humans, about 29-80 % of cholesterol, but only 2-16 % of phytosterols are absorbed (Salen *et al.*, 1970; Heinemann *et al.*, 1993; Lütjohann *et al.*, 1995; Bosner *et al.*, 1999; Ostlund *et al.*, 2002b). The absorption rates of phytostanols, i.e. sitostanol and campestanol, determined by Ostlund *et al.* (2002a) are lower than 1 %; Heinemann *et al.* (1993) described an absorption rate of 12.5 % for campestanol. The differences in the adsorption are attributed to an increased hydrophobicity of phytosterols/-stanols compared to cholesterol caused by the occurrence of an additional methyl or ethyl group in the side chain on C24 as well as by the saturated structure in case of phytostanols (Heinemann *et al.*, 1993). Another reason for the low absorption may be the poor esterification of phytosterols/-stanols in the enterocytes due to the low affinity of ACAT 2 for these compounds (Ntanios *et al.*, 1998; Lin *et al.*, 2010a).

2.1.3 Health Benefits

Cholesterol-Lowering Effects

Cardiovascular diseases (CVD) are the leading causes of death worldwide (WHO, 2013). Among others, high plasma levels of total cholesterol and low-density lipoprotein (LDL) cholesterol are associated with an increased risk of developing CVD (Wilson *et al.*, 1998; Roeters van Lennep *et al.*, 2002). The ability of phytosterols to lower cholesterol was firstly observed in the early 1950s as the dietary intake of sitosterol was shown to prevent cholesterol absorption, leading to reduced blood cholesterol levels in humans (Pollak, 1953a). Meanwhile, phytosterols/-stanols are well known to effectively lower serum concentrations of total and LDL-cholesterol (Demonty *et al.*, 2009; Talati *et al.*, 2010). However, the underlying molecular actions are still not fully understood. A competition of phytosterols/-stanols with dietary and biliary cholesterol for the intestinal solubilization into mixed micelles is considered as the most probable mechanism (Trautwein *et al.*, 2003; Mel'nikov *et al.*, 2004b; Brown *et al.*, 2010; Carr *et al.*, 2010). Free phytosterols/-stanols have a higher affinity for the micelles than cholesterol, with stigmasterol being most effective in decreasing the cholesterol solubilization in micelles in model bile (Brown *et al.*, 2010). A co-crystallization of phytosterols/-stanols with cholesterol, as originally proposed (Pollak,

1953a; Pollak, 1953b), is also discussed as a possible mechanism for the cholesterol-lowering effect, but *in vitro* studies reported inconsistent results (Christiansen *et al.*, 2003; Trautwein *et al.*, 2003; Mel'nikov *et al.*, 2004a). Further, phytosterols/-stanols may have regulatory effects on the gene expression of intestinal sterol transporters, but whether this effect is related to their cholesterol-lowering property is also unexplained (Calpe-Berdiel *et al.*, 2009; Carr *et al.*, 2010). *In vitro* studies on human-derived Caco-2 cells demonstrated that phytosterols may activate LXR accompanied by an increase of the expression of ABC transport proteins and thus resulting in an increased cholesterol efflux in enterocytes (Plat and Mensink, 2002; Plat *et al.*, 2005). Studies on Golden Syrian hamsters or mice, in turn, showed that phytosterols/-stanols decrease the cholesterol absorption independently of the intestinal sterol transport proteins ABCG5, ABCG8, and NPC1L1 (Field *et al.*, 2004; Plösch *et al.*, 2006; Jain *et al.*, 2008).

Altogether, the reduced cholesterol absorption leads to an increase of the endogenous cholesterol biosynthesis and LDL-receptor activity as well as to a decrease in the production of apoB48 and very low density lipoproteins (Ling and Jones, 1995; Brufau *et al.*, 2008; Calpe-Berdiel *et al.*, 2009). The higher expression of the LDL receptor is correlated with the serum concentration and formation of LDL-cholesterol (Plat and Mensink, 2005; Brufau *et al.*, 2008). The increase in the LDL-receptor expression and the reduced levels of apoB48 consequently result in a reduction of serum LDL-cholesterol levels despite the increase of the endogenous cholesterol biosynthesis (Plat and Mensink, 2005; Brufau *et al.*, 2008).

Several meta-studies have recently been published demonstrating the cholesterol-lowering effect of phytosterols/-stanols (Law, 2000; Katan *et al.*, 2003; Berger *et al.*, 2004; Moruisi *et al.*, 2006; AbuMweis *et al.*, 2008; Demonty *et al.*, 2009; Talati *et al.*, 2010; Musa-Veloso *et al.*, 2011). It is assumed that a daily intake of 2 g phytosterols/-stanols decreases serum LDL-cholesterol concentrations by about 10 % in hypercholesterolemic patients; high-density lipoprotein (HDL) cholesterol and triglyceride levels are not affected. It has also been reported that the supplementation form, frequency, time of intake, and baseline LDL-cholesterol levels could have an impact on the cholesterol-lowering effect of phytosterols/-stanols (Clifton, 2002; AbuMweis *et al.*, 2008; Demonty *et al.*, 2009; Carr *et al.*, 2010).

As a consequence of the cholesterol-lowering effect, phytosterols/-stanols are being incorporated into a wide range of foods, mainly margarines, spreads yogurts, cheese, or milk-based beverages (EFSA, 2008). For technological reasons, they are usually employed as fatty acid esters. In the European Union, their use falls in the scope of the Regulation (EC) No. 258/97 of the European Parliament and of the Council of January 27 1997 concerning novel foods and novel food ingredients, and the used profiles of the esterified phytosterols/-stanols have been specified by the Scientific Committee on Food (EU, 1997; SCF, 2000; SCF, 2003a; SCF, 2003b).

The cholesterol-lowering properties of food enriched with phytosteryl/-stanyl fatty acid esters have been demonstrated several times (Demonty *et al.*, 2009). However, as it has been described that intact steryl/stanyl fatty acid esters were not solubilized into mixed micelles (Brown *et al.*, 2010), their intestinal hydrolysis by digestive enzymes may be crucial for their cholesterol-lowering efficacy. *In vitro* studies have shown that phytosteryl/-stanyl fatty acid esters were accepted as substrates for hydrolysis by pancreatic cholesterol esterase (Moreau and Hicks, 2004; Brown *et al.*, 2010). Thereby, the hydrolysis rates were dependent on the esterified sterol/stanol and fatty acid moiety (Brown *et al.*, 2010). The hydrolysis of phytosteryl/-stanyl fatty acid esters has also been studied in human trials (Normén *et al.*, 2006; Nissinen *et al.*, 2007; Lubinus *et al.*, 2013). Lubinus *et al.* (2013), who investigated the metabolic fate of individual phytosteryl/-stanyl fatty acid esters, observed a significant impact of the fatty acid moiety as well as an impact of the sterol/stanol moiety on the hydrolysis rates.

Moreover, a cholesterol-lowering effect was not only shown for phytosteryl/-stanyl fatty acid esters, but also for phytosteryl/-stanyl ferulic acid esters (Rong *et al.*, 1997; Berger *et al.*, 2005; Jain *et al.*, 2008). *In vitro* and *in vivo* studies demonstrated the hydrolysis of these esters by different mammalian digestive enzymes (Miller *et al.*, 2004; Moreau and Hicks, 2004; Nyström *et al.*, 2008b; Mandak and Nyström, 2012; Lubinus *et al.*, 2013). However, it was shown that only 4-desmethylsteryl/-stanyl ferulates were accepted as substrates, but not 4,4'-dimethylsteryl ferulates (Miller *et al.*, 2004; Lubinus *et al.*, 2013).

Compared to enriched foods, the impact of phytosterol/-stanol levels naturally consumed via diets rich in plant food on the cholesterol metabolism and cholesterol-lowering effect have been less studied. However, there is clear evidence that also moderately low intakes of phytosterols/-stanols significantly influence the cholesterol absorption and serum LDL-cholesterol concentrations. Former studies reported that the cholesterol absorption in healthy humans was reduced by 12-43 % when 150-328 mg phytosterols were consumed per test meal (corn oil or wheat germ) compared to the respective sterol-free meal (Ostlund *et al.*, 2002a; Ostlund *et al.*, 2003). Lin *et al.* (2010b) observed a 26 % reduction in the cholesterol absorption for healthy humans, who consumed on average 512 mg phytosterols/d compared to the intake of only 140 mg/d. Additionally, the fecal cholesterol excretion was 79 % higher in the group consuming the phytosterol-rich diet (Lin *et al.*, 2010b). The fact that the intake of phytosterols, naturally present in normal diets, affects the cholesterol metabolism was also confirmed in another study (Sanclemente *et al.*, 2009). However, a significant increase was only detected for surrogate markers of cholesterol synthesis, but not for cholesterol absorption markers.

Further, several studies reported that an increase in the intake of naturally occurring phytosterols is associated with lower serum total and LDL-cholesterol concentrations; HDL-

cholesterol and triglyceride levels were not affected (Ågren *et al.*, 2001; Andersson *et al.*, 2004; Klingberg *et al.*, 2008a; Escurriol *et al.*, 2009b; Sanclemente *et al.*, 2012; Wang *et al.*, 2012). An overview on these results is given for selected studies in Table 1.

Table 1. Effect of increased (low versus high) natural intakes of phytosterols on serum levels of total and LDL-cholesterol.

population	mean phytosterol intake [mg/d]		reduction [%][c] in serum levels of		reference
	low	high	total cholesterol	LDL-cholesterol	
EPIC Norfolk[a], UK (22256 men and women)	178	463	4.1 for men 2.4 for women	3.5 for men 3.2 for women	Andersson *et al.* (2004)
VIP[b], Sweden (77652 men and women)	185	327	2.6 for men 3.5 for women	3.1 for men 3.2 for women	Klingberg *et al.* (2008b)
Chinese population (3940 men and women)	<206	477	6.4 for men 5.0 for women	7.1 for men 6.2 for women	Wang *et al.* (2012)
Spanish population (85 men and women)	≤459	>512	9.7	14.4	Sanclemente *et al.* (2012)

[a] European Prospective Investigation into Cancer (EPIC) population study in Norfolk, UK. [b] Västerbotten Intervention Program (VIP) in Västerbotten, Sweden. [c] Calculation of reduction is based on the comparison of total and LDL-cholesterol levels in groups with high and low daily phytosterol intake.

Anti-Oxidative Effects

Although most studies have been focused on the cholesterol-lowering ability of phytosterols, it has also been shown that they possess several other health benefits like anti-oxidative, anti-cancer, or anti-inflammatory properties.

Particularly, steryl/stanyl phenolic acid esters exhibit anti-oxidative properties due to the radical scavenging effect of the esterified phenolic acid. The donation of a hydrogen atom by the hydroxyl group of the phenolic acid results in the formation of a resonance stabilized phenoxyl radical, which is far less reactive and thus unable to initiate and propagate a radial chain reaction (Graf, 1992). Several *in vitro* studies have proven the anti-oxidative activity of steryl/stanyl ferulic acid ester mixtures extracted from rice (*γ*-oryzanol), rye, or wheat as well as of single compounds such as cycloartenyl, 24-methylene cycloartanyl, campesteryl, sitosteryl, or sitostanyl ferulate (Xu and Godber, 2001; Kikuzaki *et al.*, 2002; Wang *et al.*, 2002; Juliano *et al.*, 2005; Nyström *et al.*, 2005; Nyström *et al.*, 2007b; Tan and Shahidi, 2011). *γ*-Oryzanol further showed anti-oxidative activity against the oxidation of cholesterol (Xu *et al.*, 2001). Recently, anti-oxidative activities have also been demonstrated for various synthesized steryl phenolic acid esters other than ferulates, e.g. sinapates, vanillates, or caffeates in different model systems. Besides, the authors observed a moderate inhibitory effect of these esters on the oxidation of LDL-cholesterol (Tan and Shahidi, 2013b; Tan and Shahidi, 2013a).

In addition, free sterols were also suggested to possess anti-oxidative properties. One study, for example, indicated the anti-oxidative effects of sitosterol, campesterol, and stigmasterol against lipid peroxidation (Yoshida and Niki, 2003). The anti-oxidative effects of sitosterol and Δ^5 avenasterol were demonstrated at high temperatures, which is of particular interest for the stabilization of frying oils, but not directly relevant for humans (Kochhar, 2000; Singh, 2013). Further, an inhibitory effect on the oxidation of LDL-cholesterol, similar to that of steryl phenolic acid esters, was shown for a plant sterol mixture, which consisted of sitosterol, campesterol, sitostanol, and other sterols (Tan and Shahidi, 2013b). However, the anti-oxidative properties of free sterols have yet been fully substantiated.

Anti-Cancer Effects

There is evidence that phytosterols possess preventive effects against certain types of cancer, which have been reviewed several times (Awad and Fink, 2000; Bradford and Awad, 2007; Woyengo *et al.*, 2009; Grattan, 2013). In case-control studies it was observed that the consumption of phytosterols is associated with a reduced risk of lung, stomach, and ovarian cancer (Mendilaharsu *et al.*, 1998; De Stefani *et al.*, 2000; McCann *et al.*, 2003). *In vitro* and *in vivo* studies indicated that phytosterols also inhibited the growth and metastasis of prostate cancer cells (Awad *et al.*, 2001). In addition, a reduced tumor growth is reported for mice with estrogen-dependent breast cancer and fed with *ß*-sitosterol or for mice inherited with breast cancer and supplemented with dietary phytosterols in association with a fat-rich diet (Ju *et al.*, 2004; Llaverias *et al.*, 2013). To date, phytosterols showed, however, no proven effect on colon cancer in rats or humans (Normén *et al.*, 2001; Quilliot *et al.*, 2001). The exact mechanisms of action regarding the anti-cancer properties of phytosterols are not well known. Possibly, phytosterols reduce the production of carcinogens, inhibit metastasis, cell proliferation as well as the growth of cancer cells, and promote the apoptosis of cancer cells (Woyengo *et al.*, 2009; Bradford and Awad, 2010). However, more research is required for a clearer understanding of the anti-cancer effects of phytosterols.

Anti-Inflammatory Effects

Several animal and human studies indicated anti-inflammatory effects of phytosterols, but the exact mechanisms of actions are not fully clarified (Othman and Moghadasian, 2011; Othman and Moghadasian, 2012). Phytosterols may influence signaling pathways associated with apoptosis and immunological function. They may also have an impact on the immune system and the production of inflammatory markers (Othman and Moghadasian, 2011; Othman and Moghadasian, 2012). Preventive inflammatory effects have also been described for steryl ferulates and steryl glycosides (Ríos *et al.*, 1989; Villaseñor *et al.*, 2002; Islam *et al.*, 2011).

2.2 Natural Sources of Phytosterols/-stanols and Phytosteryl/-stanyl Esters

2.2.1 Estimated Intakes of Phytosterols/-stanols via Natural Sources

The estimated intakes of phytosterols in a normal mixed diet are dependent on several factors such as dietary patterns, population, education level, and age; they vary between 228 and 338 mg/d, as recently reported in several European studies (Normén *et al.*, 2001; Valsta *et al.*, 2004; Jiménez-Escrig *et al.*, 2006; Klingberg *et al.*, 2008b; Escurriol *et al.*, 2009a; Hearty *et al.*, 2009; Sioen *et al.*, 2011). The intakes were consistently higher for men than for women. In accordance with these findings, Wang *et al.* (2012) reported for a Chinese population mean phytosterol intakes of 311 mg/d for women and 330 mg/d for men.

Cereals and cereal-based products (e.g. bread, breakfast cereals, or pasta), added fats, spreading fats, and vegetable oils as well as vegetables and fruits are the major dietary sources of phytosterols. The rest of the phytosterols is ingested by the consumption of nuts, seeds, and other plant-based foods. For Belgian, British, Dutch, Finnish, Irish, and Chinese people, cereals and cereal-based products were the most important sources contributing up to 42 % of the total daily intake of phytosterols (Normén *et al.*, 2001; Valsta *et al.*, 2004; Klingberg *et al.*, 2008b; Hearty *et al.*, 2009; Sioen *et al.*, 2011; Wang *et al.*, 2012). Vegetable oils were the main sources of phytosterols in Spanish diets, accounting for approximately 39 % (Jiménez-Escrig *et al.*, 2006). The contribution of vegetable oils, added fats, and spreading fats to the total daily phytosterol intake ranged from approximately 12 to 40 %, those of vegetables and legumes from approximately 7 to 18 %, and those of fruits from approximately 8 to 12 % (Normén *et al.*, 2001; Valsta *et al.*, 2004; Jiménez-Escrig *et al.*, 2006; Klingberg *et al.*, 2008b; Hearty *et al.*, 2009; Sioen *et al.*, 2011; Wang *et al.*, 2012).

2.2.2 Phytosterols/-stanols and Phytosteryl/-stanyl Esters in Cereals

The consumption of whole grains contributes to the prevention of health and chronic diseases due to their significant levels of dietary fiber, proteins, vitamins, minerals, and several phytochemicals, such as phenolic compounds or phytosterols (Awika, 2011). Cereals constitute important dietary sources of phytosterols. The characterization of selected cereal grains and their contents of phytosterols and phytosteryl esters are briefly discussed in the following chapters. The overview is, however, focused on those cereal types which were analyzed in the course of the studies for this thesis.

2.2.2.1 Corn

Corn (*Zea mays* L.) belongs to the grass family *Gramineae* (synonym: *Poaceae*) and further to the sub-family *Panicoideae*, and has been ranked forth concerning the seed production in 2012 after wheat, rice, and barley (FAO, 2012). Based on grain characteristics, corn can be divided in several subspecies, e.g. dent corn, flint corn, sweet corn, or popcorn (Watson, 2003; Lieberei and Reisdorff, 2012). A comparison of the appearance of fresh kernels is shown in Figure 8. Dent corn (*Zea mays* convar. *dentiformis* KOERN.) is the commercially most important subspecies. The kernels are characterized by a vitreous and corneous endosperm at the sides and the back of the kernels, whereas the central core is soft and floury. Upon drying, the crown is sunken to give them their distinct indentation (Watson, 2003). The kernels of flint corn (*Zea mays* convar. *vulgaris* KOERN.) are smooth and rounded , and contain a thick and very hard endosperm (Watson, 2003). Both dent corn and flint corn are used as animal feed, but are also important raw materials for the food industry (Rooney and Serna-Saldivar, 2003). In sweet corn (*Zea mays* convar. *saccharata* KOERN.) the normal conversion of sugar to starch during endosperm development is prevented or reduced due to a mutation. Sweet corn kernels are primarily consumed in fresh form or as canned and frozen vegetable (Marshall and Tracy, 2003). Popcorn (*Zea mays* convar. *microsperma* KOERN.), a small flint corn type, contains a very hard endosperm and only small amounts of soft starch. These kernels are most commonly used for human consumption as freshly popped corn (Ziegler, 2003).

Figure 8. Kernels of different corn subspecies.

Studies on whole corn kernels reported total sterol/stanol contents in the range of 1.8-4.4 % of oil (Moreau *et al.*, 2001; Moreau *et al.*, 2009) and those consisted mainly of sitosterol, campesterol, stigmasterol, sitostanol, and campestanol (Harrabi *et al.*, 2008; Moreau *et al.*, 2009). Overall, 4-desmethylsterols and the saturated stanols were found to be predominant, followed by 4,4'-dimethylsterols and 4-monomethylsterols (Harrabi *et al.*, 2007; Harrabi *et al.*, 2008). The outer layers of corn kernels (pericarp) have been reported as the richest sources of sterols/stanols, and varying distribution patterns were observed between the different corn sections (Harrabi *et al.*, 2008).

The contents and compositions of individual sterol conjugates in corn have been less studied. An overview on total contents of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters in whole corn kernels is given in Table 2. The majority of sterols occurred as fatty acid esters, followed by free sterols/stanols (Moreau *et al.*, 2001; Moreau and Hicks, 2005). However, information on the composition, particularly of individual steryl/stanyl fatty acid esters, is lacking. Davis and Poneleit (1974) studied the accumulation and composition of free sterols and steryl esters in developing corn kernels. They reported sitosterol, campesterol, and stigmasterol as the major sterols, free as well as esterified, at all stages of kernel development. Sitostanyl and campestanyl ferulate, and to a lower extent sitosteryl and campesteryl ferulate were the predominant phenolic acid esters in whole corn kernels. Additionally, small amounts of sitostanyl and campestanyl *p*-coumarate have been detected (Seitz, 1989).

Table 2. Overview on total contents of free sterols/stanols and steryl/stanyl esters in whole corn kernels.

N[a]	free sterols/stanols	steryl/stanyl fatty acid esters	steryl/stanyl phenolic acid esters	reference
2	230-650 µg/g dm[c]	190-540 µg/g dm[e]	-	Davis and Poneleit (1974)
7[b]	-[d]	-	31-70 µg/g	Seitz *et al.* (1989)
3[b]	-	-	0.35-0.41 % of oil	Moreau *et al.* (1999)
49	0.66-1.39 % of oil	0.76-3.09 % of oil	0.09-0.84 % of oil	Moreau *et al.* (2001)
1[b]	0.74 % of oil	1.03 % of oil	0.38 % of oil	Moreau and Hicks (2005)

[a] Number (N) of samples. [b] Indicated as dent corn kernels. [c] Based in dry matter (dm) of ground kernels. [d] (-) Not determined. [e] Determined as esterified sterols after saponification of the respective steryl ester fraction.

It has been reported that free sterols/stanols are mainly localized in the pericarp, whereas steryl/stanyl fatty acid and phenolic acid esters were primarily found in the aleurone and fibre fraction of dissected corn kernels (Seitz, 1990; Moreau *et al.*, 2000). In both fractions, stanols were more abundant than sterols; sterols in turn were predominant in the corn germ (Moreau *et al.*, 2000). The high contents of steryl/stanyl esters in the outer layers are responsible for the comparably high levels of these compounds in corn milling fractions, such as corn bran and corn fibre. Steryl/stanyl phenolic acid esters, for instance, made up 0.2-2.6 % in oils of corn bran and related fractions, and 1.7-7.8 % in oils of different corn fibre fractions (Norton, 1995; Moreau *et al.*, 1996; Moreau *et al.*, 1999; Singh *et al.*, 2001)

2.2.2.2 Small Millets and Sorghum

The term millet is used for several small cereals which are mainly cultivated in the tropics and sub-tropics. Most millets belong to the grass family *Gramineae* and are further classified into two sub-families: *Panicoideae* and *Chloridoideae* (Belton and Taylor, 2002; Morrison, 2004). Millet plants are drought-resistant, mostly insect-resistant, and can be grown on poor soils, which enable the cultivation of these plants in semi-arid areas of African and Asian

cultures (Morrison, 2004). Major millet types are: proso millet (*Panicum miliaceum* L.), foxtail millet (*Setaria italia* (L.) P. BEAUV.), finger millet (*Eleusine coracana* (L.) GAERTN.), teff (*Eragrostis tef* (ZUCC.) TROTTER), pearl millet (*Pennisetum glaucum* L.), kodo millet (*Paspalum sorobiculatum* L.), fonio millet (*Digitaria exilis* (KIPP.) STAPF), Japanese millet (*Echinochloa frumentaceae* LINK), and little millet (*Panicum sumatrense* ROTH) (Belton and Taylor, 2002; Lieberei and Reisdorff, 2012). The first four mentioned millets were studied in this thesis and a comparison of their grains is shown in Figure 9. Millets are not generally relevant for international grain production, but their cultivation is of particular importance for the agriculture and nutrition in some areas in Asia, Central Africa, and West Africa. Teff (also called lovegrass), for example, is one of the most important cereal grains in Ethiopia, accounting for approximately 19 % of the total production of the major crops in the season 2011/2012 (Assefa *et al.*, 2011; CSA, 2012).

Sorghum (*Sorghum bicolor* (L.) MOENCH) has been ranked on place seven concerning the seed production in 2012 worldwide (FAO, 2012). Approximately one third of the sorghum grains is cultivated for human nutrition; the rest is used as animal feed and raw material for industry, e.g. for bioethanol production (Belton and Taylor, 2002; Awika and Rooney, 2004; Taylor *et al.*, 2006). The sorghum kernels are larger than those of the small millets (Figure 9), reaching on maturity approximately 2-5 mm in length and 2-3 mm in thickness (Belton and Taylor, 2002).

Figure 9. Kernels of different small millets and sorghum.

There is only little information available about contents and compositions of sterols in millets and sorghum. Previous studies reported total sterol/stanol levels of 770 µg/g for millet and of 460 and 510 µg/g for two sorghum hybrids (Piironen *et al.*, 2002; Singh *et al.*, 2003). Palmer and Bowden (1977) investigated the sterol accumulation in developing *Sorghum bicolor* grains, and 48 d after anthesis they determined a total sterol level of 314 µg/g dry matter. Another study detected approximately 1 % total sterols/stanols in sorghum grain oil (Leguizamón *et al.*, 2009). The total sterols/stanols of most millets and sorghum grains mainly consisted of sitosterol, campesterol, stigmasterol, and sitostanol (Palmer and Bowden, 1977; Avato *et al.*, 1990; Maestri *et al.*, 1996; Takatsuto and Kawashima, 1998; Takatsuto *et al.*, 1998; Takatsuto *et al.*, 2000; Piironen *et al.*, 2002; Christiansen *et al.*, 2007; Leguizamón *et al.*, 2009). Remarkably high amounts of cholesterol (6-13 % of total sterols/stanols) have been detected in proso millet (Takatsuto *et al.*, 1998). Further, cycloartenol and

citrostadienol were described as the most abundant 4,4'-dimethylsterols and 4-monomethylsterols in foxtail millet (Narumi *et al.*, 2001). Quantitative data on total sterols/stanols in teff were not available. One study reported, however, the presence of sitosterol in an ethanol extract of red teff seeds (El-Alfy *et al.*, 2011).

Data on the contents and distributions of free sterols/stanols and steryl/stanyl esters are also lacking. A summary of available data on total contents of free sterols/stanols and steryl/stanyl fatty acid esters is shown in Table 3. To date, steryl/stanyl ferulic acid esters have only been detected in sorghum, accounting for 0.03 % of oil, but the composition has not been analyzed (Singh *et al.*, 2003). In accordance with the total sterol/stanol profiles, it has been described that the free and esterified sterols of proso millet, finger millet, foxtail millet, and sorghum grains mainly consisted of sitosterol, campesterol, stigmasterol, and sitostanol (Palmer and Bowden, 1977; Mahadevappa and Raina, 1978a; Heupel *et al.*, 1986; Narumi and Takatsuto, 1999; Takatsuto *et al.*, 1999; Christiansen *et al.*, 2008).

Table 3. Overview on total contents of free sterols/stanols and steryl/stanyl esters in small millets and sorghum.

N[a]	free sterols/stanols	steryl/stanyl fatty acid esters	reference
proso millet			
1	7.8 % of nonpolar lipids	3.0 % of nonpolar lipids	Sridhar and Lakshminarayana (1994)
sorghum			
1	320 µg/g dm[b]	-[c]	Heupel *et al.* (1986)
1	4.1 % of nonpolar lipids	2.8 % of nonpolar lipids	Osagie (1987)
2	0.96 (A), 0.59 (B) % of oil	0.61 (A), 0.63 (B) % of oil	Singh *et al.* (2003)
9	≤2.5 % of total lipids	-	Christiansen *et al.* (2007)
finger millet			
1	0.091 % of seed weight	0.013 % of seed weight	Mahadevappa and Raina (1978b)
1	5.8 % of nonpolar lipids	1.2 % of nonpolar lipids	Sridhar and Lakshminarayana (1994)
foxtail millet			
1	6.0 % of nonpolar lipids	4.8 % of nonpolar lipids	Sridhar and Lakshminarayana (1994)
1	249 µg/g	132 µg/g[d]	Narumi and Takatsuto (1999)
1	330 µg/g	142 µg/g[d]	Takatsuto *et al.* (1999)

[a] Number (N) of samples. [b] Based on dry matter (dm) of ground kernels. [c] (-) Not determined. [d] Determined as esterified sterols after saponification of the respective steryl ester fraction.

In addition to the references presented in Table 3, free sterols and steryl esters have also been detected in sorghum and proso millet in a few other studies, but quantitative values have not been reported (Palmer and Bowden, 1977; Lorenz and Hwang, 1986; Christiansen *et al.*, 2008).

2.2.2.3 Other Cereals (Rye, Wheat, and Spelt)

Rye (*Secale cereale* L.), wheat (*Triticum aestivum* L.), and spelt (*Triticum spelta* L.) also belong to the grass family *Gramineae*, but in contrast to corn or millet they are further categorized into the sub-family *Pooideae*. A comparision of the appearance of the kernels is shown in

Figure 10. Rye and wheat are staple foods in human diets and are mainly processed to flours for the production of bread, biscuits, breakfast cereals, and other cereal products (Faridi and Faubion, 1995; Ragaee and Scoles, 2005). Spelt, which is also used as bread flour, is usually more resistant to weather conditions and infections than wheat (Abdel-Aal and Hucl, 2005).

Figure 10. Kernels of rye, wheat, and spelt.

Contents and compositions of total sterols/stanols in rye, wheat, and spelt have been studied several times, also with regard to the impact of cultivar, growing season, or growing location. For example, total sterols/stanols were analyzed in 16 rye, 150 wheat, and 5 spelt cultivars grown at different locations and in different years in the course of the HEALTHGRAIN cereal diversity screen research program (Ward *et al.*, 2008; Shewry *et al.*, 2013). Total sterols/stanols consisted predominantly of sitosterol, campesterol, stigmasterol, sitostanol, and campestanol. The total contents were in the range of 1098-1420 µg/g dry matter flour in rye, 670-959 µg/g in wheat, and 893-963 µg/g in spelt (Nurmi *et al.*, 2008; Nyström *et al.*, 2008a).

To date, studies on individual sterol conjugates have mainly been focused on the analysis of steryl/stanyl ferulic acid esters in rye and wheat (Table 4). Total amounts of steryl/stanyl phenolic acid esters ranged from approximately 29-114 µg/g dry matter with campestanyl, sitostanyl, campesteryl, and sitosteryl ferulate as predominant esters (Seitz, 1989; Hakala *et al.*, 2002; Nyström *et al.*, 2007c; Nurmi *et al.*, 2010; Nurmi *et al.*, 2012; Mandak and Nyström, 2013). Data on free sterols/stanols and steryl/stanyl fatty acid esters, in turn, are scarce. Free sterols/stanols in wheat and spelt comprised sitosterol, campesterol, stigmasterol, sitostanol, campestanol, and small amounts of other sterols (Pelillo *et al.*, 2003; Caboni *et al.*, 2005; Iafelice *et al.*, 2009). To the author's knowledge, information on individual intact steryl/stanyl fatty acid esters have only been reported by Caboni *et al.* (2005), who identified the palmitic, oleic, and linoleic acid esters of campesterol and sitosterol in wheat and spelt.

As described for corn (cf. 2.2.2.1), the bran fractions of rye and wheat grains are rich in phytosterols, particularly in steryl/stanyl ferulic acid esters (Hakala *et al.*, 2002; Nyström *et al.*, 2007c; Nurmi *et al.*, 2012).

Table 4. Overview on total contents of free sterols/stanols and steryl/stanyl esters in whole grains of rye, wheat and spelt.

N[a]	free sterols/stanols	steryl/stanyl fatty acid esters	steryl/stanyl phenolic acid esters	reference
rye				
1	-	1.9 % of oil	-	Zeringue and Feuge (1980)
1	-	-	29 µg/g	Seitz (1989)
2	-	-	55-64 µg/g	Hakala *et al.* (2002)
1	420 µg/g dm[d]	650 µg/g dm	70 µg/g dm	Lampi *et al.* (2004)
1	-[b]	-	44 µg/g dm	Nyström *et al.* (2007b)
5	-	-	65-74 µg/g dm	Nurmi *et al.* (2010)
wheat				
1	-	2.5 % of oil	-	Zeringue and Feuge (1980)
7	-	-	62-123 µg/g	Seitz (1989)
2	-	-	62-63 µg/g	Hakala *et al.* (2002)
1	391 µg/g dm		-	Pelillo *et al.* (2003)
5[c]	-	309-466 µg/g dm	-	Caboni *et al.* (2005)
1	-	-	52 µg/g dm	Nyström *et al.* (2007b)
5[c]	288-387 µg/g dm	219-330 µg/g dm[e]	-	Iafelice *et al.* (2009)
26	-	-	75-114 µg/g dm	Nurmi *et al.* (2010)
1	-	-	101 µg/g dm	Nurmi *et al.* (2012)
1	-	-	99 µg/g dm	Mandak and Nyström (2013)
spelt				
1	300 µg/g dm		-	Pelillo *et al.* (2003)
12[c]	-	355-493 µg/g dm	-	Caboni *et al.* (2005)
12[c]	191-294 µg/g dm	251-291 µg/g dm[e]	-	Iafelice *et al.* (2009)

[a] Number (N) of samples. [b] (-) Not determined. [c] Data reported by Caboni *et al.* (2005) and Iafelice *et al.* (2009) are based on the same sample material. [d] Based on dry matter (dm) of ground kernels. [e] Determined as esterified sterols after saponification of the respective steryl ester fraction.

2.2.3 Phytosterols/-stanols and Phytosteryl/-stanyl Esters in Tree Nuts and Peanuts

Nuts belong to different plant families; therefore, a general definition is difficult. True nuts are commonly characterized as “a dry, one-seeded fruit with an extremely hard pericarp”, whereas false nuts are defined as “any oily, edible seed, including those derived from true nuts” (Dyer, 2011). The nuts investigated in the course of this thesis (Figure 11) can be summarized under the term tree nuts, except for peanuts which are actually legumes. Their botanical classification is as follows (Lieberei and Reisdorff, 2012): almonds (*Amygdalus communis* L.; family *Rosaceae*), cashew kernels (*Anacardium occidentale* L.; family *Anacardiaceae*), Brazil nut (*Bertholletia excelsa* HUMB. et BONPL.; family *Lecythidaceae*), hazelnut (*Corylus avellana* L. family *Betulaceae*), macadamia (*Macadamia tetraphylla* L. JOHNSON; family *Proteaceae*), peanut (*Arachis hypogaea* L.; family *Papilionaceae*), pecan nut (*Carya illinoinensis* (WAGENH.) K. KOCH; family *Juglandaceae*), pine nut (*Pinus pinea* L.; family *Pinaceae*), pistachio (*Pistacia vera* L.; family *Anacardiaceae*), and walnut (*Juglans regia* L.; family *Juglandaceae*).

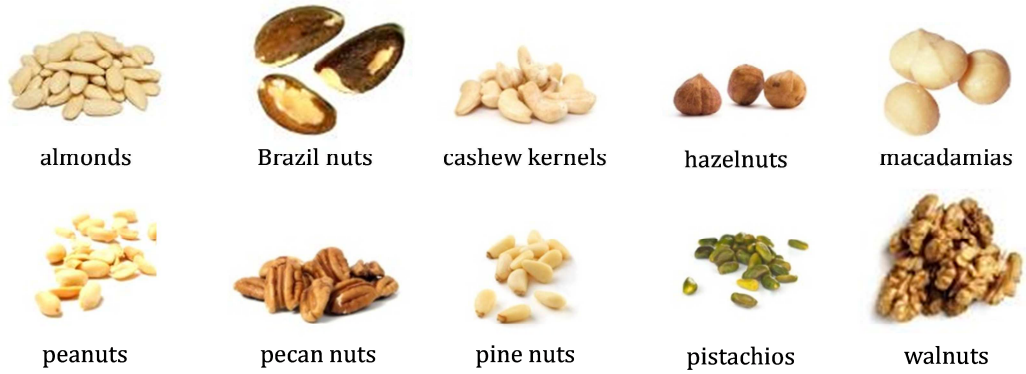

Figure 11. Kernels of various tree nuts and peanuts.

Although tree nuts and peanuts are rich in fat and energy, their regular consumption is associated with several health benefits such as a reduced incidence of coronary heart disease, cancer, type-2 diabetes, inflammation, and several other chronic diseases (Griel and Kris-Etherton, 2006; Ros, 2010; Bolling *et al.*, 2011). These positive health effects are attributed to their high amounts of unsaturated fat, dietary fiber, proteins, and minerals but also to the presence of several other phytochemicals. Besides phenols, squalene, and vitamins, tree nuts and peanuts are also rich in phytosterols.

Past research has mainly been focused on the analysis of total phytosterols/-stanols. Several studies reported either data on total phytosterols/-stanols in several kinds of nuts or investigated for example the impact of genotype, growing year, and location on phytosterols in a single type of nut. Thereby, total phytosterol/-stanol levels have been reported in the range of 0.9-3.7 mg/g nut (Phillips *et al.*, 2005; Shin *et al.*, 2010a; Robbins *et al.*, 2011; Fernández-Cuesta *et al.*, 2012) and in the range of 0.09-0.48 % of nut oil (Kaijser *et al.*, 2000; Amaral *et al.*, 2003; Bada *et al.*, 2004; Crews *et al.*, 2005a; Crews *et al.*, 2005b; Amaral *et al.*, 2006; Nasri *et al.*, 2007). Sitosterol was the most abundant sterol in all nut types, and pistachios and pine nuts usually were the richest sources of phytosterols/-stanols.

Available data concerning total amounts of free sterols/stanols and steryl/stanyl esters in nuts are presented in Table 5. For instance, free sterols and steryl esters have previously been studied in the course of lipid class analysis in different nut types, but only total amounts and not individual compositions have been analyzed (Kashani and Valadon, 1983; Miraliakbari and Shahidi, 2008). A further study investigated the lipid class compositions of almonds, hazelnuts, and walnuts as well as the sterol compositions of the isolated free sterol and steryl ester fractions (Momchilova and Nikolova-Damyanova, 2007). Total amounts of free and esterified sterols as well as the sterol compositions of both fractions have been analyzed in almond, peanut, and walnut oils (Worthington and Hitchcock, 1984; Choong *et al.*, 1999; Kalo and Kuuranne, 2001; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a; Dulf *et al.*, 2010). The contents and profiles of free sterols in nine walnut cultivars have been investigated by Verardo *et al.* (2009); steryl esters have, however, not been included in that study. Last but

not least, the ratios of free and esterified sterols have been analyzed in almonds, Brazil nuts, hazelnuts, and walnuts (Speer and Zahm, 2011). Those were reported to be approximately 70:30, except for walnuts, which exhibited ratios of approximately 50:50. In conclusion, there is still a lack of comparable data on the contents and compositions of individual free sterols/stanols and intact steryl/stanyl esters in various tree nuts and peanuts.

Table 5. Overview on total contents of free sterols/stanols and steryl/stanyl esters in tree nuts and peanuts.

N[a]	free sterols/stanols	steryl/stanyl fatty acid esters	reference
almond			
1	0.6 % of oil	not detected	Momchilova and Nikolova-Damyanova (2007)
1	0.22 % of oil	0.05 % of oil	Miraliakbari and Shahidi (2008)
almond oil			
1	0.12 %	0.08 %[b]	Dulf *et al.* (2010)
Brazil nut			
1	0.18 % of oil	0.05 % of oil	Miraliakbari and Shahidi (2008)
hazelnut			
1	0.3 % of oil	0.4 % of oil	Momchilova and Nikolova-Damyanova (2007)
1	0.21 % of oil	0.04 % of oil	Miraliakbari and Shahidi (2008)
peanut			
2	0.13-0.15 % of oil	0.05-0.07 % of oil[b]	Worthington and Hitchcock (1984)
peanut oil (refined)			
1	0.13 % of oil	0.07 % of oil[b]	Verleyen *et al*. (2002a)
1	2.7 µmol/g of oil	2.4 µmol/g of oil[b]	Kalo and Kuuranne (2001)
1	0.20 % of oil	0.62 % of oil[c]	Choong *et al*. (1999)
2	0.07-0.13 % of oil	0.02-0.10[b]	Phillips *et al.* (2002)
pecan nut			
1	0.26 % of oil	0.07 % of oil	Miraliakbari and Shahidi (2008)
pine nut			
1	0.13 % of oil	0.06 % of oil	Miraliakbari and Shahidi (2008)
pistachio			
1	10.6 mg/g	3.1 mg/g	Kashani and Valadon (1983)
1	0.19 % of oil	0.03 % of oil	Miraliakbari and Shahidi (2008)
walnut			
9	0.09-0.11 % of oil	-[d]	Verardo *et al*. (2009)
1	0.7 % of oil	1.3 % of oil	Momchilova and Nikolova-Damyanova (2007)
1	0.26 % of oil	0.09 % of oil	Miraliakbari and Shahidi (2008)
walnut oil (refined)			
1	0.08 % of oil	0.06 % oil[b]	Verleyen *et al*. (2002a)

[a] Number (N) of samples. [b] Determined as esterified sterols after saponification of the respective steryl ester fraction. [c] Amounts were calculated by subtracting the free sterol content from the total sterol content. [d] (-) Not determined.

2.2.4 Phytosterols/-stanols and Phytosteryl/-stanyl Esters in Plant Oils

Approximately a total of 24 million tons of edible plant oils, mainly palm oil, rapeseed oil, soybean oil, sunflower oil, and other oils such as olive oil have been consumed in the 27 member states of the European Union in the season 2011/2012 (Gunstone, 2012). Thereof, about 52 % was used for food purpose. However, the consumption patterns of vegetable oils

within individual countries are different. Besides a high consumption of palm oil, which is true for nearly all states, Northern countries, for example, mainly consume rapeseed oil, whereas Mediterranean countries additionally have a high intake of sunflower and soybean oil (FEDIOL, 2012).

Edible plant oils are important sources of dietary phytosterols/-stanols and their esters (Piironen *et al.*, 2000). Total sterol/stanol levels in vegetable oils have repeatedly been studied and are typically in the range of 0.04-1.5 % (Piironen *et al.*, 2000; Kamm *et al.*, 2001a). Particularly, rapeseed oil and sunflower oil as well as cereal-derived oils like corn germ oil, wheat germ oil, or rice bran oil are rich in phytosterols/-stanols and can contain up to 3.2 % phytosterols/-stanols (Piironen *et al.*, 2000). Hence, phytosterols/-stanols are essential ingredients of vegetable oils and along with the high amounts of unsaturated fatty acids and other lipid constituents such as tocopherols or squalene they significantly contribute to the health-promoting effects of vegetable oils. However, the presence of phytosterols/-stanols in oils is not only of interest due to their health benefits, their contents and distribution patterns are also useful parameters for authenticity assessments (CODEX, 1999; Aparicio and Aparicio-Ruíz, 2000; Kamm *et al.*, 2001a). As examples, rapeseed oils are rich in brassicasterol; olive oils contain high levels of sitosterol and Δ^5 avenasterol, while sunflower and safflower oils exhibit comparably high amounts of Δ^7 sitosterol (Aparicio and Aparicio-Ruíz, 2000). The sterol distribution patterns and the presence of certain sterols can thus be used for the identification of oils and for the detection of adulterations (Aparicio and Aparicio-Ruíz, 2000; Kamm *et al.*, 2001a).

The phytosterols/-stanols in edible plant oils mainly occur in free form or as esters with fatty acids (Piironen *et al.*, 2000). Both fractions are characterized by partially different sterol/stanol compositions; therefore, the combined analysis of these two classes is suggested for authenticity assessments as it provides more detailed information (Verleyen *et al.*, 2002a; Cunha *et al.*, 2006). Further, it was shown that the refining of oils influences the levels and ratios of free sterols/stanols and steryl/stanyl esters (Johansson and Hoffmann, 1979; Ferrari *et al.*, 1997; Verleyen *et al.*, 2002b).

Some studies reported contents and compositions of free sterols/stanols and steryl/stanyl esters for several types of oils (Johansson and Appelqvist, 1978; Johansson, 1979; Johansson and Hoffmann, 1979; Worthington and Hitchcock, 1984; Grob *et al.*, 1990; Ferrari *et al.*, 1997; Gordon and Miller, 1997; Choong *et al.*, 1999; Lechner *et al.*, 1999; Pasqualone and Catalano, 2000; Kalo and Kuuranne, 2001; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a; Verleyen *et al.*, 2002b; Cercaci *et al.*, 2003; Cunha *et al.*, 2006; Dulf *et al.*, 2010). An overview on total contents of free sterols/stanols and steryl/stanyl fatty acid esters of selected vegetable oils is presented in Table 6.

Table 6. Overview on total contents of free sterols/stanols and steryl/stanyl esters in edible plant oils.

N[ap]	free sterols/stanols	steryl/stanyl fatty acid esters	reference
corn germ oil			
2	0.32-0.37 %	0.95-1.42 %	Worthington and Hitchcock (1984)
1	-[b]	1.09 %	Ferrari *et al.* (1997)
4	-	0.56 % (mean)	Gordon and Miller (1997)
4	0.25-0.49 %	0.42-0.46 %[c]	Verleyen *et al.* (2002a)
1	0.44 %	0.55 %[c]	Verleyen *et al.* (2002b)
2	0.26-0.33 %	0.44-0.45%[c]	Phillips *et al.* (2002)
1	0.29 %	0.45 %[c]	Dulf *et al.* (2010)
grape seed oil			
1	0.20 %	0.09 %[c]	Dulf *et al.* (2010)
olive oil			
8	0.06-0.08 %	0.05-0.09 %	Grob *et al.* (1990)
5	-	0.08 % (mean)	Gordon and Miller (1997)
1	0.09 %	0.20 %[d]	Choong *et al.* (1999)
13	0.09-0.14 %	-	Pasqualone and Catalano (2000)
2	0.13-0.15 %	0.03-0.06 %[c]	Verleyen *et al.* (2002a)
3	0.08-0.13 %	0.04-0.07 %[c]	Phillips *et al.* (2002)
1	-	0.03 %[c]	Cercaci *et al.* (2003)
15	0.06-0.10 %	0.01-0.02 %[c]	Cunha *et al.* (2006)
rapeseed oil			
3	0.3-0.4 %	0.7-1.2 %[e]	Johansson and Appelqvist (1978)
1	-	0.76 %	Ferrari *et al*. (1997)
3	-	0.42 % (mean)	Gordon and Miller (1997)
1	6.2 µmol/g	13.5 µmol/g[c]	Kalo and Kuuranne (2001)
3	0.28-0.34 %	0.47-0.48 %[c]	Verleyen *et al.* (2002a)
5	0.23-0.31 %	0.40-0.52 %[c]	Phillips *et al*. (2002)
safflower oil			
2	-	0.10 % (mean)	Gordon and Miller (1997)
1	0.13 %	0.04 %[c]	Phillips *et al*. (2002)
sesame seed oil			
4	0.54-0.86 %	0.20-0.63 %[d]	Choong *et al*. (1999)
soybean seed oil			
2	0.31-0.34 %	0.06 %[c]	Johansson and Hoffmann (1979)
1	-	0.12 %	Ferrari *et al*. (1997)
4	-	0.06 % (mean)	Gordon and Miller (1997)
2	0.21-0.27 %	0.10-0.13 %[d]	Choong *et al*. (1999)
6	0.19-0.25 %	0.06-0.09 %[c]	Verleyen *et al.* (2002a)
1	0.25 %	0.06 %[c]	Verleyen *et al*. (2002b)
4	0.14-0.23 %	0.05-0.07 %[c]	Phillips *et al*. (2002)
sunflower oil			
1	0.34 %	0.46 %[e]	Johansson (1979)
2	-	0.21 % (mean)	Gordon and Miller (1997)
1	0.28 %	0.38 %[d]	Choong *et al*. (1999)
1	0.34 %	-	Lechner *et al*. (1999)
1	5.0 µmol/g	2.93 µmol/g[c]	Kalo and Kuuranne (2001)
6	0.19-0.29 %	0.11-0.15 %[c]	Verleyen *et al.* (2002a)
1	0.11 %	0.15 %[c]	Phillips *et al*. (2002)

[a] Number (N) of samples. [b] (-) Not determined. [c] Determined as esterified sterols after saponification of the respective steryl ester fraction. [d] Amounts were calculated by subtracting the free sterol content from the total sterol content. [e] Amounts were calculated with the assumption that steryl esters are composed entirely of sitosteryl linoleate.

However, it should be considered that most of the given levels of steryl/stanyl esters reflect amounts of esterified sterols/stanols and not of intact steryl/stanyl fatty acid esters. Esterified sterols were determined after alkaline hydrolysis of the isolated steryl/stanyl ester fractions (cf. 0). Intact steryl/stanyl fatty acid esters, in turn, have been less studied and quantified (Worthington and Hitchcock, 1984; Grob *et al.*, 1990; Ferrari *et al.*, 1997; Gordon and Miller, 1997). Most of the summarized studies demonstrated that free sterols/stanols were predominant in olive, grape seed, safflower, and soybean oils, while corn germ, rapeseed, and sunflower oils mainly contain esterified sterols/stanols (Worthington and Hitchcock, 1984; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a; Verleyen *et al.*, 2002b; Cunha *et al.*, 2006; Dulf *et al.*, 2010).

2.3 Analytical Methods

2.3.1 Analysis of Total Phytosterols/-stanols and Intact Phytosteryl/-stanyl Esters

There are three main approaches which can be applied to the analysis of phytosterols in foods: (i) hydrolysis of the sample material or lipid extract for the analysis of total phytosterols/-stanols, (ii) fractionation of the total lipid extract into several sterol classes, followed by hydrolysis of each fraction, and (iii) fractionation of the total lipid extract into several sterol classes and analysis of the intact conjugates of each fraction (Nyström, 2012). Which approach is most suitable will depend on the scientific interest and analytical target.

The most common methods for determination of total sterols/stanols involve an alkaline hydrolysis either directly of the sample material or of the lipids, which have previously been extracted usually with nonpolar solvents (Abidi, 2001; Lagarda *et al.*, 2006). The alkaline treatment liberates the sterols from their esters and the free sterols can then be extracted as part of the unsaponifiable matter. However, an alkaline hydrolysis does not hydrolyze the glycosidic bonds of steryl glycosides and acylated steryl glycosides, which thus often results in an underestimation of the total sterol/stanol contents of certain samples. Steryl glycosides can be hydrolyzed by enzymes or via acid hydrolysis. Currently, a combination of acid hydrolysis prior to the saponification step is regarded as the most appropriate method. (Toivo *et al.*, 1998; Toivo *et al.*, 2001; Piironen *et al.*, 2002; Nyström, 2012). The unsaponifiable matter is further purified and fractionated via column chromatography (CC), thin layer chromatography (TLC), or solid phase extraction (SPE) (Piironen *et al.*, 2000; Abidi, 2001; Moreau *et al.*, 2002; Lagarda *et al.*, 2006). The final analysis of sterols/stanols, either in free or derivatized form, is usually performed via capillary gas chromatography (GC) equipped with a flame ionization detector (FID). Methods based on high performance liquid chromatography (HPLC) can also be applied (Piironen *et al.*, 2000; Abidi, 2001; Lagarda *et al.*, 2006).

If various forms of sterols/stanols (e.g. free and esterified) should be analyzed, a total lipid extract as well as an efficient sample preparation for the isolation and fractionation is needed, as phytosterols/-stanols are typically minor components of the matrix. SPE, CC, TLC, or normal phase (NP)-HPLC are common techniques for the pre-separation of plant lipids (Piironen *et al.*, 2000; Moreau *et al.*, 2002; Nyström, 2012). Via a subsequent hydrolysis, the sterol/stanol compositions of the isolated fractions can be analyzed, but the loss of information on the contents and distribution patterns of individual intact steryl/stanyl fatty acid and phenolic acid esters is a crucial drawback of these approaches.

Total amounts of free sterols/stanols and intact steryl/stanyl esters can be analyzed by NP-HPLC equipped with an UV-detector or more often with an evaporative light scattering detector (ELSD) (Moreau *et al.*, 2002; Nyström, 2012). Such methods have been applied several times, e.g. to the analysis of total free sterol/stanol and steryl/stanyl ester contents in cereal grains and milling fractions or products derived thereof (Moreau *et al.*, 1996; Moreau *et al.*, 1999; Moreau *et al.*, 2001; Nyström *et al.*, 2007a; Rocha *et al.*, 2010). However, these approaches do not provide information on the distribution patterns of free sterols/stanols or steryl/stanyl esters.

For the analysis of intact steryl/stanyl fatty acid esters, various reversed phase (RP)-HPLC methods have been established (Billheimer *et al.*, 1983; Ferrari *et al.*, 1997; Mezine *et al.*, 2003; Caboni *et al.*, 2005; Barnsteiner, 2012). These approaches resulted, however, in an incomplete separation of individual esters and required mass spectrometric detection to enhance the sensitivity and to differentiate between coeluting esters and other coeluting neutral lipids. RP-HPLC/UV, in turn, has widely been applied to the analysis of individual intact steryl/stanyl phenolic acid esters (Evershed *et al.*, 1988; Seitz, 1989; Norton, 1994; Norton, 1995; Xu and Godber, 1999; Hakala *et al.*, 2002; Fang *et al.*, 2003; Nyström, 2012). However, campestanyl and sitosteryl ferulate, two main constituents of rye, wheat, or corn eluted sometimes in a merged peak (Hakala *et al.*, 2002; Nyström *et al.*, 2007c; Nurmi *et al.*, 2012). Further, a separation of *cis*- and *trans*- isomers by RP-HPLC is often not possible (Nyström, 2012). It has been reported that GC-based methods provide a better separation and enhance the sensitivity, particularly of intact steryl/stanyl fatty acid esters (Mezine *et al.*, 2003; Caboni *et al.*, 2005; Barnsteiner *et al.*, 2011). But also GC analysis of intact steryl/stanyl fatty acid esters is a challenge due to their structural similarities and high boiling points. Previous studies using non-polar (e.g. DB-1 and DB-5) or more polar stationary phases (e.g. 50%-phenyl/50 %-methyl or 50 %-phenol/25 %-methyl/25 %-X polysiloxane) resulted only in insufficient separations regarding the degree of saturation of the esterified fatty acid moiety (Evershed and Goad, 1987; Grob *et al.*, 1990; Gordon and Griffith, 1992; Gordon and Miller, 1997; Kamm *et al.*, 2001b; Caboni *et al.*, 2005; Gunawan *et al.*, 2010). Recently, the suitability of an intermediately polar thermostable stationary phase for the analysis of complex mixtures of steryl/stanyl fatty acid esters was demonstrated (Barnsteiner *et al.*, 2011; Barnsteiner *et al.*, 2012). The esters were separated according to the sterol/stanol moiety as well as according to the carbon number and degree of unsaturation of the esterified fatty acid moiety. The resolution was only hampered by a coelution of saturated and mono-unsaturated fatty acid esters of the same chain length and by a coelution of stigmasteryl esters with campesteryl/-stanyl esters. Despite these drawbacks, the employed stationary phase and the GC conditions provide very useful information on the compositions of complex mixtures of steryl/stanyl fatty acid esters (Barnsteiner *et al.*, 2012). In addition, the same

type of stationary phase was also shown to be suitable for the separation of individual steryl/stanyl ferulic acid esters (Miller *et al.*, 2003).

2.3.2 Principles and Application of On-line LC-GC

As mentioned before, sample preparation is one of the essential steps in the analysis of individual free sterols/stanols and steryl/stanyl esters, and is needed for the concentration of the analytes and the effective removal of di- and triglycerides prior to GC analysis. The on-line coupling of liquid chromatography and gas chromatography (on-line LC-GC) is an efficient and elegant alternative to laborious off-line techniques such as SPE, CC, or TLC. During on-line LC-GC analyses, the LC is used for clean-up, fractionation, and pre-concentration of the sample. The LC fraction(s) of interest can then be transferred on-line to the GC by switching a transfer valve. GC enables the final analysis of the individual composition. By using an on-line LC-GC system, the benefits of both chromatographic techniques are combined (Hyötyläinen and Riekkola, 2003). LC provides a high sample capacity and a good selectivity for clean-up and fractionation. GC, in turn, offers a high sensitivity and separation efficiency for the analysis of individual compounds. Consequently, fractionation, pre-concentration, and analysis take place in a closed and fully automated system, whereby the risks of sample loss and contamination are reduced (Hyötyläinen and Riekkola, 2003). One of the crucial steps of this technique is the evaporation of the large amount of solvent, which is transferred from LC to GC. Therefore, several techniques have been proposed, e.g. loop-type interface, on-column interface, or programmable temperature vaporizer (PTV) interface (Grob, 2000; Dugo *et al.*, 2003; Hyötyläinen and Riekkola, 2003). A schematic representation of the on-line LC-GC system used in the present study is shown in Figure 12. The system was equipped with a PTV interface operating in the solvent vent mode.

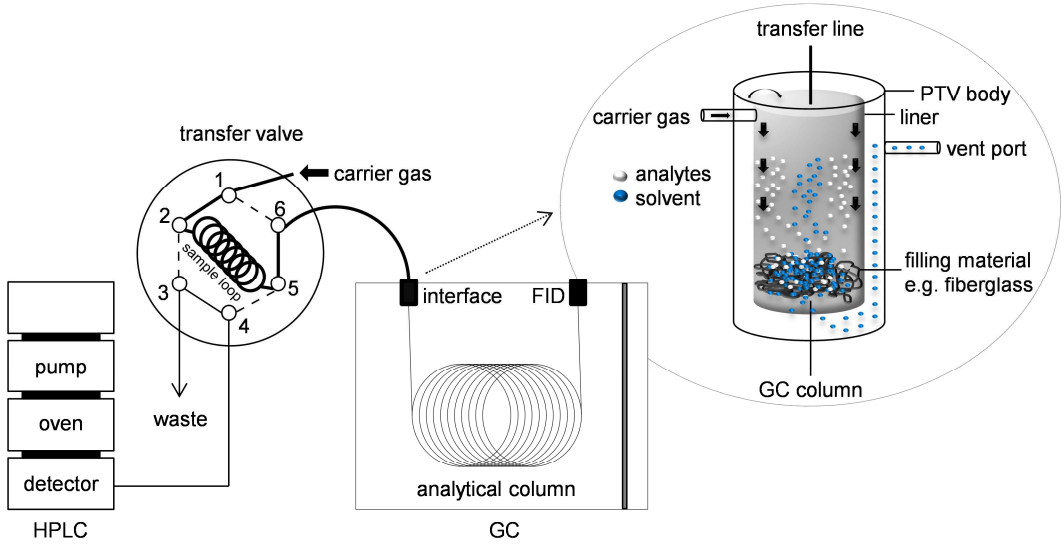

Figure 12. Schematic representation of an on-line LC-GC system equipped with a PTV interface (transfer mode).

During the transfer of the LC fraction the PTV chamber is kept close to the boiling point of the solvent resulting in a concurrent evaporation of the solvent during its introduction. Solvent vapors are then rapidly removed through the split vent port by supply of carrier gas. The loss of analytes is prevented by using a liner packed with an appropriate material, such as fiber glass. At the end of the solvent evaporation, the vent port is closed, the injector is rapidly heated up, and the trapped analytes are released into the GC column. A PTV interface enables the evaporation of the solvent in the inlet prior to the capillary GC column. The additional installation of a solvent vapor exit or pre-columns, as needed for systems with loop-type interface, is not necessary (Dugo *et al.*, 2003; Hyötyläinen and Riekkola, 2003).

On-line LC-GC has already been successfully applied to the analysis of sterols and steryl fatty acid esters in oils and fats, of steryl/stanyl ferulates in rice lipids, or of steryl/stanyl fatty acid esters in enriched fat-based foods (Grob *et al.*, 1989; Grob *et al.*, 1990; Artho *et al.*, 1993; Grob *et al.*, 1994; Lechner *et al.*, 1999; Kamm *et al.*, 2001b; Miller *et al.*, 2003; Barnsteiner *et al.*, 2011; Esche *et al.*, 2013). Usually, the lipids are fractionated on normal silica gel phases as those were shown to be very effective for the retention of triglycerides (Grob *et al.*, 1991). Also approaches based on on-line RP-LC-GC have been described and applied to the analysis of total or free sterols, partially also of other minor lipids, in vegetable oils (Villén *et al.*, 1998; Cortés *et al.*, 2006; Toledano *et al.*, 2012a; Toledano *et al.*, 2012b).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

The following chemicals were used:

Acetic anhydride (p.a., 98 %)	Sigma-Aldrich, Steinheim, Germany
Ammonium chloride (Rectapure®)	VWR International, Darmstadt, Germany
N,O-Bis(trimethylsilyl)trifluoroacetamide + 1 % trimethylchlorosilane (BSTFA/TMCS)	Sigma-Aldrich, Steinheim, Germany
Chloroform (p.a., 99 %)	VWR International, Darmstadt, Germany
5*α*-Cholestan-3*ß*-ol (~95 %)	Sigma-Aldrich, Steinheim, Germany
Cholesteryl palmitate (≥98 %)	Sigma-Aldrich, Steinheim, Germany
Trans-*p*-coumaric acid (>99 %)	Sigma-Aldrich, Steinheim, Germany
Cycloartenol (60 %)	ASM Research Chemicals, Hannover, Germany
Dichloromethane (p.a., 95 %)	Sigma-Aldrich, Steinheim, Germany
N,N'-Dicylcohexylcarbodiimide (≥99 %)	Sigma-Aldrich, Steinheim, Germany
Diethyl ether (p.a.)	VWR International, Darmstadt, Germany
4-Dimethylaminopyridine (puriss, ≥99 %)	Sigma-Aldrich, Steinheim, Germany
Ethyl acetate (puriss, ≥99.8%)	Sigma-Aldrich, Steinheim, Germany
Trans-ferulic acid (99 %)	Sigma-Aldrich, Steinheim, Germany
Heptadecanoic acid (≥98 %)	Sigma-Aldrich, Steinheim, Germany
n-Hexane (HiPerSolv Chromanorm®)	VWR International, Darmstadt, Germany
n-Hexane (AnalR Normapure®)	VWR International, Darmstadt, Germany
Hydrochloric acid (p.a., 25 %)	Sigma-Aldrich, Steinheim, Germany
Linoleic acid (99 %)	Carl-Roth GmbH, Karlsruhe, Germany
Linolenic acid (isomeric)	Carl-Roth GmbH, Karlsruhe, Germany
Magnesium sulfate (anhydrous)	Sigma-Aldrich, Steinheim, Germany
Methanol (puriss, >99 %)	Sigma-Aldrich, Steinheim, Germany
Methyl *tert*-butyl ether	Evonik Industries AG, Essen, Germany
24-Methylene cycloartanol (95 %)	ASM Research Chemicals, Hannover, Germany
Oleic acid (≥99 %)	Sigma-Aldrich, Steinheim, Germany
Palmitic acid (98 %)	Carl-Roth GmbH, Karlsruhe, Germany
Palmitoleic acid (≥99 %)	Sigma-Aldrich, Steinheim, Germany
Piperazine (99 %)	Sigma-Aldrich, Steinheim, Germany

Potassium carbonate (anhydrous)	Riedel de Häen AG, Hannover, Germany
Potassium hydroxide (≥85 %)	Sigma-Aldrich, Steinheim, Germany
iso-Propanol (Chromasolv® for HPLC)	Sigma-Aldrich, Steinheim, Germany
Pyridine (99.8 %)	Sigma-Aldrich, Steinheim, Germany
Sitostanol (~95 %)	Sigma-Aldrich, Steinheim, Germany
Sitosterol (75 %)	Acros Organics, Moris Plains, NJ, USA
Sodium hydroxide (puriss, ≥97 %)	Sigma-Aldrich, Steinheim, Germany
Sodium methylate (for synthesis, 30 % sol.)	VWR International, Darmstadt, Germany
Sodium sulfate (anhydrous, ≥99 %)	Sigma-Aldrich, Steinheim, Germany
Stearic acid (≥99 %)	Sigma-Aldrich, Steinheim, Germany
Stigmasterol (~95 %)	Sigma-Aldrich, Steinheim, Germany
Squalene (≥98 %)	Sigma-Aldrich, Steinheim, Germany
Tetrahydrofuran (anhydrous, ≥99.9 %)	Sigma-Aldrich, Steinheim, Germany
Thionyl chloride (purum)	Sigma-Aldrich, Steinheim, Germany
(±)-α-Tocopherol (95 %)	Sigma-Aldrich, Steinheim, Germany
(+)-γ-Tocopherol (≥96 %)	Sigma-Aldrich, Steinheim, Germany
(+)-δ-Tocopherol (90 %)	Sigma-Aldrich, Steinheim, Germany
Toluene (technical)	VWR International, Darmstadt, Germany
Triethylamine (>99.5 %)	Sigma-Aldrich, Steinheim, Germany

Methyl *tert*-butyl ether (MTBE) and diethyl ether were distilled prior to use.
Mixtures of phytosteryl/-stanyl fatty acid esters (Vegapure® 95E), of tall oil sterols (Generol® 867 F), and of wood stanols (Reducol® Stanol Powder) were provided by Cognis GmbH (Illertissen, Germany). A plant sterol ester mixture (rape seed sterols, STEREST 115) was provided by Raisio Group (Raisio, Finland). A fatty acid methyl ester mixture (Supelco 37 Component FAME Mix) was obtained from Sigma-Aldrich (Steinheim, Germany).

3.1.2 Synthesis of Reference Compounds

3.1.2.1 Synthesis of Steryl/Stanyl Fatty Acid Esters

Individual steryl/stanyl fatty acid esters were synthesized according to previously described procedures (Barnsteiner *et al.*, 2012). The GC purities determined for stigmasteryl palmitate and sitostanyl linoleate were 90 and 84 area%, respectively.
A mixture of steryl esters from rapeseed oil sterols and soy bean oil fatty acids was synthesized as follows: The rapeseed oil sterols were obtained by saponification of 6 g of a plant sterol ester mixture (STEREST 115) using 150 mL ethanolic KOH (3 M). The

saponification was performed under reflux for 2 h and the sterols were extracted with 3 x 50 mL of MTBE. The solvent was removed by rotary evaporation and a silylated solution was analyzed regarding the absence of fatty acids by GC-FID (3.2.4.2). The soybean oil fatty acids were obtained by alkaline saponification of 6 g oil with 150 mL ethanolic KOH (3 M) for 2 h under reflux. The lipids were extracted after acidification with 6 M HCl using 3 x 30 mL of *n*-hexane/MTBE (3:2; v/v). The solvent was removed by rotary evaporation and the residue was dried by a gentle stream of nitrogen. The esterification was performed as described elsewhere (Barnsteiner *et al.*, 2012). The GC purity was 80 area% and the mixture consisted of 24.8 % sitosteryl-18:2, 20.8 % campesteryl-18:2, 14.5 % sitosteryl-18:0/18:1, 12.0 % campesteryl-18:0/18:1, 6.3 % brassicasteryl-18:2, 4.8 % sitosteryl-16:0/16:1, 4.0 % campesteryl-16:0/16:1, 3.7 % brassicasteryl-18:0/18:1, 2.6 % sitosteryl-18:3, 2.1 % campesteryl-18:3, 1.2 % brassicasteryl-16:0/16:1, 0.9 % brassicasteryl-18:3, and 2.5 % others.

3.1.2.2 Synthesis of Steryl/Stanyl Phenolic Acid Esters

The synthesis of steryl/stanyl phenolic acid esters has been established in a previos study (Barnsteiner, 2007) and was based on published methods (Höfle *et al.*, 1978; Helm *et al.*, 1992; Grabber *et al.*, 1996; Lu and Ralph, 1998). Briefly, 2.5 g of phenolic acid was dissolved in 4.5 mL pyridine and 4.0 mL acetic anhydride, and the solution was stirred at room temperature for 4 h. The mixture was transferred into 100 mL of ice-water; the resulting precipitate was isolated by filtration via a Büchner funnel, washed with 20 mL of bi-distilled water, and recrystallized from 30 mL methanol. The acid chloride was prepared by refluxing a mixture of the prepared *O*-acetyl phenolic acid (1.2 g) with thionyl chloride at 70-75 °C for 1.5 h. The reagents were removed by rotary evaporation and the residue was dried by a gentle stream of nitrogen. For esterification, 300 mg of the *O*-acetyl acid chloride and 100 mg of sterol/stanol were dissolved in 5 mL dry 4-dimethylaminopyridine solution (2.5 mg/mL in dichloromethane) and 50 µL triethylamine. The mixture was heated to 50 °C for 16 h. The solution was washed three times with 3 mL of hydrochloric acid (0.1 M), twice with 3 mL of a saturated ammonium chloride solution, and finally with 3 mL bi-distilled water. The organic phase was dried with sodium sulfate. After filtration, the solvent was removed by a gentle stream of nitrogen. For deprotection, about 200 mg of the synthesized steryl/stanyl *O*-acetyl phenolic acid ester were dissolved in 20 mL of dry tetrahydrofuran, and 40 mL of piperazine solution (70 mg/mL in dry tetrahydrofuran) were added drop-wise at room temperature under argon. The mixture was stirred for 2 h at room temperature, diluted with 50 mL of ethyl acetate, and washed 10 times with 50 mL saturated ammonium chloride solution. After drying with magnesium sulfate and filtration, the solvent was removed by rotary evaporation.

The purification was achieved by an acid-base extraction and subsequent SPE. About 100 mg of the residue was dissolved in 20 mL of *n*-hexane, and the solution was mixed with 20 mL of sodium hydroxide (1 M). The aqueous solution was washed twice with 10 mL of *n*-hexane and the organic layer was discarded. After acidification with hydrochloric acid, the synthesized steryl/stanyl phenolic acid esters and remaining free phenolic acids were extracted twice with 20 mL of MTBE. The combined extracts were evaporated to dryness by rotary evaporation, and the residue was used for SPE. The cartridge (Chromabond C18ec, 45 µm, 500 mg, MACHEREY-NAGEL GmbH & Co. KG, Germany) was conditioned with 4 mL of methanol. After transferring of the solid onto the cartridge, the first fraction of 6 mL methanol was discarded. Elution of steryl/stanyl esters was carried out with 8 mL of MTBE. The GC purities of the obtained TMS derivatives of cholestanyl *p*-coumarate, cholestanyl ferulate, sitostanyl *p*-coumarate, and sitostanyl ferulate were 95, 93, 84, and 80 area%, respectively.
Later on, cholestanyl ferulate was synthesized based on a procedure described elsewhere (Condo *et al.*, 2001). The following modifications were performed: (i) The amounts of chemicals were 25-fold reduced and (ii) instead of preparative HPLC, a part of the final synthesis product (100 mg) was purified via SPE using a tube (60 mL) filled with 20 g of silica gel 60 (0.040-0.063 mm, Merck, Darmstadt, Germany). The elution was performed with 50 mL of *n*-hexane/dichloromethane/ethyl acetate (4:2:1, v/v/v). Fractions of 2 mL were collected and investigated regarding the presence of cholestanyl ferulate by GC-FID after silylation. The GC purity of the obtained TMS derivative of cholestanyl ferulate was 94 area%.

3.1.3 Plant Materials

Fresh dent corn and flint corn cobs were provided by Cornexo GmbH & Co. KG (Freimersheim, Germany). The plants were grown at one location (Phillipsburg, Germany) and were harvested in October 2010. Fresh cobs of sweet corn as well as several pre-packaged popcorn kernels were purchased in local stores (Freising, Germany). The characteristics and determined oil contents of the corn samples are summarized in Table 7 and Table 8.

Table 7. Characteristics and oil contents of dent corn and flint corn kernels.

	no.	cultivar, seed producer	harvest year, origin	oil content [%]
dent corn	1	MAS 31a, Maisadour	2010, Germany	3.71 ± 0.05
	2	MAS 32f, Maisadour	2010, Germany	3.92 ± 0.01
	3	Maxxis, Ragt	2010, Germany	3.94 ± 0.02
	4	Futurixx, Ragt	2010, Germany	3.83 ± 0.03
	5	315, Dekalb	2010, Germany	3.75 ± 0.13
	6	Herkuli, Caussade Saaten	2010, Germany	4.15 ± 0.11
	7	Starsky, Caussade Saaten	2010, Germany	3.61 ± 0.04
	8	CSM 9710, Caussade Saaten	2010, Germany	3.15 ± 0.02
	9	DKG 5143, Dekalb	2010, Germany	3.42 ± 0.04
	10	DKG 4490, Dekalb	2010, Germany	3.07 ± 0.02
	11	Amanda, Agromais	2010, Germany	3.39 ± 0.03
	12	PR37Y12, Pioneer	2010, Germany	3.91 ± 0.05
	13	Labouzi, Caussade Saaten	2010, Germany	4.16 ± 0.04
	14	RH 8113, Ragt	2010, Germany	4.05 ± 0.05
	15	P9494, Pioneer	2010, Germany	3.67 ± 0.05
flint corn	1	Crazi, Caussade Saaten	2010, Germany	3.77 ± 0.07
	2	Susann, Saaten Union	2010, Germany	3.89 ± 0.02
	3	Zidane, Agromais	2010, Germany	4.92 ± 0.02
	4	MAS 21d, Maisadour	2010, Germany	3.61 ± 0.04
	5	PR38Y34, Pioneer	2010, Germany	5.69 ± 0.03
	6	Symbol, KWS Saat AG	2010, Germany	4.65 ± 0.07
	7	Marcella, KWS Saat AG	2010, Germany	4.44 ± 0.05
	8	Kompromiss, KWS Saat AG	2010, Germany	4.91 ± 0.04
	9	Luigi, Caussade Saaten	2010, Germany	3.84 ± 0.03
	10	Lg 3258, Limagrain	2010, Germany	3.55 ± 0.03
	11	Suzy, Saaten Union	2010, Germany	3.92 ± 0.06

Table 8. Characteristics and oil contents of sweet corn and popcorn kernels.

	no.	supplier	harvest year, origin	oil content [%]
sweet corn	1	Vitafrisch Gemüse Vertrieb eG, Neckarsulm (D)	2010, Germany	8.83 ± 0.21
	2	Willi Sinn GmbH, Maxdorf (D)	2010, Germany	10.77 ± 0.24
	3	Schweiger's Früchte, Freising (D)	2010, Germany	8.97 ± 0.12
	4	farmer's store, Hallbergmoos (D)	2010, Germany	10.50 ± 0.20
popcorn	1	Alnatura Produktions- und Handels GmbH, Bickenbach (D)	-[a]	4.23 ± 0.14
	2	Seeberger GmbH, Ulm (D)	-	4.33 ± 0.04
	3	EfeFirat Feinkost GmbH, Achim (D)	-	4.00 ± 0.04
	4	Müller's Mühle GmbH, Gelsenkirchen (D)	-	4.04 ± 0.01
	5	Krini GmbH, Weinstadt (D)	-	3.75 ± 0.03

[a] (-) No data available.

The kernels of small millets and sorghum were either purchased in local stores (Freising, Germany) or provided by PrimaVera Naturkorn GmbH (Mühldorf, Germany), by the Chair of Brewing and Beverage Technology of the TU München (Freising, Germany), or by Biogetreidestation Krachbüchler GmbH, (Theresienfeld, Austria). The characteristics and determined oil contents are shown in Table 9.

Table 9. Characteristics and oil contents of small millet and sorghum kernels.

	no.	cultivar/supplier	harvest year, origin	oil content [%]
proso millet	1[a, b]	-[d]/real,-Handels GmbH, Düsseldorf (D)	-	4.01 ± 0.05
	2[b]	Huangmi (1)/PrimaVera Naturkorn GmbH, Mühldorf (D)	2010, China	3.05 ± 0.08
	3[a, b]	Kornberger Rispenhirse/PrimaVera Naturkorn GmbH, Mühldorf (D)	2010, Austria	3.15 ± 0.02
	4[a, b]	Kornberger Rispenhirse/PrimaVera Naturkorn GmbH, Mühldorf (D)	2011, Austria	3.51 ± 0.04
	5[a, b]	-/neuform international, Zarrentin (D)	-	4.41 ± 0.06
	6[b]	Huangmi (2)/PrimaVera Naturkorn GmbH, Mühldorf (D)	2010, China	2.04 ± 0.03
teff	1[c]	Brown/Chair of Brewing and Beverage Technology, Freising (D)	2007, USA	2.66 ± 0.02
	2[c]	Sirgaynia/Chair of Brewing and Beverage Technology, Freising (D)	2007, USA	2.82 ± 0.07
	3[c]	Dessi/Chair of Brewing and Beverage Technology, Freising (D)	2007, USA	3.06 ± 0.12
	4[c]	Ivory/Chair of Brewing and Beverage Technology, Freising (D)	2007, USA	2.60 ± 0.04
	5[c]	-/Biogetreidestation Krachbüchler GmbH, Theresienfeld (A)	2009, Germany	3.10 ± 0.07
	6[c]	Kuncho/Chair of Brewing and Beverage Technology, Freising (D)	2010	3.04 ± 0.02
sorghum	1[a, c]	-/Biogetreidestation Krachbüchler GmbH, Theresienfeld (A)	2011, Austria	3.57 ± 0.01
	2[c]	-/PrimaVera Naturkorn GmbH, Mühldorf (D)	2011	3.67 ± 0.08
	3[c]	-/PrimaVera Naturkorn GmbH, Mühldorf (D)	2011	2.96 ± 0.08
foxtail millet	1[a, c]	Pipsi/Biogetreidestation Krachbüchler GmbH, Theresienfeld (A)	2010, Austria	3.75 ± 0.02
finger millet	1[c]	-/Chair of Brewing and Beverage Technology, Freising (D)	-	1.44 ± 0.02

[a] Organic farming. [b] Seeds without husk. [c] Seeds with husk. [d] (-) No data available.

Pre-packaged whole grains of rye, wheat, and spelt were purchased in local stores (Freising, Germany). The characteristics and determined oil contents are summarized in Table 10.

Table 10. Characteristics and oil contents of rye, wheat and spelt kernels.

	no.	supplier	harvest year, origin	oil content [%]
rye	1	Donath-Mühle GmbH & Co KG, Bad Wörishofen (D)	-[b]	1.54 ± 0.01
	2[a]	Alnatura Produktions- und Handels GmbH, Bickenbach (D)	-	1.46 ± 0.02
wheat	1	Donath-Mühle GmbH & Co KG, Bad Wörishofen (D)	-	1.54 ± 0.03
	2[a]	Alnatura Produktions- und Handels GmbH, Bickenbach (D)	-	1.69 ± 0.03
spelt	1[a]	real,-Handels GmbH, Düsseldorf (D)	-	2.16 ± 0.01
	2	neuform international, Zarrentin (D)	-	2.47 ± 0.03

[a] Organic farming. [b] (-) No data available.

Pre-packaged samples of tree nuts and peanuts were obtained in local stores (Freising, Germany). The nuts were raw and unsalted, except for the peanut samples, which were dry roasted; the characteristics and determined oil contents are summarized in Table 11.

Table 11. Characteristics and oil contents of tree nut and peanut kernels.

	no.	cultivar/supplier	harvest year, origin	oil content [%]
almond	1	-[c]/Stolzenberg Nuss GmbH, Hamburg (D)	2011, Italy	48.4 ± 0.9
	2[a]	Non Pareil/ReformKontor GmbH & Co.KG., Zarrentin (D)	2011, USA	37.2 ± 1.1
	3	Non Pareil/ReformKontor GmbH & Co.KG., Zarrentin (D)	2011, USA	50.3 ± 1.8
Brazil nut	1[a]	-/Seeberger GmbH, Ulm (D)	-	62.4 ± 2.4
	2[a]	-/Stolzenberg Nuss GmbH, Hamburg (D)	2011, Brazil	66.2 ± 3.7
	3[a, b]	-/Flores Farm GmbH, Stuttgart (D)	Bolivia	60.2 ± 1.4
cashew nut	1	-/Seeberger GmbH, Ulm (D)	USA	44.8 ± 0.7
	2[b]	-/Flores Farm GmbH, Stuttgart (D)	Indonesia	43.8 ± 0.4
	3	-/ReformKontor GmbH & Co.KG., Zarrentin (D)	2011	46.0 ± 0.5
hazelnut	1[a]	Ennis/Stolzenberg Nuss GmbH, Hamburg (D)	2010, USA	46.5 ± 0.8
	2[a]	-/Unicoque, Canacon (F)	2010, France	54.6 ± 0.5
	3	Runde Römer/ReformKontor GmbH & Co.KG., Zarrentin (D)	2011, Italy	55.1 ± 2.9
macadamia	1	-/Stolzenberg Nuss GmbH, Hamburg (D)	South Africa	54.6 ± 1.3
	2[b]	-/ReformKontor GmbH & Co.KG., Zarrentin (D)	2011	60.8 ± 1.9
	3[b]	-/Flores Farm GmbH, Stuttgart (D)	Kenia	58.3 ± 1.2
peanut	1	-/Penny Markt GmbH, Köln (D)	2011, China	46.4 ± 1.1
	2	-/real,-Handels GmbH, Düsseldorf (D)	2011, Israel	47.4 ± 2.9
	3	-/Eurofood Handelsgesellschaft mbH, Ahrenburg (D)	2011, USA	46.5 ± 0.5
pecan nut	1[a]	-/Stolzenberg Nuss GmbH, Hamburg (D)	South Africa	66.6 ± 1.0
	2[a]	-/real,-Handels GmbH, Düsseldorf (D)	-	67.6 ± 0.7
	3[a, b]	-/Flores Farm GmbH, Stuttgart (D)	Peru	68.1 ± 2.0
pine nut	1[b]	-/ReformKontor GmbH & Co.KG., Zarrentin (D)	2011, Turkey	43.2 ± 1.0
	2[b]	-/ReformKontor GmbH & Co.KG., Zarrentin (D)	Turkey	46.0 ± 0.1
	3[b]	-/Alnatura Produktions- und Handels GmbH, Bickenbach (D)	Italy	38.1 ± 0.8
pistachio	1[a]	-/Kaufland Warenhandel GmbH & Co. KG, Neckersulm (D)	-	43.0 ± 1.7
	2	-/Seeberger GmbH, Ulm (D)	Iran	44.4 ± 0.1
	3	-/ReformKontor GmbH & Co.KG., Zarrentin (D)	-	45.5 ± 0.2
walnut	1[a]	Serrs/Stolzenberg Nuss GmbH, Hamburg (D)	2011, Chile	60.8 ± 1.6
	2[a]	-/Stolzenberg Nuss GmbH, Hamburg (D)	2011, Chile	57.1 ± 3.0
	3[a]	Franquette/Valcadis, Souillac (F)	2011, France	57.8 ± 2.1

[a] Kernels with testa. [b] Organic farming. [c] (-) No data available.

Edible plant oils were purchased in local stores (Freising, Germany); their brand names, suppliers, and available information on the type of processing are given in Table 12.

Table 12. Characteristics of edible plant oil samples.

	no.	brand/ supplier	processing
rapeseed oil	1	Bellasan/Bökelmann & Co. Ölmühle GmbH & Co, Hamm (D)	refined
	2	Vita D'or/Associated Oil Packers GmbH, Riesa (D)	refined
	3	Gut und Günstig/EDEKA Zentrale AG & Co. KG, Hamburg (D)	refined
	4	Holstensegen/Associated Oil Packers GmbH, Riesa (D)	refined
	5	Tip/real,-Handels GmbH, Düsseldorf (D)	refined
	6	Bonita/Penny Markt GmbH, Köln (D)	refined
	7	Kunella Rapsöl/Kunella Feinkost GmbH, Cottbus (D)	refined
	8	Mazola/Unilever, Hamburg (D)	refined
	9	Bonita pure/Penny Markt GmbH, Köln (D)	refined
	10	Brändle Vita/P. Brändle GmbH Ölmühle-Speisegroßhandel, Empfingen (D)	native
	11	Rapsgold/VPV Vereinigte Pflanzenöl Vertreibsgesellschaft Ltd, Bonn (D)	native
	12	Teutoburger Ölmühle reines Rapskernöl/Teutoburger Ölmühle GmbH & Co. KG, Ibbenbüren (D)	native
	13	real,-bio/real,-Handels GmbH, Düsseldorf (D)	native
	14	Bellasan/Ölmühle Lehen GmbH, Lehen (D)	native
	15	BioPlanète/Huilerie F. J. Moog, Bram (F)	native
sunflower oil	1	Tip/real,-Handels GmbH, Düsseldorf (D)	refined
	2	Gut und Günstig/EDEKA Zentrale AG & Co. KG, Hamburg (D)	refined
	3	Thomy/Nestlé Deutschland AG, Neuss (D)	refined
	4	Lippina/Associated Oil Packers GmbH, Riesa, Germany	refined
	5	Vita D'or/Associated Oil Packers GmbH, Riesa (D)	refined
	6	Bio Sonnenblumenöl/Ölmühle Lehen GmbH, Lehen (D)	native
	7	real,-bio/real,-Handels GmbH, Düsseldorf (D)	native
	8	enerBio/Dirk Rossmann GmbH, Burgwedel (D)	native
	9	Alnatura bio/Alnatura GmbH, Birkenbach (D)	native
	10	vivolio bio/Huilerie F.J Moog, Bram (F)	native
olive oil	1	Bertolli "Originale"/Tavarnelle Val di Pesa (I)	native
	2	Cantinelle 1/Fiorentini Firenze, Tavarnelle Val di Pesa (I)	native
	3	Cantinelle 2/Fiorentini Firenze, Tavarnelle Val di Pesa (I)	native
	4	provided by a private oil mill (GR)	native
	5	Rapunzel/Rapunzel Naturkost GmbH, Legau (D)	native
	6	Luccese/Oleifico R. M. S. p. A., Lucca (I)	native
	7	Mazola/Unilever, Hamburg (D)	native
corn germ oil	1	byodo bio/byodo Naturkost GmbH, Mühldorf (D)	native
	2	Mazola/Unilever, Hamburg (D)	refined
grape seed oil		Brändle Vita/P. Brändle GmbH Ölmühle-Speisegroßhandel, Empfingen (D)	refined
linseed oil		Schneekoppe/Schneekoppe GmbH & Co. KG, Buchholz (D)	native
safflower oil		high oleic Bellasan/Bökelmann & Co. Ölmühle GmbH & Co, Hamm (D)	refined
sesame seed oil		Mazola/Unilever, Hamburg (D)	native
soybean oil		Sojola/Vandemoortle Deutschland GmbH, Dresden (D)	refined

3.2 Methods

3.2.1 Sample Preparation

3.2.1.1 Preparation of Cereals

The kernels of six to eight fresh dent corn, flint corn, and sweet corn cobs were manually removed and dried in a cabinet drier at 40 °C for 72 h. About 200 g of the dried corn kernels as well as of all other cereal samples were frozen in liquid nitrogen and ground using a cyclone mill equipped with a 500 µm sieve (1093 Cyclotec, Foss GmbH, Rellingen, Germany). Subsequently, the flours were freeze-dried (Alpha 1-4 LSC, Christ, Osterode, Germany) for 48 h and stored in plastic bags at -18 °C until analysis.

3.2.1.2 Preparation of Tree Nuts and Peanuts

After removal of the shells, the nuts were chopped with a knife to obtain pieces of about 3 mm. About 20 g of the chopped nuts were frozen in liquid nitrogen and ground for approximately 5 sec in a coffee mill (Franz Morat GmbH & Co. KG, Eisenbach, Germany) to a fine powder (particle size <0.5 mm). The ground nut samples were stored in plastic bags at -18 °C until analysis.

3.2.2 Determination of Dry Matter and Moisture

Dry matter of the freeze-dried cereal samples was determined by drying 5 g flour in pre-weighed containers at 130 ± 2 °C until constant weight (LFGB, 2008b). To determine the dry matter of the ground nuts, 5 g of each sample was weighed in pre-weighed containers and dried at 103 ± 2 °C (approximately 8 h) until constant weight (UN, 2002). The analysis was performed in triplicate for cereals and in duplicate for nuts. Dry matter and moisture contents were calculated based on Equations 1 and 2, respectively.

Equation 1: $dry\ matter = \frac{w_3 - w_1}{w_2 - w_1} \cdot 100\ \%$

Equation 2: $moisture = 100 - dry\ matter$ in %

w_1: weight of empty container [g], w_2: weight of container with sample before drying [g], w_3: weight of container with sample after drying [g]

The residual moisture contents of the cereal flours were lower than 3 %. The nuts revealed average dry matter contents of 97.2 ± 0.2 % (almonds), 97.5 ± 0.1 % (Brazil nuts), 95.8 ± 0.1 % (cashew kernels), 96.7 ± 0.2 % (hazelnuts), 97.8 ± 0.2 % (macadamias),

97.8 ± 0.5 % (peanuts), 95.6 ± 2.6 % (pecan nuts), 95.4 ± 1.1 % (pine nuts), 96.6 ± 0.8 % (pistachios), and 97.2 ± 0.2 % (walnuts).

3.2.3 Lipid Extraction

All used devices were wrapped with aluminum foil to avoid *trans-cis* isomerization of steryl/stanyl phenolic acid esters.

3.2.3.1 Lipid Extraction of Cereal Flours

4 g of the freeze-dried flours of corn, small millet, and sorghum, respectively, and 8 g of the rye, wheat, and spelt flours, respectively, were weighed into an extraction vessel. Subsequently, the following internal standards (IS) were added: (i) 500 µL of cholesteryl palmitate (1.0 mg/mL in *n*-hexane/MTBE, 3:2, v/v), 500 µL of 5*α*-cholestan-3*ß*-ol (1.0 mg/mL in MTBE), and 80 µL of cholestanyl *p*-coumarate (1.0 mg/mL in MTBE) in case of the SPE-based approach (cf. 3.2.4); (ii) 500 µL of cholesteryl palmitate (1.0 mg/mL in *n*-hexane/MTBE, 3:2, v/v), 500 µL of 5*α*-cholestan-3*ß*-ol (1.0 mg/mL in MTBE), and 100 µL of cholestanyl ferulate (1.0 mg/mL in ethyl acetate) in case of the on-line LC-GC-based approach used for corn (cf. 3.2.5); (iii) 500 µL of cholesteryl laurate (1.0 mg/mL in *n*-hexane/MTBE, 3:2, v/v), 500 µL of 5*α*-cholestan-3*ß*-ol (1.0 mg/mL in MTBE), and 100 µL of cholestanyl ferulate (1.0 mg/mL in ethyl acetate) in case of the on-line LC-GC-based approach used for millet or sorghum (cf. 3.2.5). After adding a magnetic stir bar, the extraction was performed with 40 mL of *n*-hexane/dichloromethane (1:1, v/v) under stirring for 1 h at room temperature. After filtration of the solution (filter disc grade 389, MUNKTELL & FILTRAL GmbH, Bärenstein, Germany), the extraction vessel and the filter were washed, and the filtrates were combined in pre-dried (1 h at 103 ± 2 °C) and pre-weighed round-bottom flasks. The solvent was evaporated by rotary evaporation (Heidolph, Schwabach, Germany). The final residue was dried at 103 ± 2 °C until constant weight. The lipid contents were calculated based on Equation 3.

Equation 3: $lipid\ content = \frac{w_2 - w_1}{w_3} \cdot 100\ \%$

w_1: weight of empty and pre-dried round-bottom flask [g], w_2: weight of round-bottom flaks and oil after drying [g], w_3: weighed portion of sample [g]

3.2.3.2 Lipid Extraction of Ground Tree Nuts and Peanuts

2 g of the ground nuts were weighted into an extraction vessel and the following ISs were added: 500 µL of cholesteryl palmitate (1.0 mg/mL in *n*-hexane/MTBE, 3:2, v/v) and 500 µL

of 5*α*-cholestan-3*ß*-ol (1.0 mg/mL in MTBE). The lipids were extracted using 20 mL of *n*-hexane/dichloromethane (1:1, v/v). The remaining procedure was performed as described for cereals (cf. 3.2.3.1).

3.2.4 Solid-Phase Extraction and GC Analysis of Free Sterols/Stanols and Steryl/Stanyl Esters

3.2.4.1 Solid-Phase Extraction

100 mg of the extracted oil (cf. 3.2.3) was dissolved in 10 mL of *n*-hexane. Exactly 1 mL of this solution was loaded onto the SPE cartridge (Strata NH_2, 55 µm, 70 Å, 1 g/6 mL, Phenomenex, Aschaffenburg, Germany), which was previously activated with 2 x 5 mL of *n*-hexane. The fraction of steryl/stanyl fatty acid esters was eluted with 2 x 5 mL of *n*-hexane/diethyl ether (98:2, v/v). Subsequently, the cartridge was washed with 4 x 5 mL *n*-hexane/ethyl acetate (96:4, v/v) to remove interfering triglycerides. Free sterols/stanols were eluted with 2 x 5 mL of *n*-hexane/ethyl acetate (5:95, v/v), followed by the elution of steryl/stanyl phenolic acid esters with 2 x 5 mL of *n*-hexane/ethyl acetate (5:95, v/v) and 5 mL of MTBE. The solvents were removed by rotary evaporation and the residues of the fractions of the steryl/stanyl fatty acid esters and free sterols/stanols were dissolved in 500 µL and 1000 µL of *n*-hexane, respectively. The residue of the steryl/stanyl phenolic acid ester fraction was silylated with 20 µL of pyridine and 100 µL of BSTFA/TMCS (99:1, v/v) at 80 °C for 20 min. After silylation, the reagents were removed by a gentle stream of nitrogen and the residue was dissolved in 50 µL of *n*-hexane.

3.2.4.2 GC-FID Analysis

The analysis of the individual composition of each SPE-fraction (1 µL injection volume (i.v.)) was performed using a 6890N GC equipped with an FID (Agilent Technologies, Böblingen, Germany). The separations were carried out on a 30 m × 0.25 mm i.d., 0.1 µm film, Rtx-200MS fused silica capillary column (Restek, Bad Homburg, Germany). The temperature of the injector was set to 280 °C and hydrogen was used as carrier gas with a constant flow of 1.5 mL/min. The split flow was set to 11.2 mL/min, resulting in a split ratio of 1:7.5. The oven temperature program was as follows: initial temperature, 100 °C (2 min); programmed at 15 °C/min to 310 °C (2 min), and at 1.5 °C/min to 340 °C (3 min). The FID temperature was set to 340 or 360 °C and nitrogen was used as makeup gas with a flow of 25 mL/min. Data acquisition was performed by ChemStation B.04.03 software.

3.2.4.3 Quantification by GC-FID

Free sterols/stanols were quantified using 5*α*-cholestan-3*ß*-ol as IS with a response factor (Rf) of 1.0.

The linearity of the GC-FID responses of individual steryl/stanyl esters was determined within the calibration range of 0.1-0.5 µg/µL using a five-point calibration. The GC-FID detector was also shown to be linear up to an additionally examined concentration of 0.8 µg/µL. Steryl/stanyl fatty acid esters were quantified by generating three-point calibration functions with 0.1, 0.3, and 0.5 µg of total steryl/stanyl esters (Vegapure® 95E) and 0.05 µg cholesteryl palmitate (IS) per µL of injection. Each calibration solution was prepared in triplicate from separate stock solutions. Linear regression was confirmed in ratios of areas (area steryl or stanyl ester/area IS) and amounts (amount steryl or stanyl ester/amount IS). Steryl/stanyl fatty acid esters that were not included in the Vegapure® 95E mixture were quantified with a Rf of 1.0 in relation to the IS cholesteryl palmitate.

Steryl/stanyl phenolic acid esters were determined as their TMS-derivatives with the following Rfs in relation to the IS cholestanyl *p*-coumarate: 1.05 for stanyl *p*-coumarates and steryl ferulates, respectively, and 1.15 for stanyl ferulates.

3.2.4.4 Identification by GC-MS

Identification of the individual free sterols/stanols and steryl/stanyl esters was performed on a Finnigan Trace GC ultra coupled with a Finnigan Trace DSQ mass spectrometer (Thermo Electro Corp., Austin, TX, USA). Mass spectra were obtained by positive electron impact ionization at 70 eV in the scan mode at unit resolution from 40 to 750 Da. The interface was heated to 330 °C; the ion source to 250 °C. Gas chromatographic separations were carried out on a 30 m × 0.25 mm i.d., 0.1 µm film, Rtx-200MS fused silica capillary column (Restek, Bad Homburg, Germany). Helium was used as carrier gas with a constant flow of 1.0 mL/min. The remaining GC conditions were as described for GC-FID analysis (cf. 3.2.4.2). Data acquisition was performed by Xcalibur 1.4 SR1 software.

3.2.4.5 Validation of the SPE-Based Approach

The SPE-based approach was applied to the analysis of free sterols/stanols and steryl/stanyl esters in corn. To determine recoveries, corn flour (4 g) was spiked with defined amounts of the IS and of selected steryl/stanyl derivatives: 500 µg of 5*α*-cholestan-3*ß*-ol, stigmasterol, and sitostanol, respectively, as reference compounds for free sterols/stanols; 500 µg of cholesteryl palmitate, stigmasteryl palmitate, and sitostanyl linoleate, respectively, as reference compounds for steryl/stanyl fatty acid esters; 80 µg of cholestanyl *p*-coumarate,

sitostanyl *p*-coumarate, and sitostanyl ferulate as reference compounds for steryl/stanyl phenolic acid esters.

To confirm the repeatability of the method, a control sample (corn flour) was worked up once on each day of analysis (in total, 10 replicates).

The limits of detection (LODs) and the limits of quantification (LOQs) were determined via the linear regression methodology according to official German procedures and criteria (DIN, 2008). Each regression analysis was performed in triplicate; residual standard deviations (s_y), LODs and LOQs were calculated using Equation 4, 5, and 6, respectively:

Equation 4: $$s_{y,x} = \sqrt{\frac{\sum_{i=1}^{n}(\bar{y}_i - y_i)}{n-2}}$$

$s_{y,x}$: residual standard deviation, $\bar{y}_i$: calculated peak area (area ratio) based on calibration function ($y_i = a + bx_i$), y_i: measured peak area (area ratio), n...number of measured values

Equation 5: $$LOD = \frac{s_{y,x}}{b} \cdot t_{f,\alpha} \cdot \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{\bar{x}^2}{Q_x}}$$

$s_{y,x}$: residual standard deviation, b: slope of the regression line, $t_{f,\alpha}$: t-value (f = n – 2, α = 0.05), m: number of calibration standards, n: number of parallel determinations, $\bar{x}$: mid-working range, Q_x: deviance

Equation 6: $$LOQ = k \cdot \frac{s_{y,x}}{b} \cdot t_{f,\alpha} \cdot \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(3 \cdot LOD - \bar{x})^2}{Q_x}}$$

$s_{y,x}$: residual standard deviation, b: slope of the regression line, $t_{f,\alpha}$: t-value (f = n – 2, α = 0.05), m: number of calibration standards, n: number of parallel determinations, $\bar{x}$: mid-working range, Q_x: deviance, k = 3

3.2.5 On-line LC-GC Analysis of Free Sterols/Stanols and Steryl/Stanyl Esters

3.2.5.1 Sample Preparation

100 mg of the extracted cereal grain oils (cf. 3.2.3) were dissolved in 10 mL of *n*-hexane; 50 mg of the nut oils and edible plant oils, respectively, were dissolved in 5 mL of *n*-hexane. The edible plant oils were previously spiked with the following ISs: 100 µL of 5*α*-cholestan-3*ß*-ol (1.0 mg/mL in MTBE), 100 µL (50 µL in case of olive oils) of cholesteryl palmitate (1.0 mg/mL in *n*-hexane/MTBE, 3:2, v/v), 50 µL hexadecanoic acid (1.0 mg/mL in MTBE), and 50 µL *trans*-cholestanyl ferulate (1.0 mg/mL in ethyl acetate).

250 µL of each solution was transferred into a 1.5 mL vial. The solvent was removed by a gentle stream of nitrogen and the residue was silylated with 75 µL of pyridine and 150 µL of BSTFA/TMCS (99:1, v/v) at 80 °C for 20 min. After silylation, the reagents were removed by a

gentle stream of nitrogen and the residue was dissolved in 250 µL of *n*-hexane/MTBE/*iso*-propanol (96:4:0.1, v/v/v).

3.2.5.2 On-line LC-GC-FID Analysis

For on-line LC-GC analysis, a 1220 Infinity LC was coupled with a 7890A GC equipped with an FID. Coupling was achieved via a 1200 Infinity Series 2-position/6-port switching valve fitted with a 200 µL sample loop (Agilent Technologies, Waldbronn, Germany).

LC separations were carried out on a 250 mm x 2 mm, 5 µm, Eurospher-100 Si column (Knauer, Berlin, Germany) at 27 °C using *n*-hexane/MTBE/*iso*-propanol (96:4:0.1, v/v/v) as eluent. Detection was performed with an UV detector set to 205 nm for free sterols/stanols and steryl/stanyl fatty acid esters and set to 325 nm for steryl/stanyl phenolic acid esters. The transfer valve was switched 3.7 min after injection for transfer 1 (free sterols/stanols and steryl/stanyl fatty acid esters; flow: 0.25 mL/min; i.v.: 5 µL for cereals, rapeseed oils, corn germ oils, and sunflower oils, 10 µL for olive oils, soybean oils, safflower oils, sesame seed oils, grape seed oils, and linseed oils, and 20 µL for nuts) and 6.75 min after injection for transfer 2 (*trans*-steryl/stanyl ferulic acid esters; flow: 0.20 mL/min; i.v.: 20 µL for cereals or edible plant oils and 40 µL for nuts).

The evaporation of the solvent was performed via the programmable multimode inlet in the solvent vent mode. The injector was set to 50 °C (0.5 min) and then heated up with 900 °C/min to 350 °C. Vent flow was adjusted to 1000 mL/min and vent pressure was hold at 4 psi for 0.5 min. Purge flow to split vent (2.5 mL/min) was started 0.5 min after transfer. To avoid a pushing back of the solvent vapors into the transfer line, a stainless steel transfer line was installed between the valve and the inlet. This line was pressure-controlled as follows: 5 psi (0.3 min); programmed at 10 psi/min to 20 psi.

GC separations were carried out on a 30 m × 0.25 mm i.d., 0.1 µm film, Rtx-200MS fused silica capillary column (Restek, Bad Homburg, Germany). Hydrogen was used as carrier gas with a constant flow 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 (2 min), and at 1.5 °C/min to 340 °C (3 min). The detector temperature was set to 340 or 360 °C and nitrogen was used as makeup gas with a flow of 25 mL/min. Data acquisition was performed by ChemStation B.04.03 software.

3.2.5.3 Quantification by On-line LC-GC-FID

The TMS-derivatives of free sterols/stanols were quantified in relation to the IS 5α-cholestan-3β-ol with a Rf of 1.0.

Steryl/stanyl fatty acid esters in cereals were quantified by generating three-point calibration functions with 0.1, 0.3, and 0.5 µg of total esters (Vegapure® 95E) per µL of injection. The fatty acid esters in edible oils were calculated based on three-point calibration functions prepared either of a commercially obtained steryl/stanyl ester mixture (range: 0.03-0.17 µg or 0.1-0.5 µg Vegapure® 95E per µL of injection) or of a steryl ester mixture synthesized from rapeseed oil sterols and soybean oil fatty acids (range: 0.3-0.6 µg steryl esters per µL of injection). Each calibration solution contained 0.05 µg/µL cholesteryl palmitate as IS. In nuts, the steryl/stanyl fatty acid esters were quantified via three-point calibration functions generated either of the synthesized steryl ester mixture or of the Vegapure® 95E mixture (range: 0.8-14.2 or 0.8-20.8 ng steryl/stanyl esters per µL of injection, respectively) in the presence of 5 ng/µL cholesteryl palmitate. At first, the responses of individual steryl/stanyl esters were determined within each calibration range using five-point calibration functions; they showed always good linearity (coefficients of determination (R^2) ≥0.99). On this basis, steryl/stanyl fatty acid esters were subsequently quantitated by generating three-point calibration functions. Each calibration solution was prepared in triplicate from separate stock solutions. Linear regression was confirmed in ratio of areas (area steryl or stanyl ester/area IS) and amounts (amount steryl or stanyl ester/amount IS). Esters that were not included in the calibration solutions were quantified with a Rf of 1.0 in relation to the IS cholesteryl palmitate.

Steryl/stanyl ferulates were determined as their TMS-derivatives in relation to the IS *trans*-cholestanyl ferulate with the following Rfs: 1.01 for stanyl ferulates and 1.15 for steryl ferulates.

Tocopherols and squalene, which were additionally quantified in nuts and edible oils, were determined via 5*α*-cholestan-3*ß*-ol with a Rf of 1.2 and 0.92, respectively.

The free fatty acids in edible plant oils were determined via hexadecanoic acid as IS with a Rf of 1.0.

3.2.5.4 Identification by On-line LC-GC-MS

The LC fractions of free sterols/stanols and steryl/stanyl esters of the sweet corn and popcorn samples were collected after visualization at 205 and 325 nm; after concentration they were analyzed by GC-MS (cf. 3.2.4.4).

During the further analysis, an on-line LC-GC-MS system was used for the identification. The GC part was coupled via a transfer line to an inert 5975C mass spectrometer with a 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 40 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 carried out on a 30 m × 0.25 mm i.d., 0.1 µm film, Rtx-200MS fused silica capillary column (Restek, Bad Homburg, Germany). Hydrogen was used as carrier gas with constant flow (1.5 mL/min). The remaining GC conditions were as described for the on-line LC-GC-FID analysis (cf. 3.2.5.2). Data acquisition was performed by MSD Productivity ChemStation E.02.02 and Masshunter B.05.00 software.

3.2.5.5 Validation of the On-line LC-GC-Based Approach

The on-line LC-GC-based approach was applied to the analysis of free sterols/stanols and steryl/stanyl esters in sweet corn, popcorn, small millets, sorghum, tree nuts, peanuts, and edible plant oils.

The limits of detection (LODs) and the limits of quantification (LOQs) were determined via the linear regression methodology according to official German procedures and criteria (DIN, 2008). Each regression analysis was performed in triplicate and LODs and LOQs were calculated using Equation 5 and 6, respectively (cf. 3.2.4.5).

Validation Experiments for Cereals

To determine recoveries for cereals as matrix, 4 g flour (corn flour and proso millet flour) was spiked with defined amounts of selected steryl/stanyl derivatives: 500 µg of 5*α*-cholestan-3*ß*-ol, stigmasterol, and sitostanol, respectively, as reference compounds for free sterols/stanols; 500 µg of cholesteryl palmitate, stigmasteryl palmitate, and sitostanyl linoleate, respectively, as reference compounds for steryl/stanyl fatty acid esters; 100 µg of cholestanyl ferulate and sitostanyl ferulate, respectively, as reference compound for *trans*-steryl/stanyl ferulic acid esters. The recovery experiments were performed in triplicate for both corn and proso millet flour.

To confirm the repeatability of the method, control samples (corn flour and proso millet flour) were worked up once on each day of analysis (in total, 5 replicates for corn and 10 replicates for proso millet).

Validation Experiments for Tree Nuts and Peanuts

The recoveries of selected steryl/stanyl derivatives were determined by spiking 2 g of ground walnut, hazelnut, and peanut, respectively, with defined amounts of selected steryl/stanyl derivatives: 100 µg stigmasterol and 350 µg sitosterol as reference compounds for free sterols/stanols; 100 µg of stigmasteryl palmitate as reference compound for steryl/stanyl fatty acid esters; 50 µg of *α*- and *δ*-tocopherol, respectively, as reference compounds for tocopherols. The recovery experiments were performed in triplicate for each type of nut.

The repeatability of the method was verified by analysis of a control sample (ground walnut) once on each day of analysis (in total, 10 replicates)

Validation Experiments for Edible Plant Oils

For edible plant oils, recoveries were determined by spiking rapeseed oil, corn germ oil, and sunflower oil with two different amounts of selected steryl/stanyl derivatives: 10 and 40 µg stigmasterol as well as 150 and 300 µg sitosterol as reference compounds for free sterols/stanols; 10 and 80 µg of stigmasteryl palmitate as reference compound for steryl/stanyl fatty acid esters; 10 and 40 µg of *α*- and *δ*-tocopherol, respectively, as reference compounds for tocopherols; 10 and 40 µg palmitic and stearic acid, respectively, as reference compounds for free fatty acids. The recovery experiments were performed in triplicate for each oil type.
The method repeatability was confirmed by a 10-fold analysis of rapeseed oil, corn germ oil, and sunflower oil, respectively, on two different days.

3.2.6 GC Analysis of Fatty Acid Methyl Esters (FAME)

3.2.6.1 Preparation of FAME

The FAME composition of each sample was determined according to an official German method (LFGB, 2008a). The extraction of lipids with *n*-hexane/dichloromethane (1:1, v/v) was performed as previously described (cf. 3.2.3), but without previous addition of IS. Edible plant oils were directly used for the analysis.
About 60 mg of each oil was weighed in a 11 mL vial and dissolved in 4 mL of *n*-hexane. After adding 200 µL of methanolic KOH (2 M), the mixture was vigorously shaken and left standing for 10 min at room temperature. Subsequently, the solution was neutralized using 2 N HCl and the upper phase was diluted 1:5 with *n*-hexane for GC-FID analysis.

To determine the fatty acid composition of steryl/stanyl fatty acid esters, the extracted oils were fractionated via SPE as previously described (cf. 3.2.4). The fraction of steryl/stanyl fatty acid esters was collected and the solvent was removed by a gentle stream of nitrogen. In case of the nuts and some edible plant oils, about 20 SPE fractions were collected to obtain a more concentrated solution. The combined and dried steryl/stanyl fatty acid ester fractions were dissolved in 100 µL of *n*-hexane. After adding 100 µL of sodium methylate (30 % in methanol), the solutions were vigorously shaken and left standing for 1 h at room temperature. The samples were neutralized with 6 M HCl and the upper phase was directly used for GC-FID analysis.

3.2.6.2 GC-FID Analysis

GC analysis of FAME (1 µL i.v.) was performed on a 6890N GC equipped with an FID (Agilent Technologies, Böblingen, Germany). Separations were carried out on a 60 m × 0.32 mm i.d., 0.25 µm film, DB-1 fused silica capillary column (J&W Scientific columns, Agilent Technologies, Folsom, CA, USA). The temperature of the injector was set to 280 °C and hydrogen was used as carrier gas with constant flow (1.5 mL/min). The split flow was set to 15.0 mL/min, resulting in a split ratio of 1:10. The oven temperature program was as follows: initial temperature, 100 °C; programmed at 4 °C/min to 320 °C. The FID temperature was set to 320 °C and nitrogen was used as makeup gas with a flow of 25 mL/min. Data acquisition was performed by ChemStation B.04.03 software.
Peak assignments were performed either via comparison of retention times to a FAME reference mix (Supelco 37 component FAME mix) or by GC-MS.

3.2.6.3 GC-MS Analysis

GC-MS analysis was carried out on a Finnigan Trace GC ultra coupled with a Finnigan Trace DSQ mass spectrometer (Thermo Electro Corp., Austin, TX, USA). Mass spectra were obtained by positive electron impact ionization at 70 eV in the scan mode at unit resolution from 40 to 400 Da. The interface was heated to 300 °C; the ion source to 250 °C. GC separations were carried out on a 60 m × 0.32 mm i.d., 0.25 µm film, DB-1 fused silica capillary column (J&W Scientific columns, Agilent Technologies, Folsom, CA, USA). Helium was used as carrier gas with constant flow (1.0 mL/min). The remaining GC conditions were as described for GC-FID analysis (cf. 3.2.6.2). Data acquisition was performed by Xcalibur 1.4 SR1 software.

3.2.7 Data Analysis

Each experiment was performed in triplicate, if not indicated otherwise, and results represent the means ± standard deviations. Principal component analysis (PCA) and box-whisker plots were created using XLSTAT 2013 (Addinsoft, Paris, France). The whiskers of the boxplots were defined with maximum 1.5x interquartile range. Outliers between 1.5x interquartile range and 3x interquartile range were classified as mild outliers; outliers above 3x interquartile range were classified as strong outliers. Equality of variances was tested with Levene's test; normal distribution with Shapiro-Wilk test. Either student's *t*-test, Mann-Whitney *U*-test, or Welch *t*-test were used to test for equality of means. The difference were considered as statistically significant at $p<0.05$. Statistical analyses were performed using XLSTAT 2013 (Addinsoft, Paris, France).

4 RESULTS AND DISCUSSION

4.1 Method Development for the Analysis of Free Sterols/Stanols and Intact Steryl/Stanyl Esters

Despite the benefits of a phytosterol-rich diet for humans, comprehensive data concerning individual steryl/stanyl conjugates in natural sources are lacking. Particularly, information on the contents and compositions of intact steryl/stanyl fatty acid esters is rare, also due to the lack of appropriate methods. The primary aim of the present study was the development of analytical approaches, which enable the qualitative and quantitative determination of free sterols/stanols and of intact steryl/stanyl fatty acid and phenolic acid esters within one single working up procedure. Therefore, two methods were established. The first method was based on SPE for the fractionation of the extracted plant lipids and subsequent GC analysis; the second approach was based on on-line LC-GC. Both procedures were validated concerning recovery, repeatability, limits of detection, and limits of quantification.

4.1.1 Establishment and Validation of an Approach Based on SPE and GC

4.1.1.1 SPE and GC Analysis

The high amounts of triglycerides in plant lipids are crucial as they interfere with the direct GC analysis of free sterols/stanols and steryl/stanyl esters. Using corn as example, an efficient method based on SPE was established for the purification, fractionation, and isolation of free sterols/stanols and steryl/stanyl esters from plant lipids. As the removal of triglycerides with a normal silica phase was not sufficient, an aminopropyl-modified silica phase was employed. This material has already been described to be suitable for the fractionation of lipids, e.g. for the separation of microbial lipids (Pinkart *et al.*, 1998). In the present study, triglycerides were effectively removed with a mixture of *n*-hexane/ethyl acetate (96:4, v/v) subsequent to the elution of steryl/stanyl fatty acid esters with a mixture of *n*-hexane/diethyl ether (98:2, v/v). Free sterols/stanols were eluted with a mixture of *n*-hexane/ethyl acetate (5:95, v/v), directly followed by the elution of steryl/stanyl phenolic acid esters with a mixture of *n*-hexane/ethyl acetate (5:95, v/v) and MTBE.

The procedure, finally employed for the fractionation of plant lipids into free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters is presented in Figure 13.

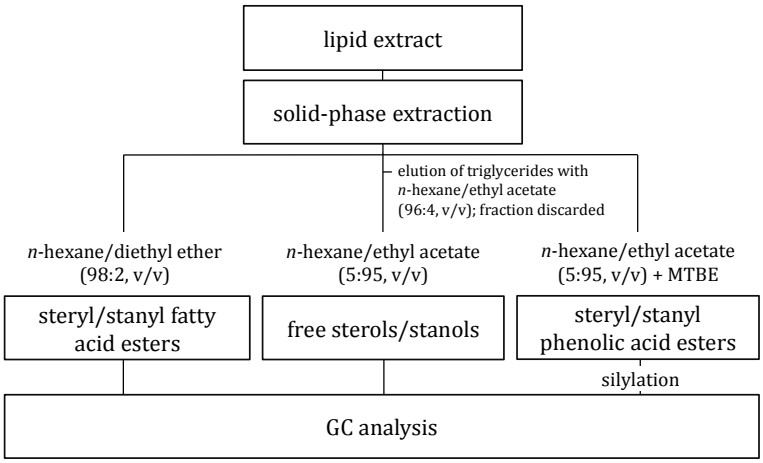

Figure 13. Outline of the procedure for the fractionation of plant lipids into free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic esters.

The SPE fractions were qualitatively and quantitatively analyzed by means of GC-FID and GC-MS. The GC separations were achieved using an intermediately polar capillary column coated with a film of trifluoropropylmethyl polysiloxane, which has been shown to be suitable for the analysis of intact steryl/stanyl fatty acid esters and steryl/stanyl ferulic acid esters (Miller *et al.*, 2003; Barnsteiner *et al.*, 2011; Barnsteiner *et al.*, 2012).

The GC analyses of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters isolated from a wholegrain flour of corn are shown in Figure 14.

The GC separation of steryl/stanyl fatty acid esters was achieved according to the carbon number and the degree of saturation of the esterified fatty acid. Furthermore, steryl esters could be distinguished from their respective stanyl esters. However, the resolution was hampered by the coelution of esters of saturated and monounsaturated fatty acids of the same chain length and by a coelution of stigmasteryl esters with campesteryl linoleate and campestanyl esters. Compared to the analysis of intact steryl/stanyl esters on non-polar stationary phases (e.g. DB-1 or DB-5), where a separation of main esters like oleates and linoleates was not possible (Kamm *et al.*, 2001b; Caboni *et al.*, 2005; Gunawan *et al.*, 2010), the employed GC conditions provide very detailed information on the individual composition of naturally occurring steryl/stanyl fatty acid esters.

Free sterols/stanols were directly analyzed by means of GC-FID without further derivatization; steryl/stanyl phenolic acid esters were transferred into their TMS-derivatives to improve the resolution and sensitivity. The employed stationary phase allowed the analysis and separation of *cis*- and *trans*-ferulic acid esters as well as of coumaric acid esters within one single run. Ferulic or coumaric acid esters of stanols eluted just after their respective steryl esters.

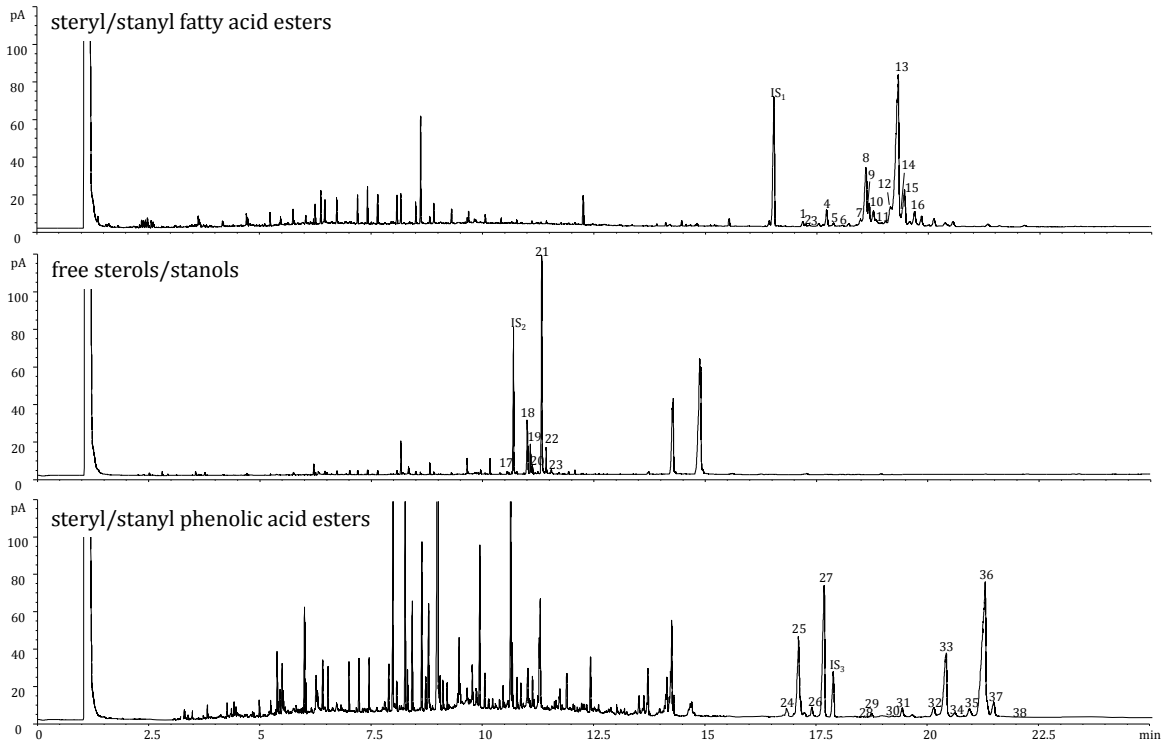

Figure 14. GC-FID analysis of the SPE fractions of steryl/stanyl fatty acid esters, free sterols/stanols, and steryl/stanyl phenolic acid esters extracted from corn.
Steryl/stanyl fatty acid esters: (1) campesteryl-16:0/16:1, (2) stigmasteryl-16:0/16:1, (3) campestanyl-16:0/16:1, (4) sitosteryl-16:0/16:1, (5) sitostanyl-16:0/16:1, (6) Δ^7 sitosteryl-16:0/16:1, (7) campesteryl-18:0/18:1, (8) campesteryl-18:2 + stigmasteryl-18:0/18:1, (9) stigmasteryl-18:2 + campestanyl-18:0/18:1, (10) campestanyl-18:2, (11) Δ^7 campesteryl-18:2, (12) sitosteryl-18:0/18:1, (13) sitosteryl-18:2, (14) sitostanyl-18:0/18:1, (15) sitostanyl-18:2, (16) Δ^7 sitosteryl-18:2, and (IS_1) cholesteryl-16:0; free sterols/stanols: (17) cholesterol, (18) campesterol, (19) stigmasterol, (20) campestanol, (21) sitosterol, (22) sitostanol, (23) Δ^7 sitosterol and unknown sterol, and (IS_2) 5*α*-cholestan-3*ß*-ol; steryl/stanyl phenolic acid esters: (24) *cis*-campesteryl ferulate, (25) *cis*-campestanyl ferulate, (26) *cis*-sitosteryl ferulate, (27) *cis*-sitostanyl ferulate, (28) *trans*-campesteryl *p*-coumarate, (29) *trans*-campestanyl *p*-coumarate, (30) *trans*-sitosteryl *p*-coumarate, (31) *trans*-sitostanyl *p*-coumarate, (32) *trans*-campesteryl ferulate, (33) *trans*-campestanyl ferulate, (34) *trans*-Δ^7 campesteryl ferulate, (35) *trans*-sitosteryl ferulate, (36) *trans*-sitostanyl ferulate, (37) *trans*-Δ^7 sitosteryl ferulate, (38) *trans*-24-methylene cycloartanyl ferulate, and (IS_3) *trans*-cholestanyl *p*-coumarate.

4.1.1.2 Validation of the SPE and GC-Based Approach

The approach was validated in terms of recovery, LOD, LOQ, and repeatability. Recoveries, LODs, and LOQs of the ISs as well as of various reference compounds representative for each sterol class are summarized in Table 13. Recoveries, which were determined by spiking corn flour with known amounts of selected steryl/stanyl derivatives, were >92 %. To confirm the repeatability of the method during workup, a control sample (corn flour) was analyzed once on each day of analysis. The relative standard deviations of ten replicate analyses of the total contents of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters were <9 %, indicating a good repeatability.

Table 13. Recoveries, LODs, and LOQs for ISs and selected steryl/stanyl derivatives determined via GC-FID.

	recovery [%]	LOD [µg/mL][a]	LOQ [µg/mL][a]
internal standards			
5*α*-cholestan-3*β*-ol	98.0 ± 2.0	0.03	0.07
cholesteryl palmitate	97.9 ± 1.9	0.04	0.09
cholestanyl *p*-coumarate	93.6 ± 5.3	1.48	3.36
free sterols/stanols and steryl/stanyl esters			
stigmasterol	99.1 ± 0.6	0.03	0.07
sitostanol	95.2 ± 0.3	0.03	0.07
stigmasteryl palmitate	96.7 ± 1.9	0.13	0.34
sitostanyl linoleate	102.9 ± 1.4	0.16	0.52
sitostanyl *p*-coumarate	96.9 ± 4.5	1.10	3.30
sitostanyl ferulate	92.8 ± 3.7	3.60	7.01

[a] LODs and LOQs expressed as µg/mL of i.v. (GC-FID).

4.1.2 Establishment and Validation of an Approach Based on On-line LC-GC

4.1.2.1 On-line LC-GC Analysis

The on-line coupling of LC and GC is an efficient and elegant alternative to off-line techniques such as isolation and fractionation via SPE or TLC and subsequent GC analysis. On-line LC-GC has already been successfully applied to the analysis, e.g. of intact steryl/stanyl fatty acid esters (Grob *et al.*, 1989; Grob *et al.*, 1990; Grob *et al.*, 1994; Lechner *et al.*, 1999; Kamm *et al.*, 2001b; Barnsteiner *et al.*, 2011; Esche *et al.*, 2013), of free sterols (Grob and Lanfranchi, 1989; Grob *et al.*, 1989; Grob *et al.*, 1990; Grob *et al.*, 1994), and of steryl ferulic acid esters (Miller *et al.*, 2003) in complex food matrices containing considerable amounts of triglycerides. However, the coupling of LC and GC is not trivial and method development requires the establishment of an appropriate LC method, of a suitable interface technique for the evaporation of the large solvent amounts, which are transferred from LC to GC, and thus also of suitable GC conditions.

The primary objective was the implementation of an on-line LC-GC method for the analysis of individual free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid ester in plant lipids. Therefore, a fully automated on-line LC-GC system with PTV interface was used (cf. 2.3.2). The LC separations were carried out on a normal silica phase as this material was shown to have a high capacity to retain triglycerides (Grob *et al.*, 1991; Miller *et al.*, 2003; Barnsteiner *et al.*, 2011). LC conditions were optimized in terms of mobile phase composition and flow rate.

First attempts to analyze the lipid extracts directly without derivatization failed as the retention times of free sterols/stanols were not constant under the employed LC conditions (Figure 15); thus, an automated transfer would have not been possible.

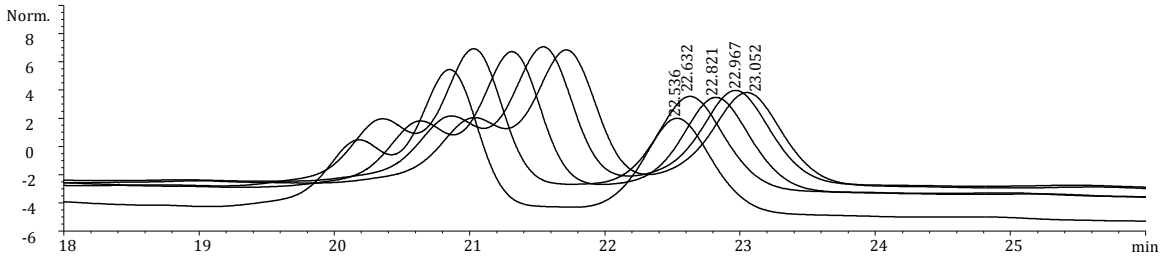

Figure 15. LC-UV analysis of underivatized free sterols/stanols at 205 nm after 5-fold injection of a non silylated corn sample (eluent: *n*-hexane/*iso*-propanol 99:1, flow: 0.20 mL/min).

Therefore, the lipid extracts and references substances were silylated prior to on-line LC-GC analysis. Through the TMS-derivatization, free sterols/stanols and steryl/stanyl fatty acid esters eluted at the same time, when a mixture of *n*-hexane/MTBE/*iso*-Propanol (96:4:0.1, v/v/v) was used as eluent with a flow of 0.25 mL/min, and could thus be transferred together to the GC. In addition to free sterols/stanols and steryl/stanyl fatty acid esters, steryl/stanyl phenolic acid esters should be transferred in a second step. However, *cis*-steryl/stanyl ferulic acid esters and *trans*-steryl/stanyl coumaric acid esters were separated from *trans*-steryl/stanyl ferulic acid esters under the employed LC conditions and the separation of these compounds from other matrix constituents was not sufficient. They eluted in a merged peak closely after steryl/stanyl fatty acid esters and free sterols/stanols, so that the on-line transferred fraction always contained small amounts of co-transferred steryl/stanyl fatty acid esters. Variations of the polarity of the mobile phase did not yield sufficient improvements as it either resulted in a broadening of the LC peak accompanied by a loss in resolution or in a coelution with triglycerides. Thus, only the *trans*-steryl/stanyl ferulates could be analyzed as their TMS-derivatives in a second transfer. An overlay of the LC separations of a silylated corn lipid extract detected at 205 and 325 nm is shown in Figure 16.

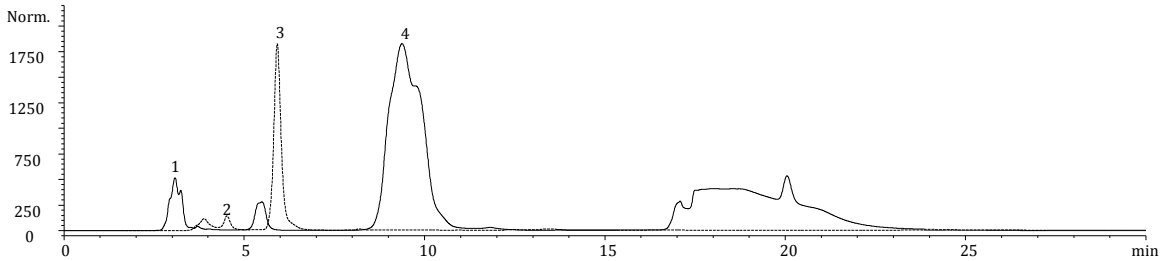

Figure 16. LC-UV analysis of a silylated corn sample detected at 205 nm (–) and 325 nm (--).
(1) free sterols/stanols and steryl/stanyl fatty acid esters, (2) *trans*-steryl/stanyl *p*-coumaric acid esters and *cis*-steryl/stanyl ferulic acid esters, (3) *trans*-steryl/stanyl ferulic acid esters, and (4) triglycerides; 15 min after injection the column was rinsed with MTBE (For conditions cf. 3.2.5.2.).

The final GC analysis of the transferred fraction(s) was performed via the intermediately polar stationary phase previously shown to be suitable for the analysis of free sterols/stanols and complex mixtures of steryl/stanyl esters in corn (cf. 4.1.1.1).

An outline of the applied on-line LC-GC-based approach is presented in Figure 17; on-line LC-GC analyses of free sterols/stanols and steryl/stanyl fatty acid esters as well as of *trans*-steryl/stanyl ferulic acid esters are exemplarily shown for a corn lipid extract in Figure 18 and Figure 19.

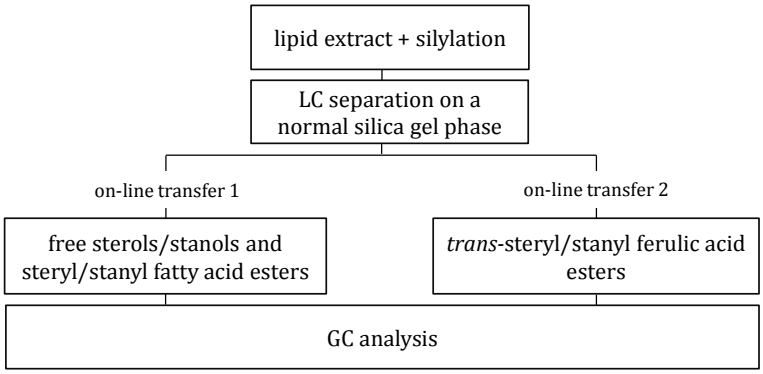

Figure 17. Outline of the procedure for the analysis of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl ferulic esters in plant lipids via on-line LC-GC.

The separations of individual free sterols/stanols and steryl/stanyl esters were comparable to those obtained by analysis of the SPE fractions via GC-FID (cf. 4.1.1.1). However, the final GC chromatograms revealed fewer interferences and the employed on-line LC-GC conditions enabled the simultaneous analysis of free sterols/stanols and steryl/stanyl fatty acid esters.

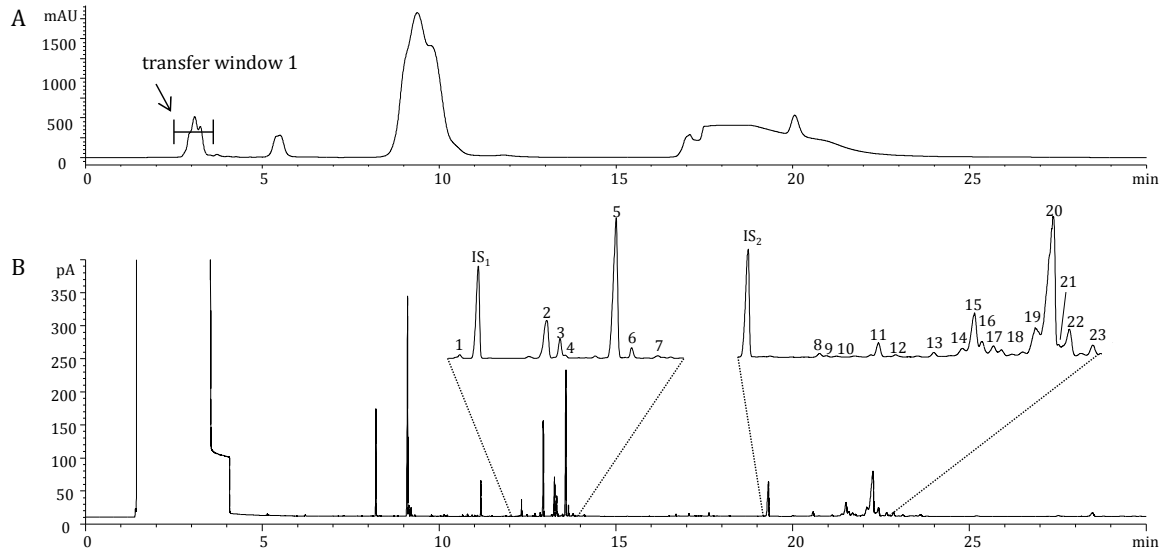

Figure 18. On-line LC-GC analysis of free sterols/stanols and steryl/stanyl fatty acid esters in corn. (**A**) LC-UV chromatogram at 205 nm and (**B**) GC-FID chromatogram of the transferred LC-fraction.
(1) cholesterol, (2) campesterol, (3) stigmasterol, (4) campestanol, (5) sitosterol, (6) sitostanol, (7) Δ^7 sitosterol + unknown sterol, (8) campesteryl-16:0/16:1, (9) stigmasteryl-16:0/16:1, (10) campestanyl-16:0/16:1, (11) sitosteryl-16:0/16:1, (12) sitostanyl-16:0/16:1, (13) Δ^7 sitosteryl-16:0/16:1, (14) campesteryl-18:0/18:1, (15) campesteryl-18:2 + stigmasteryl-18:0/18:1, (16) campestanyl-18:0/18:1 + stigmasteryl-18:2, (17) campestanyl-18:2, (18) Δ^7 campesteryl-18:2, (19) sitosteryl-18:0/18:1, (20) sitosteryl-18:2, (21) sitostanyl-18:0/18:1, (22) sitostanyl-18:2, (23) Δ^7 sitosteryl-18:2, (IS_1) 5α-cholestan-3β-ol, and (IS_2) cholesteryl-16:0 (For conditions cf. 3.2.5.2.).

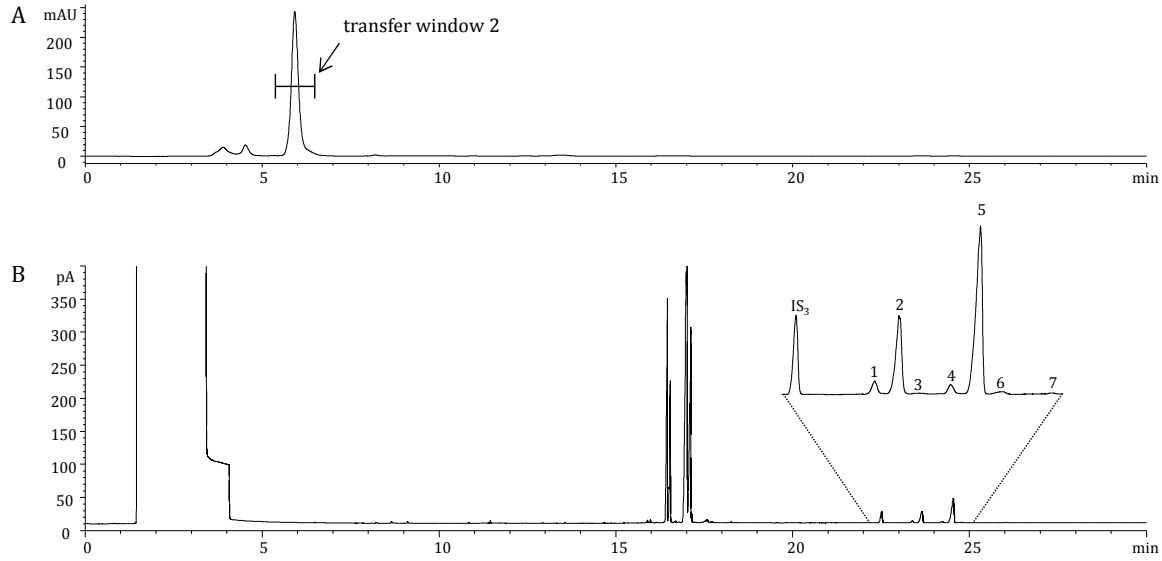

Figure 19. On-line LC-GC analysis of *trans*-steryl/stanyl ferulic acid esters in corn. (**A**) LC-UV chromatogram at 325 nm and (**B**) GC-FID chromatogram of the transferred LC-fraction.
(1) *trans*-campesteryl ferulate, (2) *trans*-campestanyl ferulate, (3) *trans*-Δ^7 campesteryl ferulate, (4) *trans*-sitosteryl ferulate, (5) *trans*-sitostanyl ferulate, (6) *trans*-Δ^7 sitosteryl ferulate, (7) *trans*-24-methylene cycloartanyl ferulate, and (IS_3) *trans*-cholestanyl ferulate (For conditions cf. 3.2.5.2.).

4.1.2.2 Validation of the On-line LC-GC-Based Approach

Method validation was performed by determination of recoveries, LODs, LOQs, and repeatability. Recoveries were determined by spiking corn flour with known amounts of selected steryl/stanyl derivatives. The results obtained for the analysis of recoveries, LODs, and LOQs are summarized in Table 14.

Table 14. Recoveries, LODs, and LOQs for ISs and selected steryl/stanyl derivatives determined via on-line LC-GC-FID.

	recovery [%]	LOD [µg/mL][a]	LOQ [µg/mL][a]
internal standards			
5*α*-cholestan-3*β*-ol	92.0 ± 0.5	0.01	0.02
cholesteryl palmitate	92.6 ± 1.1	0.08	0.16
cholestanyl ferulate	91.4 ± 1.2	0.47	0.94
free sterols/stanols and steryl/stanyl esters			
stigmasterol	103.8 ± 1.0	0.02	0.03
sitostanol	95.1 ± 2.3	0.03	0.09
stigmasteryl palmitate	101.5 ± 0.4	0.18	0.38
sitostanyl linoleate	83.3 ± 8.8	0.14	0.29
sitostanyl ferulate	94.5 ± 1.2	0.59	1.18

[a] LODs and LOQs expressed as µg/mL of i.v. (on-line LC-GC-FID); determined on the basis of 5 µL i.v. for free sterols/stanols and steryl/stanyl fatty acid esters, respectively, and 20 µL i.v. for *trans*-steryl/stanyl ferulic acid esters.

The repeatability of the method, including lipid extraction and on-line LC-GC analysis, was confirmed by working up a control sample once on each day of analysis. The total contents of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters of

five replicate analyses of a corn flour as example resulted in relative standard deviations of 1.2 % for free sterols/stanols, 6.7 % for steryl/stanyl fatty acid esters, and 8.5 % for *trans*-steryl/stanyl ferulic acid esters, which indicates a good repeatability.

4.1.3 Identification of Compounds via On-line LC-GC-MS

On-line LC-GC-MS was accomplished by replacing the FID of the on-line LC-GC by a mass selective detector. As the solvent evaporation was insufficient when helium was used as carrier gas for the gas chromatographic part, the analysis was performed with hydrogen. Furthermore, by using hydrogen as carrier gas improved resolutions and peak shapes could be achieved, and the background noise was lower as shown in Figure 20.

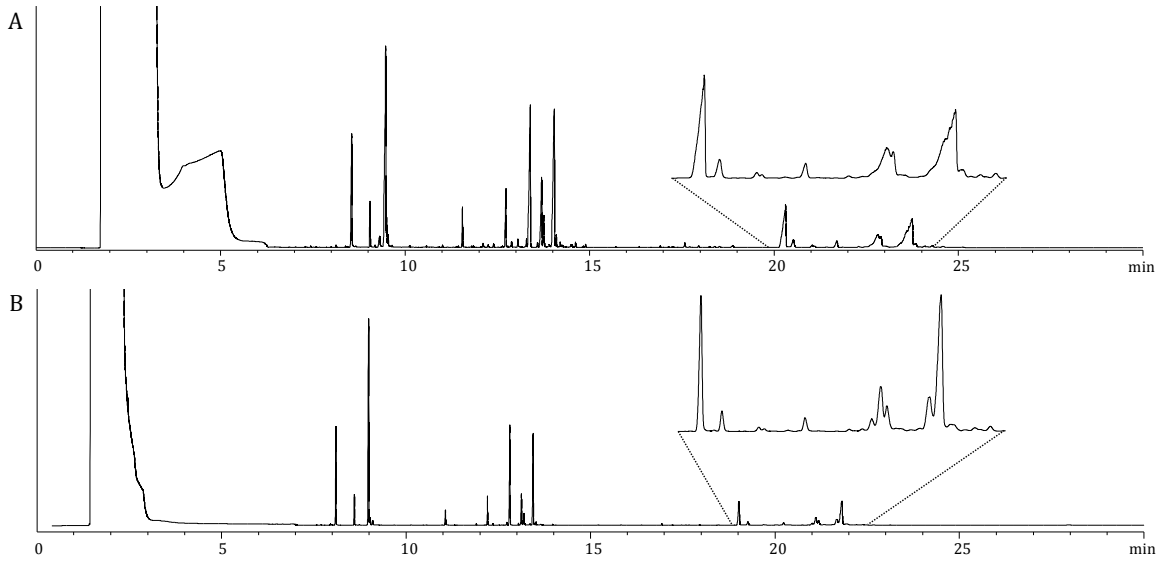

Figure 20. On-line LC-GC-MS analysis of a plant lipid extract: GC chromatograms with zoom of the elution area of steryl/stanyl fatty acid esters by using (**A**) helium and (**B**) hydrogen as carrier gas. (For conditions cf. 3.2.5.4).

Mass spectrometric analyses were performed in the positive electron impact ionization mode. Mass spectra of the reference compounds obtained by GC-MS and on-line LC-GC-MS revealed the same characteristic fragments; only slight differences were observed in the intensities of certain mass fragments. A comparison of the mass spectra obtained by the single GC-MS and on-line LC-GC-MS system is exemplarily shown in Figure 21 for the TMS-ether of sitosterol.

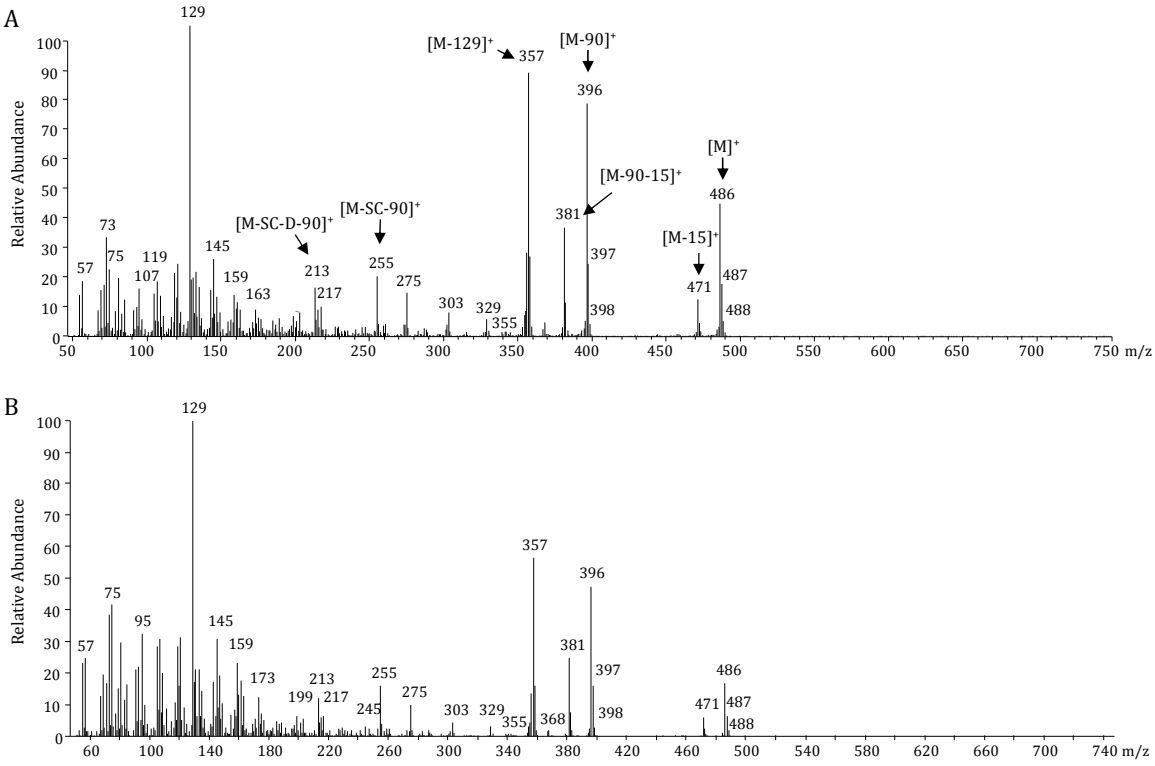

Figure 21. Comparison of mass spectra (EI) of the TMS-ether of sitosterol obtained (**A**) by GC-MS and (**B**) by on-line LC-GC-MS. (M) molecular ion, (SC) side chain, and (D) D-ring of the basic sterol structure.

Free sterols/stanols were identified based on chromatographic and mass spectral data either compared to those of commercially obtained reference compounds or to literature data (Rahier and Benveniste, 1989; Kamal-Eldin *et al.*, 1992; Pelillo *et al.*, 2003). Individual steryl/stanyl fatty acid esters of plant origin are not commercially available and thus identification was performed based on synthesized reference compounds. The synthesis and fragmentation patterns of steryl/stanyl fatty acid esters have previously been described in detail (Barnsteiner *et al.*, 2011; Barnsteiner *et al.*, 2012). The fragmentation patterns of the TMS-ethers of steryl/stanyl ferulic acid esters were compared to those of synthesized esters and as well as to data described in the literature (Evershed *et al.*, 1988). GC-MS data of intact steryl/stanyl coumaric acid esters are not reported. Identification of these compounds was performed by comparison of the fragmentation patterns to synthesized references (Barnsteiner, 2007).

A compilation of the steryl/stanyl derivatives, which were identified in cereals, nuts and edible plant oils is given in Table 15. Additionally, free fatty acids, tocopherols, and squalene were identified based on mass spectral data of commercially obtained reference compounds.

Table 15. Compounds identified via GC-MS and on-line LC-GC-MS.

compound	id.[a]	compound	id.[a]
free sterols/stanols		*steryl/stanyl fatty acid esters*	
α-amyrin	C	campestanyl-16:0/16:1	B
β-amyrin	C	campestanyl-18:0/18:1	B
Δ^5 avenasterol	C	campestanyl-18:2	B
campestanol	A	campesteryl-16:0/16:1	B
campesterol	A	campesteryl-18:0/18:1	B
cholesterol	A	campesteryl-18:2	B
citrostadienol	C	campesteryl-18:3	B
clerosterol	C	Δ^7 campesteryl-16:0/16:1	D
cycloartanol	C	Δ^7 campesteryl-18:0/18:1	D
cycloartenol	A	Δ^7 campesteryl-18:2	D
24-methylene cycloartanol	A	cholesteryl-16:0/16:1	A
sitostanol	A	cholesteryl-18:0/18:1	A
sitosterol	A	cholesteryl-18:2	A
Δ^7 sitosterol	C	cycloartenyl-16:0/16:1	B
stigmasterol	A	cycloartenyl-18:0/18:1	B
		cycloartenyl-18:2	B
steryl/stanyl phenolic acid esters		cycloartanyl-18:2	B
cis-campestanyl ferulate	D	cycloartanyl-18:3	B
cis-campesteryl ferulate	D	24-methylene cycloartanyl-16:0/16:1	B
cis-24-methylene cycloartanyl ferulate	D	24-methylene cycloartanyl-18:0/18:1	B
cis-sitostanyl ferulate	D	24-methylene cycloartanyl-18:2	B
cis-sitosteryl ferulate	D	24-methylene cycloartanyl-18:3	B
trans-campestanyl *p*-coumarate	B	sitostanyl-16:0/16:1	B
trans-campesteryl *p*-coumarate	B	sitostanyl-18:0/18:1	B
trans-sitostanyl *p*-coumarate	B	sitostanyl-18:2	B
trans-sitosteryl *p*-coumarate	B	sitosteryl-16:0/16:1	B
trans-campestanyl ferulate	B	sitosteryl-18:0/18:1	B
trans-campesteryl ferulate	B	sitosteryl-18:2	B
trans-Δ^7 campesteryl ferulate	D	sitosteryl-18:3	B
trans-24-methylene cycloartanyl ferulate	A	Δ^7 sitosteryl-16:0/16:1	D
trans-sitostanyl ferulate	B	Δ^7 sitosteryl-18:0/18:1	D
trans-sitosteryl ferulate	B	Δ^7 sitosteryl-18:2	D
trans-Δ^7 sitosteryl ferulate	D	stigmasteryl-16:0/16:1	B
		stigmasteryl-18:0/18:1	B
		stigmasteryl-18:2	B

[a] Identification of the compounds was performed as follows: (A) identified via mass spectral data and retention times of commercially obtained reference compounds, (B) identified via mass spectral data and retention times of synthesized reference compounds, (C) tentatively identified according to literature data, and (D) tentatively identified according to the respective Δ^5- or *trans*-derivative.

4.1.4 Comparison of Methods

The lipid extracts of five different corn flours were analyzed by means of GC after fractionation of the lipids via SPE as well as by means of on-line LC-GC. Total contents determined for free sterols/stanols and steryl/stanyl fatty acid esters were not significantly different on a significance level (α) of 0.05 (Figure 22). The total contents of *trans*-steryl/stanyl ferulic acid esters were slightly higher, with the exception of sample 1, when

applying the on-line LG-GC-based approach. The recoveries determined for sitostanyl ferulate were comparable between both methods (cf. Table 13 and Table 14); thus, a systematic error during sample preparation can be excluded.

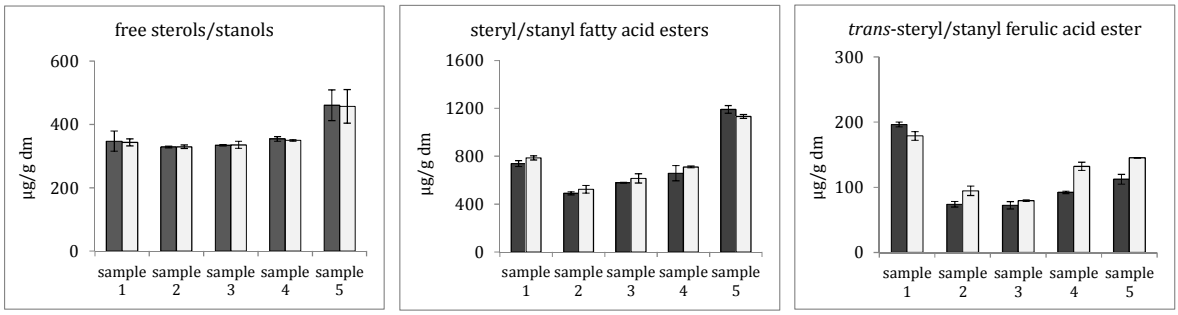

Figure 22. Comparison of the methods for the quantitative analysis of free sterols/stanols, steryl/stanyl fatty acid esters, and *trans*-steryl/stanyl ferulic acid esters: SPE-based approach (dark gray bar) and on-line LC-GC-based approach (light gray bar).

4.1.5 Summary

An approach based on SPE for the effective fractionation of lipids into free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters was established. The isolated steryl/stanyl derivatives were subsequently analyzed in their intact form by means of GC-FID. In addition, an on-line LC-GC system with PTV interface was implemented. The lipid fractionation was performed on a normal silica gel phase and the subsequent GC analysis of the on-line transferred fraction(s) enabled the simultaneous qualitative and quantitative determination of free sterols/stanols and intact steryl/stanyl fatty acid esters. The *trans*-derivatives of steryl/stanyl ferulic acid esters were analyzed by means of a second transfer.

Both analytical methods enable a comprehensive analysis of free sterols/stanols and of intact steryl/stanyl esters within one working up procedure. Each method exhibits certain advantages depending on the analytical target. While the approach based on SPE and GC provides more detailed information on steryl/stanyl phenolic acid esters, the advantages of on-line LC-GC are, in particular, the far less complex sample preparation and the automated analysis. Compared to methods commonly used for the determination of sterols/stanols and steryl/stanyl esters in cereals and other natural foods, the established methodologies show distinct advantages. They allow the simultaneous analysis of various substance classes and especially the achieved GC separations enable a detailed qualitative and quantitative analysis of individual intact steryl/stanyl esters as it has not been reported so far.

4.2 Analysis of Free Sterols/Stanols and Steryl/Stanyl Esters in Various Dent Corn and Flint Corn Cultivars

The developed SPE-based approach (cf. 4.1.1) was applied to determine the natural variability of contents and compositions of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters in corn. Overall, 26 corn cultivars (dent corn and flint corn) grown at the same location and in the same year were studied, thus enabling a comparison independent from differences caused by environmental conditions. Representative GC chromatograms of the separations of free sterols/stanols and steryl/stanyl esters extracted from a corn sample have already been shown in Figure 14.

4.2.1 Compositions and Contents of Free Sterols/Stanols and Steryl/Stanyl Esters

Free Sterols/Stanols

The contents and compositions of free sterols/stanols determined in the 26 different corn cultivars revealed almost no variation (Table 16). Sitosterol was dominating in all samples, accounting for 59.6-70.8 % of total free sterols/stanols, followed by campesterol, and stigmasterol. These three sterols made up together >85 % of total free sterols/stanols. Free stanols, i.e. sitostanol and campestanol, represented on average 8.9 ± 0.9 % in the dent corn cultivars and 6.9 ± 1.5 % in the flint corn cultivars. As the distributions of sterols and stanols in corn kernels have most commonly been determined after alkaline hydrolysis (Normén *et al.*, 2002; Harrabi *et al.*, 2007; Harrabi *et al.*, 2008; Moreau *et al.*, 2009; Harrabi *et al.*, 2011), literature data concerning the profiles of free sterols/stanols are rare. The accumulation of free sterols/stanols in developing corn kernels has been investigated after isolation of the free sterol fraction via serial elution column chromatography (Davis and Poneleit, 1974; Davis and Poneleit, 1975). Sitosterol, campesterol, and stigmasterol were described as the major free sterols. This is in agreement with the data obtained in the present study. The observed profiles are also generally comparable to data reported for free sterols/stanols in commercial corn germ oils (Phillips *et al.*, 2002; Dulf *et al.*, 2010). Cholesterol, the predominant sterol in animals, was present in all corn samples; however, the amounts were lower than 0.8 %. Cholesterol in plants is thought to serve as precursor for the synthesis of steroidal saponins and alkaloids, and amounts of 1-5 % of total phytosterols are reported as not uncommon (Moreau *et al.*, 2002).

Most of the investigated corn cultivars revealed total free sterol/stanol levels in the range of 300-400 µg/g dry matter flour. Only four cultivars exhibited amounts of >400 µg/g and two

cultivars of <300 μg/g. The contents calculated on the basis of extracted oil averaged 900.4 ± 107.5 μg/100 mg oil, being highest in Starsky (dent corn, no. 7) and lowest in Zidane (flint corn, no. 3). The amounts determined in the extracted oils are in good agreement with earlier reported data for several corn kernel oils (Moreau *et al.*, 2001).

Table 16. Contents and compositions of free sterols/stanols in dent corn and flint corn.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	sitosterol [%]	campesterol [%]	stigmasterol [%]	sitostanol [%]	campestanol [%]	Δ^7 sitosterol[b] [%]	cholesterol [%]
dent corn	1	347.1 ± 31.6	936.1 ± 78.1	63.4 ± 0.6	19.0 ± 0.2	6.2 ± 0.1	6.8 ± 0.3	2.7 ± 0.1	1.5 ± 0.1	0.5 ± 0.0
	2	354.3 ± 7.2	933.8 ± 36.3	63.9 ± 0.4	18.0 ± 0.1	6.6 ± 0.1	6.1 ± 0.2	3.4 ± 0.1	1.7 ± 0.1	0.2 ± 0.0
	3	336.4 ± 2.6	853.1 ± 5.2	62.5 ± 0.3	19.4 ± 0.1	6.4 ± 0.1	6.4 ± 0.3	3.1 ± 0.1	1.8 ± 0.2	0.4 ± 0.0
	4	385.9 ± 2.6	1007.0 ± 6.2	62.3 ± 0.1	21.0 ± 0.3	6.5 ± 0.0	5.4 ± 0.3	3.0 ± 0.1	1.3 ± 0.1	0.5 ± 0.1
	5	364.3 ± 9.0	971.9 ± 11.3	65.4 ± 0.1	18.4 ± 0.1	7.1 ± 0.0	5.3 ± 0.1	2.4 ± 0.1	1.0 ± 0.0	0.5 ± 0.1
	6	352.2 ± 13.7	849.4 ± 19.8	63.7 ± 0.5	20.1 ± 0.2	6.8 ± 0.1	5.2 ± 0.3	2.7 ± 0.1	1.0 ± 0.2	0.6 ± 0.1
	7	388.3 ± 15.8	1086.7 ± 23.9	61.5 ± 0.4	17.8 ± 0.1	6.5 ± 0.0	6.6 ± 0.2	3.0 ± 0.0	3.9 ± 0.3	0.7 ± 0.2
	8	310.6 ± 2.0	986.5 ± 1.0	64.6 ± 0.1	18.4 ± 0.0	7.0 ± 0.0	5.5 ± 0.1	2.8 ± 0.1	1.3 ± 0.0	0.4 ± 0.0
	9	333.9 ± 2.3	976.5 ± 11.1	61.4 ± 0.1	20.7 ± 0.0	7.0 ± 0.0	5.7 ± 0.1	3.1 ± 0.2	1.6 ± 0.1	0.4 ± 0.0
	10	328.7 ± 2.2	1071.4 ± 2.9	61.3 ± 0.2	20.5 ± 0.0	7.4 ± 0.0	5.5 ± 0.1	3.1 ± 0.1	1.9 ± 0.1	0.3 ± 0.0
	11	353.7 ± 6.2	1044.3 ± 8.3	64.7 ± 0.0	19.7 ± 0.1	5.8 ± 0.0	5.6 ± 0.1	2.6 ± 0.1	1.3 ± 0.0	0.4 ± 0.0
	12	419.3 ± 6.3	1071.6 ± 5.9	61.0 ± 0.1	21.9 ± 0.0	6.5 ± 0.0	5.9 ± 0.1	2.8 ± 0.0	1.5 ± 0.1	0.4 ± 0.0
	13	325.3 ± 7.1	782.1 ± 14.1	59.6 ± 0.1	19.9 ± 0.0	7.3 ± 0.0	7.4 ± 0.1	3.6 ± 0.0	1.6 ± 0.1	0.5 ± 0.0
	14	345.7 ± 4.6	853.6 ± 19.6	62.4 ± 0.3	20.1 ± 0.2	6.6 ± 0.1	6.0 ± 0.2	3.3 ± 0.1	1.4 ± 0.1	0.3 ± 0.0
	15	366.0 ± 2.8	996.2 ± 18.5	62.0 ± 0.1	20.5 ± 0.0	7.9 ± 0.1	5.1 ± 0.1	2.9 ± 0.0	1.4 ± 0.1	0.2 ± 0.0
flint corn	1	296.3 ± 1.2	787.0 ± 13.4	68.1 ± 0.3	18.8 ± 0.1	6.3 ± 0.0	3.6 ± 0.2	1.7 ± 0.1	0.9 ± 0.1	0.5 ± 0.1
	2	339.5 ± 8.3	872.7 ± 23.0	64.3 ± 0.4	17.6 ± 0.3	7.0 ± 0.0	6.6 ± 0.5	2.6 ± 0.1	1.3 ± 0.1	0.6 ± 0.0
	3	341.8 ± 1.5	694.3 ± 4.5	67.3 ± 0.2	20.4 ± 0.0	5.3 ± 0.0	4.0 ± 0.0	1.9 ± 0.3	0.7 ± 0.0	0.5 ± 0.0
	4	319.3 ± 1.9	883.9 ± 5.9	63.9 ± 0.2	20.0 ± 0.1	5.9 ± 0.1	5.9 ± 0.2	2.5 ± 0.1	1.1 ± 0.1	0.5 ± 0.0
	5	432.9 ± 6.2	762.9 ± 7.4	66.2 ± 0.5	20.4 ± 0.3	5.2 ± 0.0	4.6 ± 0.5	2.2 ± 0.1	1.0 ± 0.1	0.4 ± 0.1
	6	410.2 ± 5.2	881.5 ± 3.5	69.4 ± 0.1	18.0 ± 0.0	4.7 ± 0.0	4.4 ± 0.1	1.7 ± 0.0	1.2 ± 0.0	0.5 ± 0.0
	7	342.2 ± 3.6	770.6 ± 7.0	70.8 ± 0.0	16.9 ± 0.1	5.6 ± 0.0	4.0 ± 0.0	1.4 ± 0.0	0.9 ± 0.1	0.5 ± 0.0
	8	424.7 ± 6.5	865.1 ± 8.2	70.6 ± 0.3	17.3 ± 0.1	5.2 ± 0.0	3.9 ± 0.1	1.4 ± 0.0	0.7 ± 0.1	0.8 ± 0.0
	9	324.1 ± 0.6	843.3 ± 9.0	64.5 ± 0.1	19.3 ± 0.1	6.2 ± 0.1	5.6 ± 0.1	2.5 ± 0.1	1.3 ± 0.1	0.6 ± 0.0
	10	298.0 ± 5.5	838.8 ± 8.7	67.6 ± 0.3	15.9 ± 0.2	5.6 ± 0.1	6.2 ± 0.1	2.4 ± 0.2	1.9 ± 0.1	0.4 ± 0.0
	11	309.6 ± 3.5	790.8 ± 17.3	66.7 ± 0.3	18.9 ± 0.2	6.2 ± 0.0	4.3 ± 0.1	2.2 ± 0.2	0.9 ± 0.1	0.8 ± 0.1

[a] Based on dry matter (dm) of ground kernels. [b] Coelution with an unidentified sterol.

Steryl/Stanyl Fatty Acid Esters

In total, 16 individual steryl/stanyl fatty acid esters could be determined in the 26 investigated corn cultivars (Table 17). The overall percentage distribution showed only little variation. Steryl esters of linoleic acid were most abundant, with sitosteryl-18:2 as predominant ester, followed by campesteryl-18:2 (coeluted with stigmasteryl-18:0/18:1) and in some cases by sitosteryl-18:0/18:1. However, the amounts of sitosteryl-18:0/18:1 were generally lower in the dent corn cultivars. Another linoleic acid ester, namely sitostanyl-18:2, made up 6.5-11.0 % of total steryl/stanyl fatty acid esters. The predominance of linoleic acid esters has already been described for corn germ oil, but also for other vegetable oils (Worthington and Hitchcock, 1984; Ferrari *et al.*, 1997). About 3-5 % of total steryl/stanyl fatty acid esters occurred as esters of C16-fatty acids. Stanyl esters represented approximately 20 %. To the author's knowledge, this is the first time that the individual steryl/stanyl fatty acid ester composition was analyzed in whole flours of different corn cultivars.

The study of Dulf *et al.* (2010), who investigated the sterol composition of steryl/stanyl fatty acid esters after transesterification of the esters of corn germ oil, indicated the presence of cholesteryl and Δ^5 avenasteryl esters, which is in contrast to the present study, where these esters could not be detected. Analyses of the tissue distribution of steryl fatty acid esters in 10-day old corn seedlings revealed that sitosteryl-18:2 was the major steryl ester of the scutellum and the endosperm; palmitic, palmitoleic, oleic, and linoleic acid esters, in turn, were dominating in the root (Kemp and Mercer, 1968). This suggests a varying substrate availability as well as possibly distinct enzyme specificities in different corn tissues.

As previously described, the resolution of individual steryl/stanyl esters was hampered under the employed GC conditions for saturated and monounsaturated fatty acids of the same chain length, i.e. esters of palmitic/palmitoleic acid and of stearic/oleic acid (cf. 4.1.1.1). The distributions of these acids were therefore determined by GC-FID as fatty acid methyl esters after methanolysis of the respective steryl/stanyl fatty acid ester fraction isolated by SPE from two samples as representatives for each corn subspecies. Palmitoleic acid could only be detected in one of the four investigated corn cultivars (ratio palmitic/palmitoleic acid 30:1), thus indicating a predominance of palmitic acid esters. Furthermore, oleic acid was more abundant than stearic acid, with a 4- to 14-fold higher amount.

The total contents of steryl/stanyl fatty acid esters showed large differences between the 26 corn cultivars. A 2.7-fold margin was observed between the highest and the lowest level determined in dry matter flour (average: 674.0 ± 134.5 µg/g). In total extracted lipids, steryl/stanyl fatty acid esters made up 1.2-2.1 %, which is in line with data reported for 49 corn accessions (Moreau *et al.*, 2001).

Table 17. Contents and compositions of steryl/stanyl fatty acid esters in dent corn and flint corn.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	sitosteryl-18:2 [%]	campesteryl-18:2 [%][b]	sitosteryl-18:0/18:1 [%]	sitostanyl-18:2 [%]	campestanyl-18:0/18:1 [%][c]	Δ^7 sitosteryl-18:2 [%][d]	campesteryl-18:0/18:1 [%]
dent corn	1	738.4 ± 24.6	1992.0 ± 38.9	45.8 ± 2.9	14.2 ± 0.4	8.5 ± 2.7	8.9 ± 0.1	4.9 ± 0.2	3.3 ± 0.1	3.0 ± 0.1
	2	658.6 ± 64.1	1730.6 ± 76.2	48.3 ± 1.9	12.4 ± 0.1	8.7 ± 0.8	8.7 ± 0.6	4.4 ± 0.3	3.2 ± 0.1	2.6 ± 0.2
	3	722.9 ± 6.0	1833.2 ± 6.6	47.4 ± 0.9	14.6 ± 0.1	9.6 ± 0.6	8.8 ± 0.2	3.7 ± 0.1	3.5 ± 0.1	2.9 ± 0.0
	4	639.6 ± 8.3	1668.9 ± 19.6	47.2 ± 0.4	16.2 ± 0.0	9.6 ± 0.3	8.2 ± 0.1	4.1 ± 0.0	3.4 ± 0.1	2.0 ± 0.2
	5	618.9 ± 8.9	1652.3 ± 69.0	46.8 ± 1.0	16.2 ± 0.3	3.4 ± 0.6	10.5 ± 0.1	5.7 ± 0.1	3.2 ± 0.2	1.9 ± 0.1
	6	675.5 ± 16.9	1629.0 ± 12.3	49.3 ± 0.1	15.5 ± 0.2	9.0 ± 0.1	7.7 ± 0.0	4.0 ± 0.1	2.7 ± 0.1	2.2 ± 0.2
	7	650.6 ± 9.0	1821.5 ± 35.2	52.4 ± 0.4	14.8 ± 0.1	3.5 ± 0.2	9.3 ± 0.0	5.5 ± 0.1	3.2 ± 0.0	2.0 ± 0.3
	8	573.1 ± 26.5	1820.2 ± 77.5	48.8 ± 0.3	13.9 ± 0.0	9.1 ± 0.6	8.3 ± 0.1	4.3 ± 0.1	3.4 ± 0.1	1.8 ± 0.1
	9	579.1 ± 2.0	1693.5 ± 18.4	49.3 ± 0.4	16.2 ± 0.1	7.0 ± 0.3	8.4 ± 0.1	4.0 ± 0.1	3.2 ± 0.0	2.1 ± 0.1
	10	490.0 ± 13.1	1597.3 ± 50.8	45.8 ± 0.5	16.2 ± 0.0	8.1 ± 0.4	8.6 ± 0.1	4.4 ± 0.1	3.6 ± 0.1	2.5 ± 0.1
	11	688.8 ± 89.6	2032.3 ± 247.4	48.0 ± 4.0	12.6 ± 1.4	7.7 ± 1.0	7.9 ± 0.4	3.7 ± 0.1	3.0 ± 0.4	2.3 ± 0.3
	12	577.5 ± 3.9	1474.2 ± 14.8	45.4 ± 0.3	16.2 ± 0.0	9.3 ± 0.0	9.0 ± 0.3	3.7 ± 0.0	3.4 ± 0.0	2.1 ± 0.1
	13	602.7 ± 3.5	1443.7 ± 2.9	45.7 ± 0.9	15.6 ± 0.1	7.8 ± 0.5	9.9 ± 0.0	3.8 ± 0.1	3.6 ± 0.1	2.1 ± 0.1
	14	700.4 ± 21.8	1716.5 ± 55.1	44.7 ± 0.7	14.0 ± 0.1	9.3 ± 1.0	9.2 ± 0.0	4.4 ± 0.1	3.5 ± 0.1	2.5 ± 0.0
	15	689.0 ± 13.3	1875.6 ± 56.4	47.3 ± 0.3	16.3 ± 0.1	7.6 ± 0.3	7.7 ± 0.0	4.6 ± 0.1	3.6 ± 0.0	2.5 ± 0.2
flint corn	1	510.0 ± 11.0	1355.2 ± 50.9	53.2 ± 1.5	16.6 ± 0.2	4.9 ± 0.8	7.4 ± 0.2	4.1 ± 0.0	2.9 ± 0.2	2.4 ± 0.0
	2	642.3 ± 44.4	1651.6 ± 121.4	45.0 ± 0.8	11.7 ± 0.3	11.9 ± 0.6	9.7 ± 0.3	4.2 ± 0.1	3.8 ± 0.3	2.5 ± 0.3
	3	642.1 ± 30.1	1650.8 ± 84.8	44.9 ± 0.4	14.1 ± 0.7	12.9 ± 0.7	9.7 ± 0.2	3.1 ± 0.1	2.3 ± 0.2	3.6 ± 0.2
	4	562.9 ± 7.0	1558.2 ± 20.8	45.6 ± 0.6	12.5 ± 0.1	10.0 ± 1.1	9.2 ± 0.1	4.2 ± 0.1	3.6 ± 0.2	3.3 ± 0.1
	5	1186.8 ± 44.6	2081.6 ± 74.0	47.9 ± 0.3	13.5 ± 0.0	10.6 ± 0.4	8.6 ± 0.0	2.9 ± 0.0	3.6 ± 0.0	2.8 ± 0.1
	6	636.2 ± 43.6	1635.7 ± 119.4	45.4 ± 0.8	11.8 ± 0.3	12.0 ± 0.6	9.8 ± 0.2	4.2 ± 0.1	3.0 ± 0.3	2.5 ± 0.3
	7	618.0 ± 14.3	1391.2 ± 19.4	52.3 ± 0.5	14.9 ± 0.1	7.4 ± 0.5	8.0 ± 0.1	3.1 ± 0.2	2.5 ± 0.1	2.2 ± 0.2
	8	784.0 ± 79.7	1596.3 ± 152.6	53.5 ± 2.3	12.0 ± 0.6	11.2 ± 0.9	6.5 ± 0.1	2.8 ± 0.1	2.4 ± 0.3	2.6 ± 0.2
	9	581.0 ± 23.2	1511.2 ± 43.2	47.0 ± 0.4	12.0 ± 0.1	12.6 ± 0.2	8.8 ± 0.1	4.1 ± 0.1	2.4 ± 0.0	2.7 ± 0.2
	10	430.4 ± 28.1	1211.7 ± 80.2	48.4 ± 0.8	10.6 ± 0.4	8.1 ± 0.4	11.0 ± 0.3	3.6 ± 0.1	4.6 ± 0.1	1.8 ± 0.1
	11	624.4 ± 46.5	1593.8 ± 96.9	47.7 ± 0.3	11.6 ± 0.1	11.7 ± 0.8	8.3 ± 0.2	4.5 ± 0.2	3.4 ± 0.1	2.4 ± 0.2

Table 17. continued.

	no.	campestanyl-18:2 [%]	sitostanyl-18:0/18:1 [%]	sitosteryl-16:0/16:1 [%]	Δ7 campesteryl-18:2 [%][d]	Δ7 sitosteryl-16:0/16:1 [%][d]	campesteryl-16:0/16:1 [%]	sitostanyl-16:0/16:1 [%]	stigmasteryl-16:0/16:1 [%]	campestanyl-16:0/16:1 [%][d]
dent corn[a]	1	3.3 ± 0.1	2.4 ± 0.6	1.9 ± 0.1	1.4 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.2 ± 0.0
	2	2.5 ± 0.1	2.5 ± 0.2	1.9 ± 0.2	2.3 ± 0.9	0.6 ± 0.1	0.5 ± 0.0	0.8 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
	3	2.2 ± 0.0	2.1 ± 0.1	1.5 ± 0.0	1.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	4	2.4 ± 0.1	1.4 ± 0.1	1.7 ± 0.0	1.7 ± 0.1	0.5 ± 0.0	0.7 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
	5	3.8 ± 0.1	2.1 ± 0.3	1.6 ± 0.8	1.8 ± 0.1	0.5 ± 0.2	0.6 ± 0.1	1.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
	6	2.8 ± 0.1	1.5 ± 0.1	1.9 ± 0.0	1.2 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
	7	2.7 ± 0.0	1.5 ± 0.3	2.0 ± 0.2	0.7 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
	8	2.3 ± 0.1	3.0 ± 0.2	1.7 ± 0.1	1.4 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
	9	2.6 ± 0.1	1.8 ± 0.1	1.6 ± 0.0	1.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
	10	2.6 ± 0.1	2.0 ± 0.2	1.7 ± 0.0	1.8 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.9 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
	11	1.8 ± 0.2	8.3 ± 7.6	1.2 ± 0.5	1.3 ± 0.1	0.4 ± 0.2	0.5 ± 0.1	0.8 ± 0.3	0.3 ± 0.1	0.2 ± 0.0
	12	2.7 ± 2.7	2.4 ± 0.0	1.6 ± 0.0	1.6 ± 0.1	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
	13	3.1 ± 0.1	2.6 ± 0.2	1.7 ± 0.0	1.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
	14	3.2 ± 0.0	2.2 ± 0.4	1.9 ± 0.1	1.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	0.4 ± 0.1	0.4 ± 0.0
	15	3.3 ± 0.0	1.0 ± 0.1	1.7 ± 0.1	1.6 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.8 ± 0.1	0.4 ± 0.0	0.3 ± 0.1
flint corn[a]	1	1.6 ± 0.0	1.4 ± 0.3	1.9 ± 0.1	1.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.1
	2	2.9 ± 0.1	2.8 ± 0.2	2.2 ± 0.5	1.2 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
	3	2.2 ± 0.1	2.8 ± 0.3	1.5 ± 0.8	1.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
	4	1.9 ± 0.1	4.2 ± 0.0	1.8 ± 0.0	1.7 ± 0.3	0.3 ± 0.2	0.6 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	5	1.9 ± 0.0	2.2 ± 0.1	1.9 ± 0.0	1.5 ± 0.1	0.9 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
	6	3.0 ± 0.1	2.8 ± 0.2	2.2 ± 0.6	1.2 ± 0.2	0.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
	7	1.9 ± 0.1	1.6 ± 0.2	2.2 ± 0.0	1.4 ± 0.1	0.5 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
	8	1.9 ± 0.0	1.8 ± 0.3	2.4 ± 0.5	1.1 ± 0.2	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	0.2 ± 0.0	0.1 ± 0.0
	9	1.7 ± 0.0	3.8 ± 0.7	1.7 ± 0.0	1.3 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.7 ± 0.2	0.3 ± 0.0	0.2 ± 0.0
	10	2.0 ± 0.0	3.1 ± 0.2	2.1 ± 0.0	1.6 ± 0.1	0.8 ± 0.0	0.5 ± 0.0	1.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
	11	2.3 ± 0.1	2.9 ± 0.3	2.0 ± 0.0	1.0 ± 0.1	0.7 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.1 ± 0.0

[a] Based on dry matter (dm) of ground kernels. [b] Coelution with stigmasteryl-18:0/18:1. [c] Coelution with stigmasteryl-18:2. [d] Compound calculated with Rf = 1.

Steryl/Stanyl Phenolic Acid Esters

In all dent corn and flint corn cultivars, ferulic acid esters were predominating (>93 %), with *trans*-sitostanyl ferulate as most abundant ester (Table 18). This is in agreement with previously reported data for corn (Seitz, 1989; Norton, 1995). The main part of the phenolic acids occurred as esters of sitostanol and campestanol; steryl esters, in turn, were only minor constituents. *Trans*-24-methylene cycloartanyl ferulate, one of the main compounds of the steryl/stanyl ferulate mixture in rice (Miller and Engel, 2006; Cho *et al.*, 2012), constituted up to 3.2 %. Overall, a much greater variation was observed for the distributions of the individual steryl/stanyl phenolic acid esters than for free sterols/stanols or steryl/stanyl fatty acid esters. *Cis*-ferulic acid esters made up 3.5-39.1 %, which corresponds to a cis/trans ratio of 0.04-0.64. Comparable *cis*/*trans*-ratios have already been reported for other cereals (Nyström *et al.*, 2008b). It has been described that the *trans*-form of phenolic acids can be partially converted into their *cis*-form by UV light and daylight (Hartley and Jones, 1975; Fenton *et al.*, 1978). However, as no *cis*-derivative of the IS was detected neither by GC-FID nor by GC-MS, *trans*-*cis*-isomerization during sample preparation can be excluded. Thus, naturally occurring photoisomerization during plant growth may be the cause of the occurrence of *cis*-steryl/stanyl ferulates. Additionally, up to 7 % of total steryl/stanyl phenolic acid esters were present as steryl/stanyl coumaric acid esters. Steryl/stanyl coumaric acid esters have firstly been identified and quantified in corn in 1989 and later in corn bran and unrefined corn oils (Seitz, 1989; Norton, 1994; Norton, 1995). The reported distributions are generally in agreement with those determined in the present study.

On average, 101.2 ± 38.2 µg/g dry matter flour and 260.4 ± 103.2 µg/100 mg extracted oil consisted of steryl/stanyl phenolic acid esters. The highest amounts were detected in the cultivar MAS 31a (dent corn, no. 1), which were 5.2-fold above the levels determined in Crazi (flint corn, no. 1). The total amounts based on extracted oils are comparable to those reported for three dent corn samples as well as for several other corn kernel oils (Moreau *et al.*, 1999; Moreau *et al.*, 2001). The total contents based on dry matter flour of the dent corn kernels, in turn, were little higher than those described for seven other dent corn samples (Seitz, 1989).

Table 18. Contents and compositions of steryl/stanyl phenolic acid esters in dent corn and flint corn.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	*trans*-sitostanyl ferulate [%]	*trans*-campestanyl ferulate [%]	*cis*-sitostanyl ferulate [%]	*trans*-Δ⁷ sitosteryl ferulate [%]	*cis*-campestanyl ferulate [%]	*trans*-campesteryl ferulate [%]	*trans*-sitosteryl ferulate [%]
dent corn	1	240.2 ± 6.1	646.2 ± 27.5	52.4 ± 1.4	20.2 ± 0.4	11.4 ± 2.7	2.9 ± 1.3	4.8 ± 1.0	2.6 ± 0.0	2.5 ± 0.1
	2	100.3 ± 2.2	264.2 ± 9.5	52.9 ± 0.4	23.6 ± 0.4	3.7 ± 0.0	5.7 ± 0.4	1.8 ± 0.1	3.6 ± 0.1	3.7 ± 0.0
	3	93.6 ± 5.9	237.2 ± 14.2	50.0 ± 0.9	24.5 ± 2.5	9.4 ± 1.9	1.7 ± 0.3	4.9 ± 0.5	3.3 ± 0.8	2.9 ± 0.7
	4	124.1 ± 6.2	323.7 ± 13.4	41.7 ± 0.4	22.6 ± 0.6	12.1 ± 1.0	2.6 ± 0.4	7.4 ± 0.5	4.1 ± 0.2	3.6 ± 0.1
	5	68.0 ± 8.6	181.0 ± 16.8	44.2 ± 0.6	21.3 ± 0.4	16.1 ± 0.8	2.1 ± 0.3	8.7 ± 0.4	3.3 ± 0.0	3.1 ± 0.0
	6	133.5 ± 5.4	322.1 ± 12.7	51.7 ± 0.0	21.1 ± 0.4	10.4 ± 1.0	1.5 ± 0.3	4.5 ± 0.3	2.8 ± 0.1	2.1 ± 0.1
	7	157.5 ± 13.3	441.0 ± 35.8	37.2 ± 2.3	13.8 ± 0.7	22.5 ± 2.1	2.8 ± 0.6	14.9 ± 0.8	1.8 ± 0.1	1.9 ± 0.2
	8	82.4 ± 6.3	261.6 ± 18.8	46.0 ± 0.9	23.2 ± 0.7	10.0 ± 1.2	3.6 ± 0.6	5.7 ± 0.7	3.8 ± 0.1	3.7 ± 0.2
	9	80.2 ± 6.1	234.5 ± 17.3	50.6 ± 0.5	24.0 ± 0.2	4.1 ± 0.2	5.4 ± 0.1	2.2 ± 0.1	4.0 ± 0.1	3.8 ± 0.1
	10	80.5 ± 4.7	262.3 ± 16.6	48.5 ± 0.6	27.0 ± 0.3	3.5 ± 0.2	4.6 ± 0.4	2.2 ± 0.1	5.1 ± 0.1	4.2 ± 0.1
	11	117.0 ± 10.8	345.5 ± 32.9	49.7 ± 0.8	20.3 ± 0.7	12.4 ± 1.2	2.1 ± 0.2	5.7 ± 0.4	2.9 ± 0.1	2.9 ± 0.1
	12	68.9 ± 6.4	176.0 ± 14.9	44.7 ± 0.5	20.8 ± 0.4	12.5 ± 0.6	2.5 ± 0.1	6.8 ± 0.2	2.1 ± 0.1	3.5 ± 0.1
	13	71.0 ± 0.9	170.7 ± 2.9	58.9 ± 4.0	25.6 ± 4.5	4.8 ± 0.4	<LOQ[b]	2.7 ± 0.4	<LOQ	3.0 ± 0.6
	14	59.2 ± 2.3	146.2 ± 3.8	49.4 ± 0.9	24.4 ± 0.5	3.5 ± 0.3	8.4 ± 0.9	<LOQ	3.1 ± 0.2	3.6 ± 0.1
	15	90.5 ± 2.5	246.4 ± 8.6	52.7 ± 0.5	23.4 ± 0.0	3.5 ± 0.3	4.6 ± 0.4	1.7 ± 0.1	3.8 ± 0.1	3.6 ± 0.0
flint corn	1	46.1 ± 4.0	122.4 ± 12.1	41.8 ± 0.4	18.4 ± 0.4	14.4 ± 0.1	3.8 ± 0.1	6.9 ± 0.2	7.0 ± 0.1	4.6 ± 0.2
	2	113.4 ± 5.3	291.5 ± 12.7	53.7 ± 0.7	22.1 ± 0.4	7.5 ± 0.6	3.0 ± 0.7	3.1 ± 0.2	3.0 ± 0.1	3.5 ± 0.1
	3	135.9 ± 9.5	276.0 ± 19.1	46.1 ± 1.0	18.7 ± 0.3	7.5 ± 0.2	4.2 ± 0.4	3.4 ± 0.1	6.5 ± 0.4	4.2 ± 0.2
	4	88.2 ± 1.4	244.1 ± 1.9	46.1 ± 0.8	20.0 ± 0.6	12.7 ± 0.9	3.9 ± 0.4	6.2 ± 0.2	3.0 ± 0.0	3.3 ± 0.1
	5	123.2 ± 8.5	216.7 ± 15.5	55.3 ± 1.5	22.5 ± 0.6	4.3 ± 0.4	5.6 ± 1.0	2.0 ± 0.1	3.0 ± 0.1	2.7 ± 0.2
	6	89.5 ± 5.8	214.3 ± 38.9	52.1 ± 1.8	19.7 ± 1.2	8.4 ± 1.9	3.5 ± 0.3	<LOQ	4.6 ± 1.1	3.7 ± 0.8
	7	77.0 ± 2.0	174.2 ± 6.3	41.7 ± 0.4	16.2 ± 0.1	16.6 ± 0.7	2.2 ± 0.1	7.4 ± 0.4	6.8 ± 0.2	4.2 ± 0.1
	8	109.0 ± 18.9	222.1 ± 39.1	47.8 ± 1.0	18.2 ± 0.2	9.3 ± 1.2	2.0 ± 0.9	3.9 ± 0.1	6.8 ± 0.4	4.1 ± 0.2
	9	96.2 ± 5.5	250.3 ± 17.3	50.4 ± 0.9	22.1 ± 0.6	9.4 ± 2.1	3.9 ± 0.8	4.8 ± 0.8	2.5 ± 0.2	2.5 ± 0.1
	10	91.0 ± 6.3	256.2 ± 17.2	57.8 ± 0.8	20.7 ± 0.2	4.1 ± 0.3	6.3 ± 0.7	1.8 ± 0.1	2.7 ± 0.1	2.9 ± 0.0
	11	95.5 ± 7.2	244.1 ± 21.8	51.6 ± 0.3	21.3 ± 0.1	6.4 ± 0.2	4.4 ± 1.0	2.5 ± 0.2	5.1 ± 1.2	3.7 ± 0.5

Table 18. continued.

	no.	*trans*-sitostanyl *p*-coumarate [%]	*trans*-campestanyl *p*-coumarate [%]	*trans*-Δ^7 campesteryl ferulate [%]	*trans*-24-meth.-cycl.[d] ferulate [%]	*cis*-sitosteryl ferulate [%]	*cis*-campesteryl ferulate [%]	*trans*-campesteryl *p*-coumarate [%]	*trans*-sitosteryl *p*-coumarate [%]
dent corn[a]	1	1.3 ± 0.1	0.7 ± 0.1	1.1 ± 0.4	<LOQ	-	<LOQ	<LOQ	<LOQ
	2	1.3 ± 0.0	1.2 ± 0.5	2.6 ± 0.1	<LOQ	-	-	-	-
	3	2.0 ± 1.3	1.2 ± 0.4	<LOQ	-	-	<LOQ	-	-
	4	1.7 ± 0.0	1.0 ± 0.1	<LOQ	-	1.3 ± 0.0	2.0 ± 0.1	<LOQ	<LOQ
	5	1.2 ± 0.1	-[c]	-	-	<LOQ	<LOQ	-	-
	6	2.9 ± 1.1	1.0 ± 0.0	-	2.1 ± 0.1	<LOQ	<LOQ	<LOQ	<LOQ
	7	1.7 ± 0.2	0.7 ± 0.1	0.9 ± 0.2	<LOQ	1.7 ± 0.1	<LOQ	<LOQ	<LOQ
	8	1.1 ± 0.1	1.2 ± 0.1	1.6 ± 0.3	<LOQ	<LOQ	<LOQ	-	<LOQ
	9	2.2 ± 0.1	1.1 ± 0.0	2.6 ± 0.1	-	-	-	-	<LOQ
	10	1.5 ± 0.1	0.9 ± 0.0	2.6 ± 0.2	<LOQ	-	-	-	-
	11	1.8 ± 0.2	0.9 ± 0.1	<LOQ	-	<LOQ	1.3 ± 0.1	<LOQ	<LOQ
	12	4.5 ± 0.5	2.4 ± 0.3	<LOQ	-	-	-	-	-
	13	3.3 ± 1.6	1.6 ± 0.5	-	-	-	-	-	-
	14	2.2 ± 0.0	1.3 ± 0.1	3.9 ± 0.3	<LOQ	-	-	-	-
	15	3.1 ± 0.1	1.4 ± 0.1	2.1 ± 0.2	-	-	-	-	-
flint corn[a]	1	<LOQ	<LOQ	<LOQ	-	<LOQ	3.0 ± 0.2	-	-
	2	2.2 ± 0.1	1.2 ± 0.1	<LOQ	<LOQ	<LOQ	-	<LOQ	0.7 ± 0.1
	3	2.6 ± 0.1	1.2 ± 0.1	1.7 ± 0.1	2.1 ± 0.6	<LOQ	1.7 ± 0.0	<LOQ	<LOQ
	4	1.7 ± 0.0	1.3 ± 0.3	<LOQ	<LOQ	<LOQ	1.7 ± 0.2	<LOQ	-
	5	1.5 ± 0.1	0.9 ± 0.0	2.2 ± 0.3	<LOQ	-	-	-	-
	6	3.2 ± 0.0	1.7 ± 0.7	<LOQ	3.2 ± 0.5	-	-	<LOQ	<LOQ
	7	1.1 ± 0.1	-	-	-	<LOQ	3.7 ± 0.1	-	-
	8	3.7 ± 0.6	1.6 ± 0.3	-	2.6 ± 0.1	<LOQ	<LOQ	<LOQ	<LOQ
	9	1.9 ± 0.0	0.9 ± 0.1	1.6 ± 0.2	-	-	<LOQ	-	-
	10	1.5 ± 0.1	<LOQ	2.2 ± 0.3	-	-	-	-	-
	11	2.1 ± 0.0	1.1 ± 0.0	1.7 ± 0.3	<LOQ	-	<LOQ	<LOQ	<LOQ

[a] Based on dry matter (dm) of ground kernels. [b] Content below limit of quantification (Table 13). [c] (-) Content below limit of detection (Table 13). [d] 24-Methylene cycloartanyl.

4.2.2 Distribution Patterns of Free and Esterified Sterols/Stanols

The distribution patterns of sterols/stanols which occurred free or esterified to fatty acids were almost comparable. Sitosterol and campesterol were dominating in both fractions, which is in accordance with other studies on corn seedlings and corn germ oil (Kemp *et al.*, 1967; Kemp and Mercer, 1968; Verleyen *et al.*, 2002a; Dulf *et al.*, 2010). The only obvious differences were that the proportions of campestanol and sitostanol were slightly higher in the fractions of steryl/stanyl fatty acid esters, whereas the proportion of stigmasterol was higher in the fractions of free sterols/stanols. A dominance of free over esterified stigmasterol in corn has also been previously described (Kemp *et al.*, 1967; Milkova *et al.*, 1977); other studies detected almost equal proportions in corn germ oils (Phillips *et al.*, 2002; Verleyen *et al.*, 2002a; Dulf *et al.*, 2010).

The percentage composition of sterols/stanols esterified to phenolic acids differed significantly from those of free sterols/stanols and steryl/stanyl fatty acid esters. The majority (85-89 %) of phenolic acids in corn was esterified to stanols. Stigmasterol, which was detected free as well as esterified to fatty acids, could not be detected as phenolic acid ester. These observations agree with earlier reported results concerning the steryl/stanyl phenolic acid ester distribution of corn (Evershed *et al.*, 1988; Seitz, 1989; Norton, 1995; Iwatsuki *et al.*, 2003).

Possible reasons for the differences observed for the distribution patterns of sterols/stanols esterified to phenolic acids compared to the profile of free sterols/stanols or sterols/stanols esterified to fatty acid might be varying substrate availability or different enzyme specificities. Furthermore, previous studies have shown that steryl/stanyl phenolic acid esters were mainly found in the aleurone layer of corn kernels (Seitz, 1990; Moreau *et al.*, 2000) and that the pericarp, which consists of the outer layers (including aleurone layer) of corn kernels, contains comparably high amounts of stanols (Harrabi *et al.*, 2008). This might contribute to the prevalence of stanol phenolic acid esters.

4.2.3 Distribution Patterns of Total Fatty Acids and of Fatty Acids Esterified to Sterols/Stanols

The total fatty acid compositions of the 26 corn cultivars were almost equal (Appendix Table 39). Linoleic acid (51-64 %), oleic acid (23-36 %), and palmitic acid (9-13 %) were the main fatty acids, which agrees very well with earlier reported data (Goffman and Böhme, 2001; Harrabi *et al.*, 2009; Moreau *et al.*, 2009).

The fatty acids esterified to sterols/stanols consisted also mainly of linoleic acid, followed by oleic/stearic acid and palmitic/palmitoleic acid. However, linoleic acid was found to a higher

degree esterified to sterols/stanols compared to the total fatty acids, whereas the amounts of palmitic/palmitoleic acid and oleic/stearic acid were lower. This distribution pattern was observed for all cultivars and has also been described for crude and refined corn germ oils (Worthington and Hitchcock, 1984; Ferrari *et al.*, 1997).

4.2.4 Proportions of Free Sterols/Stanols and Steryl/Stanyl Esters

The sum of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters determined in dry matter flour was the highest in the cultivar PR38Y34 with 1.74 mg/g and the lowest in Lg 3258 with 0.82 mg/g, both belonging to the subspecies flint corn (Figure 23A). The amounts based on extracted oils ranged from 2.26 to 3.57 mg/100 mg, being highest in the dent corn cultivar MAS 31a and lowest in the flint corn cultivar Crazi (Figure 23B).

Significant positive correlations were observed for the total contents of free sterols/stanols and steryl/stanyl fatty acid esters ($r = 0.64$, $p = 0.001$) and for the total contents of steryl/stanyl fatty acid esters and steryl/stanyl phenolic acid esters ($r = 0.56$, $p = 0.004$) based on dry matter flour. The same trends were detected for the total amounts calculated on the basis of extracted oils, but the correlations were not significant ($p < 0.05$)

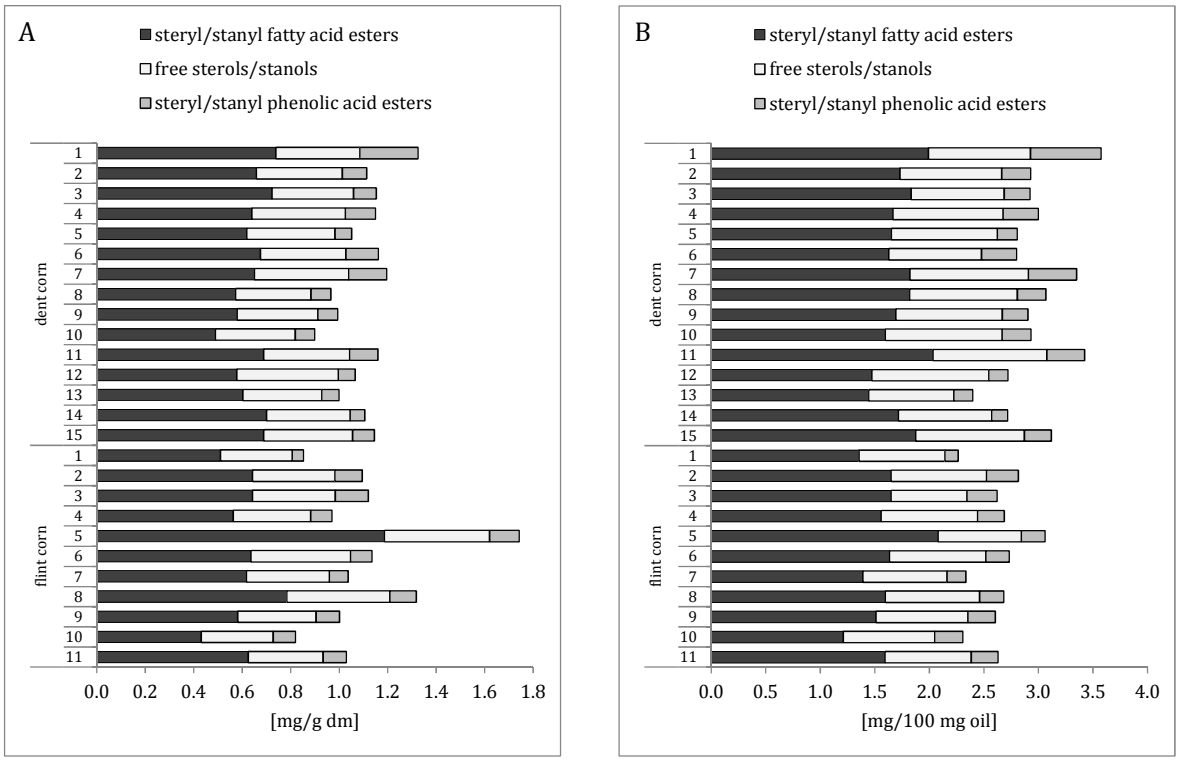

Figure 23. Sums of total contents of steryl/stanyl fatty acid esters, free sterols/stanols, and steryl/stanyl phenolic acid esters of the dent corn and flint corn cultivars determined (**A**) in dry matter flour and (**B**) in extracted oil. (The numberings of the samples correspond to those in Table 7.)

The mean percentage distribution of the three sterol classes (sum of total contents of intact conjugates) revealed a very similar pattern for all 26 cultivars. The majority (on average about 60 %) of sterols was present in form of their fatty acid esters. Approximately one third occurred as free sterols/stanols and 5-18 % as phenolic acid esters. The dominance of steryl/stanyl fatty acid esters in corn kernels and oils has also been observed in a few other studies (Worthington and Hitchcock, 1984; Moreau *et al.*, 2001; Phillips *et al.*, 2002; Moreau and Hicks, 2005; Dulf *et al.*, 2010).

4.2.5 Comparison of Dent Corn and Flint Corn

The dent corn and flint corn plants were grown at one location and the cobs were harvested at the same time. Therefore, differences owing to environmental conditions could largely be excluded and the influence of the subspecies on the total contents of free sterols/stanols and steryl/stanyl esters could be studied. The mean total contents of the three sterol classes determined in the dent corn and flint corn cultivars are illustrated in Figure 24.

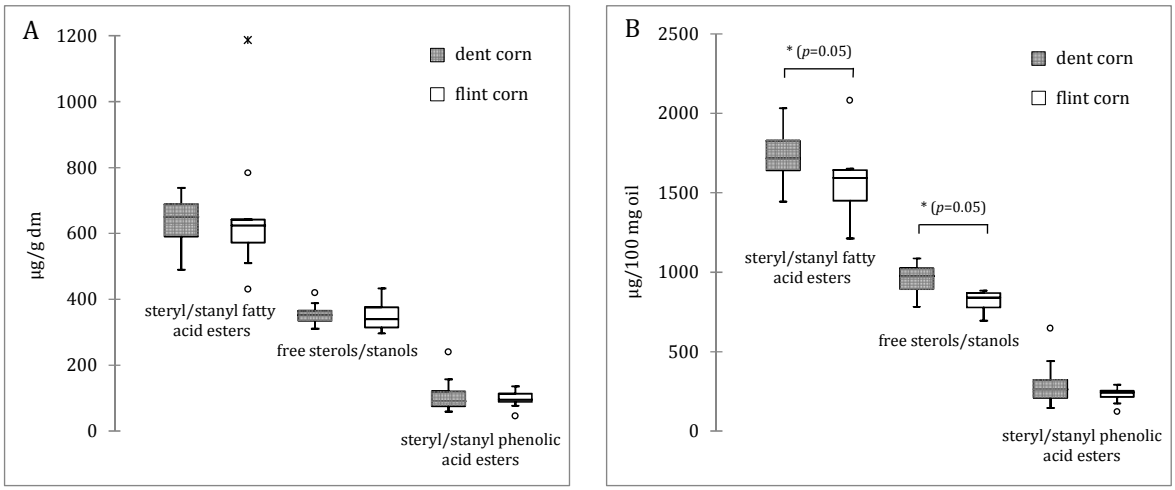

Figure 24. Boxplot diagrams of total contents of free sterols/stanols and steryl/stanyl esters determined (**A**) in dry matter flour and (**B**) in extracted oil of dent corn and flint corn.

The mean total contents of free sterols/stanols and of steryl/stanyl fatty acid esters determined in the dent corn and flint corn oils were significantly different (p = 0.05). In turn, the mean total contents of steryl/stanyl phenolic acid esters in the oils as well as the levels of all sterol classes calculated on the basis of dry matter flour revealed no significant differences (p = 0.05). The oil contents of the 26 cultivars ranged from 3.07 to 5.69 % (Table 7), being on average slightly lower in the dent corn cultivars (3.72 ± 0.34 %) than in the flint corn cultivars (4.29 ± 0.68 %). However, the difference between both subspecies was not significant (p = 0.05).

4.3 Analysis of Free Sterols/Stanols and Steryl/Stanyl Esters in Sweet Corn and Popcorn

The described on-line LC-GC-based approach (cf. 4.1.2) was applied to the analysis of two other corn subspecies, namely sweet corn and popcorn. In total, four fresh sweet corn and five popcorn samples, all commercially obtained, were subjected to on-line LC-GC analysis for the determination of the contents and compositions of free sterols/stanols and steryl/stanyl esters. Identification was performed by GC-MS after multiple manual collection of the LC fractions of interest. On-line LC-GC chromatograms of free sterols/stanols and steryl/stanyl esters have already been shown for a popcorn sample in Figure 18 and Figure 19.

4.3.1 Compositions and Contents of Free Sterols/Stanols and Steryl/Stanyl Esters

Free Sterols/Stanols

The profiles of free sterols/stanols showed almost no variation within as well as between both subspecies (Table 19). Sitosterol was throughout predominating, accounting for 59.3-65.1 % of total free sterols/stanols, followed by campesterol and stigmasterol. Free stanols made up on average 5.6 ± 2.2 % in sweet corn and 7.2 ± 0.9 % in popcorn. Cholesterol represented 0.2-2.4 % of total free sterols/stanols. To the author's knowledge, literature data on the qualitative and quantitative composition of free sterols/stanols in sweet corn and popcorn are not available. However, the detected profiles agree with already published data on total or free sterols/stanols in corn kernels or corn oils (Normén *et al.*, 2002; Harrabi *et al.*, 2007; Harrabi *et al.*, 2008; Moreau *et al.*, 2009; Harrabi *et al.*, 2011), and are also comparable to the compositions analyzed in several dent corn and flint corn cultivars (cf. 4.2.1).

Total contents of free sterols/stanols determined on the basis of dry matter sweet corn flour were in the range of 870.7-1049.1 µg/g and thus approximately 2- to 3.6-times higher than those of the popcorn flours (273.7-395.8 µg/g). The amounts calculated for the extracted oils averaged 0.99 ± 0.09 % in sweet corn and 0.78 ± 0.10 % in popcorn. A comparison of the amounts to those in dent corn and flint corn (cf. 4.2.1) shows that sweet corn has by far the highest total contents of free sterols/stanols among the investigated corn subspecies.

Steryl/Stanyl Fatty Acid Esters

The determined contents and percentage compositions of steryl/stanyl fatty acid esters in the sweet corn and popcorn samples are summarized in Table 20. The majority of sterols/stanols occurred as esters of C18-fatty acids, with sitosterol and campesterol as main esterified

sterols. Steryl/stanyl esters of C16-fatty acids made up about 4-5 % in sweet corn and 5-11 % in popcorn. Stanyl esters accounted for approximately 20 % in popcorn, which is comparable to the amounts detected in dent corn and flint corn (cf. 4.2.1), but higher than the levels analyzed in sweet corn (8.1 ± 0.7 %).

The determination of the distributions of palmitic/palmitoleic acid and stearic/oleic acid esters, which coeluted under the employed GC conditions via analysis of the respective fatty acid methyl esters, revealed a predominance of palmitic acid and oleic acid esters. No palmitoleic acid could be detected in the investigated popcorn samples; in the sweet corn samples the ratios of palmitic/palmitoleic acid were 21:1 and 138:1. The ratios of oleic/stearic acid were in the range of 7:1 to 14:1. This is in accordance with the data obtained for two dent corn and flint corn cultivars (cf. 4.2.1).

The average total contents of steryl/stanyl fatty acid esters based on dry matter sweet corn flour were 1.7- to 4.4-fold above the levels determined in the flours of popcorn and also above those detected in the dent corn and flint corn flours (cf. 4.2.1). The higher amounts of steryl/stanyl fatty acid esters as well as of free sterols/stanols in the sweet corn kernels can be explained by their relatively high oil contents. The oil contents of the sweet corn samples averaged 9.77 ± 1.01 % and were thus more than twice as high as those determined in the popcorn, dent corn, or flint corn samples (Table 7 and Table 8). Comparable high fat contents based on dry matter of matured sweet corn kernels have already been described (Sanderson *et al.*, 1979).

Steryl/Stanyl Phenolic Acid Esters

As shown in Table 21, sitostanyl and campestanyl ferulate were dominating within the fraction of *trans*-steryl/stanyl ferulic acid esters in sweet corn and popcorn. These esters accounted together for 74.4-90.3 % of total *trans*-steryl/stanyl ferulic acid esters. Additionally, ferulic acid esters of sitosterol, campesterol, and 24-methylene cycloartanol were detected. One obvious difference was that the analyzed popcorn samples exhibited a higher degree of esterified stanols than the samples of sweet corn.

The total contents of *trans*-steryl/stanyl ferulic acid esters in the flours and oils of sweet corn were on average about 1.4- to 13.7-times lower, respectively, than those determined in popcorn. Additionally, a 3-fold margin was observed between the lowest and highest content of these esters in sweet corn (no. 1 and no. 2, respectively), whereas the total contents of the four popcorn samples were in the same order of magnitude. Even though the on-line LC-GC-based approach does not enable the analysis of steryl/stanyl coumaric acid esters and *cis*-derivatives of ferulic acid esters, the total amounts determined in popcorn were also higher than those quantified in the dent corn and flint corn cultivars (cf. 4.2.1).

Table 19. Contents and compositions of free sterols/stanols in sweet corn and popcorn.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	sitosterol [%]	campesterol [%]	stigmasterol [%]	sitostanol [%]	Δ7 sitosterol[b] [%]	campestanol [%]	cholesterol [%]
sweet corn	1	993.1 ± 15.7	1125.2 ± 17.5	59.7 ± 0.5	22.4 ± 0.2	7.8 ± 0.1	2.3 ± 0.0	5.6 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
	2	1049.1 ± 10.2	974.3 ± 31.1	62.6 ± 0.8	19.7 ± 0.3	6.6 ± 0.3	3.7 ± 0.1	5.1 ± 0.1	1.5 ± 0.3	0.9 ± 0.1
	3	870.7 ± 11.6	971.2 ± 4.7	61.7 ± 0.6	20.8 ± 0.2	7.4 ± 0.3	3.7 ± 0.1	4.3 ± 0.6	1.5 ± 0.0	0.7 ± 0.1
	4	946.5 ± 20.9	901.6 ± 27.7	59.3 ± 0.5	22.6 ± 0.2	6.8 ± 0.1	6.1 ± 0.3	1.9 ± 0.3	2.6 ± 0.1	0.6 ± 0.1
popcorn	1	395.8 ± 14.2	935.1 ± 7.6	65.1 ± 0.2	18.7 ± 0.1	7.7 ± 0.4	4.6 ± 0.0	1.7 ± 0.0	1.3 ± 0.0	0.9 ± 0.0
	2	331.0 ± 5.5	804.0 ± 19.0	64.9 ± 0.1	18.9 ± 0.2	6.4 ± 0.1	6.1 ± 0.2	1.8 ± 0.1	1.7 ± 0.2	0.2 ± 0.0
	3	273.7 ± 9.1	683.3 ± 16.8	63.5 ± 0.7	19.7 ± 0.1	6.5 ± 0.3	5.7 ± 0.1	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0
	4	276.4 ± 0.7	683.8 ± 5.5	62.4 ± 0.1	19.7 ± 0.1	6.6 ± 0.1	5.7 ± 0.0	1.6 ± 0.0	1.5 ± 0.1	2.4 ± 0.1
	5	290.2 ± 1.5	773.4 ± 8.3	64.2 ± 0.2	18.3 ± 0.0	6.2 ± 0.1	6.4 ± 0.0	1.8 ± 0.0	1.7 ± 0.1	1.3 ± 0.1

[a] Based on dry matter (dm) of ground kernels. [b] Coelution with an unidentified sterol.

Table 20. Contents and compositions of steryl/stanyl fatty acid esters in sweet corn and popcorn.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	sitosteryl-18:2 [%]	campesteryl-18:2 [%][b]	sitosteryl-18:0/18:1 [%]	sitostanyl-18:2 [%]	campestanyl-18:0/18:1 [%][c]	Δ7 sitosteryl-18:2 [%][d]	campesteryl-18:0/18:1 [%]
sweet corn	1	822.6 ± 35.3	932.1 ± 40.2	47.0 ± 0.2	16.7 ± 0.2	13.0 ± 0.2	4.7 ± 0.1	3.7 ± 0.2	5.5 ± 0.5	4.6 ± 0.3
	2	1067.8 ± 20.0	991.3 ± 20.7	53.7 ± 1.4	14.1 ± 0.2	11.8 ± 0.1	4.6 ± 0.4	2.9 ± 0.4	4.7 ± 0.3	3.2 ± 0.5
	3	905.1 ± 27.8	1009.5 ± 25.7	53.6 ± 0.6	16.6 ± 0.6	10.5 ± 0.1	4.3 ± 0.4	2.7 ± 0.2	5.3 ± 0.3	3.1 ± 0.3
	4	932.4 ± 52.4	887.6 ± 39.2	54.3 ± 0.9	15.6 ± 0.2	10.6 ± 0.2	4.6 ± 0.6	3.0 ± 0.2	3.8 ± 0.5	2.8 ± 0.1
popcorn	1	493.3 ± 16.4	1165.6 ± 7.5	45.4 ± 0.9	10.3 ± 0.2	13.5 ± 0.6	9.1 ± 0.2	6.0 ± 0.1	2.2 ± 0.3	2.1 ± 0.1
	2	343.3 ± 5.3	792.8 ± 5.3	43.2 ± 0.4	10.9 ± 0.1	14.9 ± 0.3	9.8 ± 0.2	5.5 ± 0.1	2.5 ± 0.1	1.9 ± 0.1
	3	240.0 ± 2.0	599.5 ± 9.0	33.8 ± 1.1	10.7 ± 0.2	22.3 ± 0.0	7.3 ± 0.2	6.4 ± 0.1	2.4 ± 0.0	2.5 ± 0.2
	4	246.2 ± 1.5	609.2 ± 4.3	41.1 ± 0.2	11.0 ± 0.0	12.4 ± 0.8	8.1 ± 0.3	5.4 ± 0.1	2.8 ± 0.1	3.0 ± 0.1
	5	276.6 ± 6.4	736.9 ± 12.0	44.2 ± 1.3	11.1 ± 0.1	7.7 ± 1.1	10.8 ± 0.1	6.1 ± 0.1	3.4 ± 0.1	1.6 ± 0.1

Table 20. continued.

	no.	campestanyl-18:2 [%]	sitostanyl-18:0/18:1 [%]	sitosteryl-16:0/16:1 [%]	Δ^7 campesteryl-18:2 [%][d]	Δ^7 sitosteryl-16:0/16:1 [%][d]	campesteryl-16:0/16:1 [%]	sitostanyl-16:0/16:1 [%]	stigmasteryl-16:0/16:1 [%]	campestanyl-16:0/16:1 [%][d]
sweet corn[b]	1	<LOQ[e]	<LOQ	3.0 ± 0.2	-[f]	-	1.0 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
	2	<LOQ	<LOQ	3.0 ± 0.0	-	-	1.1 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.0
	3	<LOQ	<LOQ	2.7 ± 0.0	-	-	0.8 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	4	<LOQ	<LOQ	3.2 ± 0.1	-	-	1.0 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
popcorn[b]	1	2.6 ± 0.1	1.8 ± 0.3	2.7 ± 0.1	0.6 ± 0.1	2.0 ± 0.4	0.7 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	0.4 ± 0.1
	2	3.1 ± 0.1	2.2 ± 0.4	2.4 ± 0.1	0.7 ± 0.0	0.9 ± 0.1	0.7 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.3 ± 0.0
	3	2.0 ± 0.1	1.9 ± 0.3	3.6 ± 0.0	0.6 ± 0.0	2.1 ± 0.0	0.8 ± 0.1	0.4 ± 0.0	2.7 ± 0.4	0.4 ± 0.0
	4	2.0 ± 0.1	3.3 ± 0.2	5.0 ± 0.0	0.7 ± 0.1	2.3 ± 0.1	0.8 ± 0.0	0.5 ± 0.0	1.3 ± 0.0	0.4 ± 0.1
	5	2.5 ± 0.0	3.2 ± 0.5	4.3 ± 0.1	0.7 ± 0.1	2.0 ± 0.0	0.7 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.5 ± 0.0

[a] Based on dry matter (dm) of ground kernels. [b] Coelution with stigmasteryl-18:0/18:1. [c] Coelution with stigmasteryl-18:2. [d] Compound calculated with Rf = 1. [e] Content below limit of quantification (Table 14). [f] (-) Content below limit of detection (Table 14).

Table 21. Contents and compositions of *trans*-steryl/stanyl ferulic acid esters in sweet corn and popcorn.

	no.	$\sum$ [µg/g dm[a]]	$\sum$ [µg/100 mg oil]	*trans*-sitostanyl ferulate [%]	*trans*-campestanyl ferulate [%]	*trans*-sitosteryl ferulate [%]	*trans*-campesteryl ferulate [%]	*trans*-Δ^7 sitosteryl ferulate [%]	*trans*-Δ^7 campesteryl ferulate [%]	*trans*-24-meth.-cycl.[d] ferulate [%]
sweet corn	1	33.5 ± 2.8	37.9 ± 2.4	43.2 ± 1.4	31.2 ± 1.5	13.0 ± 0.6	7.4 ± 0.6	<LOQ[b]	-[c]	5.3 ± 1.1
	2	111.7 ± 7.0	103.6 ± 4.4	42.2 ± 0.9	36.9 ± 0.5	9.5 ± 0.6	9.8 ± 0.1	1.6 ± 0.2	-	<LOQ
	3	47.6 ± 1.3	53.0 ± 0.8	41.9 ± 0.3	36.1 ± 0.3	9.9 ± 0.2	6.9 ± 0.5	2.4 ± 0.1	-	2.7 ± 0.4
	4	47.6 ± 0.8	45.3 ± 1.4	42.1 ± 0.4	36.4 ± 0.5	10.0 ± 0.2	6.6 ± 0.9	2.5 ± 0.0	-	2.5 ± 0.3
popcorn	1	202.6 ± 6.2	503.3 ± 11.1	60.7 ± 0.2	28.2 ± 0.2	4.0 ± 0.1	4.9 ± 0.1	1.3 ± 0.1	0.6 ± 0.0	0.3 ± 0.0
	2	165.4 ± 3.4	401.8 ± 6.2	60.7 ± 0.0	28.7 ± 0.1	3.1 ± 0.1	4.1 ± 0.1	1.8 ± 0.2	0.7 ± 0.0	0.9 ± 0.1
	3	185.4 ± 5.5	466.6 ± 7.9	60.3 ± 0.1	30.0 ± 0.4	3.1 ± 0.3	4.1 ± 0.3	1.4 ± 0.1	0.7 ± 0.1	0.3 ± 0.0
	4	173.0 ± 6.0	433.8 ± 7.8	59.7 ± 0.2	30.3 ± 0.0	3.0 ± 0.0	4.1 ± 0.0	1.5 ± 0.1	0.9 ± 0.1	0.5 ± 0.1
	5	186.5 ± 4.3	521.0 ± 18.0	63.0 ± 0.6	25.0 ± 0.3	4.3 ± 0.2	4.6 ± 0.5	1.5 ± 0.3	0.9 ± 0.2	0.6 ± 0.0

[a] Based on dry matter (dm) of ground kernels. [b] Content below limit of quantification (Table 14). [c] (-) Content below limit of detection (Table 14). [d] 24-Methylene cycloartanyl.

4.3.2 Distribution Patterns of Free and Esterified Sterols/Stanols

In popcorn and sweet corn the sterols/stanols which occurred free and esterified to fatty acids revealed the same distribution patterns as those of dent corn and flint corn (cf. 4.2.2). Briefly, the compositions of free sterols/stanols and of sterols/stanols esterified to fatty acids were comparable, revealing only slightly higher proportions of stanols and, in turn, lower amounts of stigmasterol in the fractions of steryl/stanyl fatty acid esters. The majority of ferulic acid in popcorn and sweet corn was esterified to stanols, i.e. sitostanol and campestanol. However, the proportions of esterified stanols in sweet corn (~77 %) were slightly lower than those of popcorn (~85 %).

4.3.3 Distribution Patterns of Total Fatty Acids and of Fatty Acids Esterified to Sterols/Stanols

The total fatty acid compositions of the popcorn oils were equal to those of the dent corn and flint corn oils (Appendix Table 39). Linoleic acid was predominant (56-63 %), followed by oleic acid (22-29 %), and palmitic acid (10-12 %). The fatty acid compositions of the sweet corn oils differed from those of the other corn subspecies as higher proportions of oleic acid (35-40 %) and, in turn, lower proportions of linoleic acid (42-46 %) were detected. Comparable distributions have also been described for the fatty acid composition of whole sweet corn kernels and of several high oil producing corn lines (Weber, 1969; Weber and Alexander, 1975; Harrabi *et al.*, 2009)

The fatty acids in sweet corn and popcorn, which were esterified to sterols/stanols were composed mainly of linoleic acid, followed by oleic/stearic acid and palmitic/palmitoleic acid. However, linoleic acid was detected to a higher degree esterified to sterols/stanols as in the whole oils, whereas palmitic/palmitoleic acid and oleic/stearic acid were found to a lower degree. This is in agreement with the data obtained for dent corn and flint corn (cf. 4.2.3).

4.3.4 Proportions of Free Sterols/Stanols and Steryl/Stanyl Esters

As illustrated in Figure 25A, the sums of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters calculated on the basis of dry matter flour were higher in the sweet corn samples (1.82-2.22 mg/g) than in the popcorn samples (0.70-1.09 mg/g) and also above the levels analyzed in the dent corn and flint corn cultivars (cf. 4.2.4, Figure 23A). The amounts based on extracted oils ranged from 1.83 to 2.09 mg/100 mg in sweet corn and from 1.73 to 2.60 mg/100 mg in popcorn (Figure 25B).

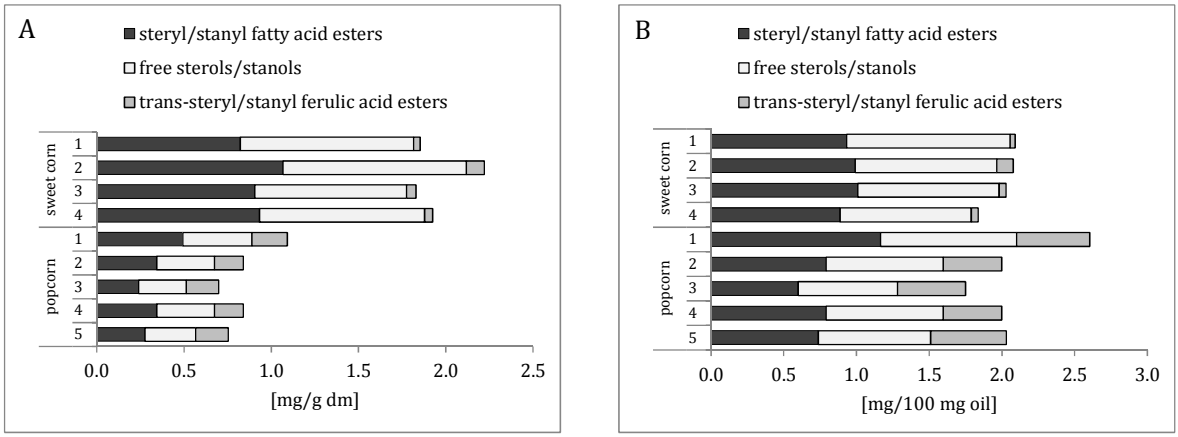

Figure 25. Sums of total contents of steryl/stanyl fatty acid esters, free sterols/stanols, and steryl/stanyl phenolic acid esters of the sweet corn and pocorn samples determined (**A**) in dry matter flour and (**B**) in extracted oil. (The numberings of the samples correspond to those in Table 8.)

Considering the mean percentage distribution of the three sterol classes, free sterols/stanols and intact steryl/stanyl fatty acid esters made up almost the same proportion in sweet corn (approximately 49 and 48 %, respectively). Popcorn revealed a higher proportion of *trans*-steryl/stanyl ferulic acid esters (about 23 %). Steryl/stanyl fatty acid esters and free sterols/stanols made up on average 39 % and 38 %, respectively. Thus, in both subspecies the amounts of steryl/stanyl fatty acid esters were in the same order of magnitude as the respective levels of free sterols/stanols. This is in contrast to the distributions observed for dent corn and flint corn (cf. 4.2.4), where steryl/stanyl fatty acid esters were always more abundant than free sterols/stanols.

4.4 Analysis of Free Sterols/Stanols and Steryl/Stanyl Esters in Small Millets and Sorghum

Despite the relevance of small millets and sorghum for human nutrition, particularly in Asian and African cultures, data on individual free sterols/stanols and steryl/stanyl esters are lacking (cf. 2.2.2.2). In the course of the present work, certain types of small millets and three cultivars of sorghum grains were analyzed regarding their contents and compositions of free sterols/stanols, steryl/stanyl fatty acid esters, and *trans*-steryl/stanyl ferulic acid esters. The approach based on on-line LC-GC was applied. During the work up of the samples, the system originally equipped with an FID was extended and an on-line LC-GC-MS system was introduced, which simplified the identification of the individual compounds.

4.4.1 On-line LC-GC-Based Approach

On-line LC-GC chromatograms of free sterols/stanols and steryl/stanyl fatty acid esters extracted from a proso millet sample are exemplarily shown in Figure 26.

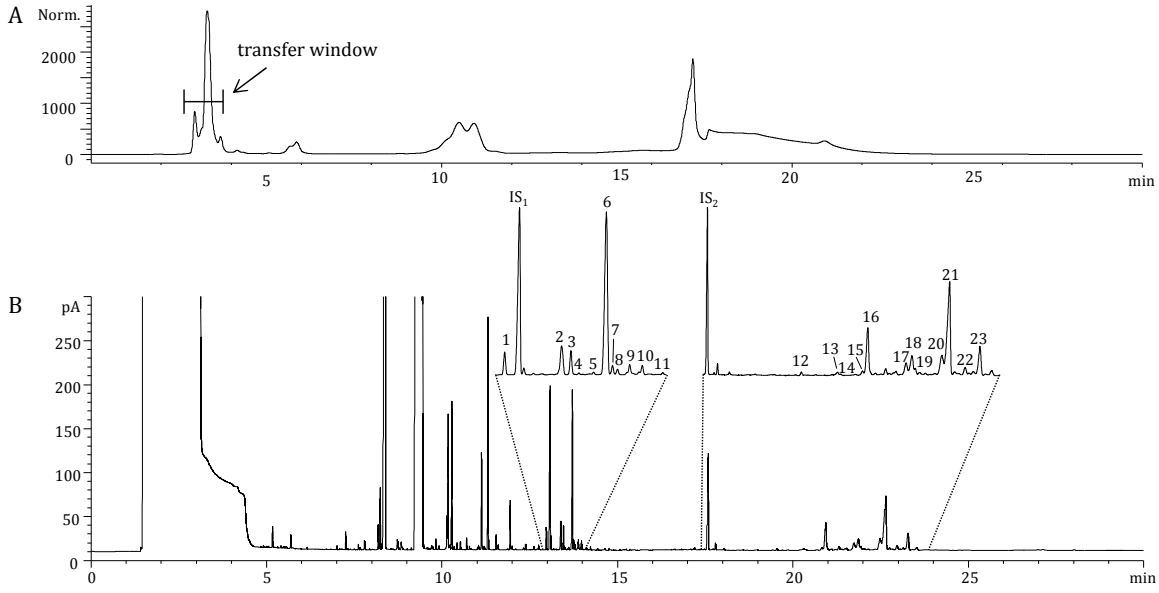

Figure 26. On-line LC-GC analysis of free sterols/stanols and steryl/stanyl fatty acid esters in proso millet. (**A**) LC-UV chromatogram at 205 nm and (**B**) GC-FID chromatogram of the transferred LC-fraction.
(1) cholesterol, (2) campesterol, (3) stigmasterol, (4) campestanol, (5) clerosterol, (6) sitosterol, (7) sitostanol, (8) Δ^5 avenasterol, (9) cycloartanol, (10) cycloartenol, (11) 24-methylene cycloartanol, (12) cholesteryl-16:0/16:1, (13) campesteryl-16:0/16:1, (14) stigmasteryl-16:0/16:1, (15) cholesteryl-18:0/18:1, (16) cholesteryl-18:2 + sitosteryl-16:0/16:1, (17) campesteryl-18:0/18:1, (18) campesteryl-18:2 + stigmasteryl-18:0/18:1, (19) stigmasteryl-18:2, (20) sitosteryl-18:0/18:1, (21) sitosteryl-18:2, (22) cycloartanyl-18:2, (23) cycloartenyl-18:2, (IS_1) 5α-cholestan-3β-ol, and (IS_2) cholesteryl-12:0.

The analysis of a proso millet flour revealed the natural presence of cholesteryl palmitate. This compound has been used as IS for the quantification of steryl/stanyl fatty acid esters in all other cereal grains. It was replaced by cholesteryl laurate in case of the small millet and sorghum samples.

The methodology was re-validated for the new matrix in terms of recovery and repeatability. Recoveries were determined by spiking a proso millet flour with known amounts of selected plant sterols/stanols and steryl/stanyl esters. The experiments resulted in recoveries of 92.7 ± 2.9 % for stigmasterol, 98.5 ± 1.8 % for sitostanol, 95.4 ± 2.9 % for stigmasteryl-16:0, and 86.1 ± 3.1 % for sitostanyl-18:2, thus being comparable to those obtained for the corn spiking experiments (cf. 4.1.2.2).

The analysis, including lipid extraction and on-line LC-GC analysis, of five replicates from a control sample (proso millet flour) resulted in relative standard deviations of 1.2 % for free sterols/stanols and of 3.3 % for steryl/stanyl fatty acid esters, indicating a good repeatability of the approach.

4.4.2 Compositions and Contents of Free Sterols/Stanols and Steryl/Stanyl Esters

Free Sterols/Stanols

Total contents and percentage compositions of free sterols/stanols determined in the small millet and sorghum samples are presented in Table 22. Within a certain type of grain, the distribution patterns of free sterols/stanols were almost comparable, but considerable differences were observed between the various grains.

All millet and sorghum samples had in common that sitosterol was the predominant free sterol, followed by campesterol. This is in agreement with previous findings observed for free sterols in sorghum, foxtail millet, and finger millet (Mahadevappa and Raina, 1978a; Heupel *et al.*, 1986; Narumi and Takatsuto, 1999; Takatsuto *et al.*, 1999). However, the percentage amounts of sitosterol were lower in sorghum (41.0-45.4 %) compared to the seeds of small millets (55.5-68.9 %). The three investigated sorghum samples, in turn, revealed much higher proportions of campesterol and stigmasterol. This is in line with observations reported for the compositions of major free sterols in developing sorghum kernels (Palmer and Bowden, 1977; Heupel *et al.*, 1986). Overall, 4-desmethylsterols were most abundant, accounting for 92.0 ± 1.6 % in the seeds of proso millet, 82.6 ± 3.9 % in the teff samples, 90.5 ± 1.9 % in the sorghum kernels, 81.1 % in foxtail millet, and 88.9 % in finger millet. 4,4'-dimethylsterols represented 1.8-8.3 % of total free sterols/stanols. The highest percentage amount of free stanols was detected in foxtail millet (11.2 %). Sitostanol has already been reported as a major component in foxtail millet not only of free but also of total sterols, accounting for 17

and 26 %, respectively (Takatsuto and Kawashima, 1998; Narumi and Takatsuto, 1999). The teff samples contained relatively high amounts of *α*-amyrin and *β*-amyrin. Both made up 9.5-15.7 % of total free sterols/stanols. *β*-Amyrin is known as the major triterpene in teff seeds (El-Alfy *et al.*, 2011). *α*-Amyrin and *β*-amyrin do not belong to the group of phytosterols, but to the triterpene alcohols (Moreau *et al.*, 2002). Their chemical structures are shown in Figure 27. Nevertheless, due to the structural similarities of *α*-amyrin and *β*-amyrin to 4,4'-dimethylsterols (Figure 2), they are often dealt together with sterols.

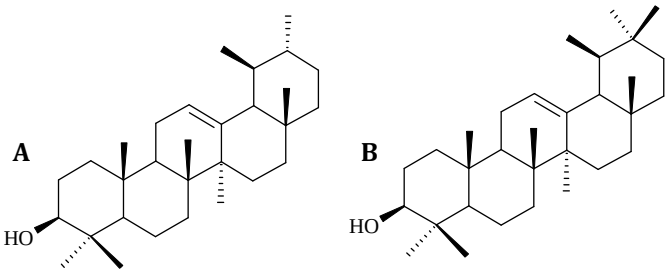

Figure 27. Chemical structures of (**A**) *α*-amyrin and (**B**) *β*-amyrin.

α-Amyrin and *β*-amyrin were also detected in sorghum, foxtail millet, and finger millet with amounts of up to 5.2 %, but not in the seeds of proso millet. Both compounds have also been identified within the fraction of 4,4'-dimethylsterols in foxtail millet in an earlier study (Narumi *et al.*, 2001).

Cholesterol could be detected in almost all of the investigated samples. However, the levels were particularly high in the seeds of proso millet, where it accounted for 3.6-9.1 % of total free sterols/stanols. Comparable cholesterol amounts (6.7 % of free sterols) have also been analyzed in another species of *Panicum*, namely *Panicum coloratum* (Bowden and Williams, 1971). Furthermore, during analysis of total sterols in proso millet it has been shown that cholesterol represented 3-16 % (Takatsuto *et al.*, 1998). All other small millet and sorghum seeds investigated in the present study revealed cholesterol amounts of <0.7 %.

The total contents of free sterols/stanols exhibited large variations. Total amounts based on dry matter flour ranged from 149.0-259.8 µg/g in proso millets, from 228.4-544.1 µg/g in teff, and from 266.4-347.2 µg/g in sorghum. The foxtail millet and finger millet sample revealed total free sterol/stanol levels of 421.2 and 146.2 µg/g, respectively, which thus were amounts in the upper and lower range of all investigated small millets.

The highest amounts based on extracted oil were determined in three of the six investigated teff samples (no. 1, 5, and 6), in the foxtail millet, and the finger millet sample. Here, free sterols/stanols made up >1 % of total extracted lipids, being by far the highest in the teff cultivars Brown (no. 1) and Kuncho (no. 6) with amounts of 1.75 and 1.79 %, respectively. The lowest amounts were detected in the seeds of proso millet 0.44-0.74 %. Previously, free

sterols have been investigated in a finger millet sample via a combination of preparative CC and TLC (Mahadevappa and Raina, 1978a). The sterol composition was further investigated via GC-FID. Even if they only detected sitosterol and stigmasterol, the total amount of free sterols reported for this finger millet cultivar was higher than that of the present study. However, a comparison is difficult not only because of the possible impact of the cultivar, but also because of differences in analytical performance. Due to the lack of information on free sterols in small millets, a further comparison to literature data is not possible. The total contents of free sterols/stanols determined in the three sorghum samples were in the same order of magnitude, averaging 0.92 ± 0.02 %, and are in good agreement with earlier results (Singh *et al.*, 2003; Christiansen *et al.*, 2007).

Table 22. Contents and compositions of free sterols/stanols in small millets and sorghum.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	sitosterol [%]	campesterol [%]	cholesterol [%]	stigmasterol [%]	cycloartenol [%]	cycloartanol [%]
proso millet	1	208.5 ± 2.1	518.4 ± 6.1	58.4 ± 0.2	12.2 ± 0.0	9.1 ± 0.2	5.7 ± 0.0	4.0 ± 0.1	3.2 ± 0.1
	2	188.3 ± 3.5	613.3 ± 3.4	64.2 ± 0.6	11.6 ± 0.1	6.0 ± 0.2	6.9 ± 0.6	2.6 ± 0.2	3.1 ± 0.0
	3	211.5 ± 3.0	671.6 ± 13.1	65.7 ± 0.7	12.1 ± 0.1	6.7 ± 0.2	5.9 ± 1.4	3.3 ± 0.3	2.7 ± 0.2
	4	259.8 ± 3.8	741.8 ± 5.2	61.0 ± 0.3	12.1 ± 0.1	7.4 ± 0.1	5.4 ± 0.1	2.9 ± 0.1	4.4 ± 0.2
	5	190.8 ± 13.4	439.0 ± 10.0	61.9 ± 0.2	11.4 ± 0.3	7.9 ± 0.1	7.3 ± 0.2	4.2 ± 0.0	1.7 ± 0.0
	6	149.0 ± 1.3	730.1 ± 12.1	66.7 ± 0.6	12.8 ± 0.1	3.6 ± 0.1	7.0 ± 0.4	2.3 ± 0.0	2.7 ± 0.0
teff	1	464.8 ± 6.3	1748.1 ± 25.6	59.6 ± 1.0	15.6 ± 0.2	0.3 ± 0.0	6.4 ± 0.1	0.6 ± 0.2	-[b]
	2	228.4 ± 4.4	790.2 ± 68.2	56.4 ± 0.8	12.2 ± 0.1	0.2 ± 0.0	6.3 ± 0.4	0.6 ± 0.1	-
	3	250.9 ± 2.4	819.8 ± 39.0	61.4 ± 0.2	13.0 ± 0.1	0.2 ± 0.0	4.6 ± 0.2	0.6 ± 0.1	-
	4	230.6 ± 1.5	888.7 ± 19.5	58.3 ± 0.3	12.3 ± 0.1	0.2 ± 0.0	5.0 ± 0.6	0.4 ± 0.1	-
	5	349.8 ± 22.7	1127.4 ± 67.2	59.3 ± 0.1	15.9 ± 0.2	0.2 ± 0.0	7.1 ± 0.8	0.7 ± 0.1	-
	6	544.1 ± 1.6	1791.0 ± 19.0	57.1 ± 0.9	9.2 ± 0.2	0.2 ± 0.0	3.8 ± 0.0	0.7 ± 0.1	-
sorghum	1	331.7 ± 7.9	930.1 ± 20.1	41.9 ± 0.4	26.5 ± 0.1	0.2 ± 0.0	14.1 ± 0.2	1.3 ± 0.1	-
	2	347.2 ± 4.1	945.3 ± 33.0	45.4 ± 2.8	24.9 ± 2.2	-	14.7 ± 0.9	1.0 ± 0.1	-
	3	266.4 ± 5.6	899.0 ± 37.7	41.0 ± 0.5	24.5 ± 1.5	0.1 ± 0.0	21.6 ± 0.5	1.6 ± 0.2	-
foxtail millet		421.2 ± 5.2	1358.2 ± 46.3	55.5 ± 0.4	12.1 ± 0.3	0.7 ± 0.1	8.2 ± 0.1	1.8 ± 0.1	-
finger millet		146.2 ± 7.6	1017.5 ± 61.9	68.9 ± 0.6	12.6 ± 0.3	0.7 ± 0.0	2.5 ± 0.1	2.1 ± 0.1	-

Table 22. continued.

	no.	Δ^5 avenasterol [%]	sitostanol [%]	24-meth.-cycl.[c] [%]	clerosterol [%]	α-amyrin [%]	β-amyrin [%]	Δ^7 sitosterol[e] [%]	campestanol [%]
proso millet	1	3.8 ± 0.1	1.2 ± 0.2	1.1 ± 0.0	0.8 ± 0.0	-	-	-	0.6 ± 0.1
	2	2.7 ± 0.1	1.4 ± 0.2	<LOQ[d]	0.9 ± 0.1	-	-	-	0.6 ± 0.1
	3	2.4 ± 0.1	1.0 ± 0.1	<LOQ	0.8 ± 0.0	-	-	-	<LOQ
	4	3.6 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.8 ± 0.0	-	-	-	<LOQ
	5	2.5 ± 0.1	1.8 ± 0.3	0.5 ± 0.1	0.8 ± 0.1	-	-	-	-
	6	2.8 ± 0.1	1.3 ± 0.0	-	0.8 ± 0.1	-	-	-	-
teff	1	0.7 ± 0.2	1.0 ± 0.1	1.7 ± 0.3	1.5 ± 0.3	6.8 ± 0.1	2.7 ± 0.1	3.0 ± 0.2	-
	2	0.6 ± 0.1	1.2 ± 0.1	2.9 ± 0.1	1.1 ± 0.1	11.7 ± 1.4	3.7 ± 0.0	3.2 ± 0.1	-
	3	0.4 ± 0.0	1.0 ± 0.1	1.9 ± 0.1	1.3 ± 0.0	9.1 ± 0.0	3.4 ± 0.1	3.0 ± 0.1	-
	4	0.7 ± 0.1	1.0 ± 0.1	3.1 ± 0.1	1.2 ± 0.0	11.3 ± 0.2	3.0 ± 0.1	3.5 ± 0.2	-
	5	0.4 ± 0.0	0.6 ± 0.2	1.1 ± 0.1	0.9 ± 0.2	8.0 ± 0.7	3.2 ± 0.3	2.6 ± 0.2	-
	6	0.7 ± 0.0	0.8 ± 0.0	5.7 ± 0.2	1.3 ± 0.0	11.1 ± 0.5	4.6 ± 0.2	4.6 ± 0.0	-
sorghum	1	1.2 ± 0.0	2.6 ± 0.1	2.3 ± 0.1	0.7 ± 0.0	3.7 ± 0.1	1.4 ± 0.2	3.7 ± 0.2	0.4 ± 0.0
	2	1.3 ± 0.2	2.7 ± 0.9	1.7 ± 0.2	0.8 ± 0.1	2.2 ± 0.1	1.2 ± 0.1	4.1 ± 0.2	-
	3	0.5 ± 0.0	2.4 ± 0.6	2.8 ± 0.6	1.1 ± 0.2	1.3 ± 0.1	-	3.1 ± 0.2	-
foxtail millet		0.6 ± 0.0	9.3 ± 0.2	3.8 ± 0.2	0.6 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	3.4 ± 0.1	1.9 ± 0.2
finger millet		0.7 ± 0.1	1.3 ± 0.1	2.6 ± 0.2	1.1 ± 0.0	4.2 ± 0.4	1.0 ± 0.2	2.4 ± 0.2	-

[a] Based on dry matter (dm) of ground kernels. [b] (-) Content below limit of detection (Table 14). [c] 24-Methylene cycloartanol. [d] Content below limit of quantification (Table 14). [e] Coelution with an unidentified sterol.

Steryl/Stanyl Fatty Acid Esters

The total contents and individual compositions of steryl/stanyl fatty acid esters determined in the seeds of proso millet, teff, sorghum, foxtail millet, and finger millet are presented in Table 23.

Sitosteryl esters were dominating in proso millet with sitosteryl-18:2 as most abundant ester, accounting for 37.2 ± 7.9 %. Overall, steryl esters of linoleic acid were predominant, followed by oleic and palmitic acid esters. This is in agreement with data reported by Sridhar and Lakshminarayana (1994), who determined the fatty acid composition of steryl esters isolated via TLC for a single proso millet cultivar. Furthermore, cholesterol, which was shown to represent up to 9.1 % of free sterols/stanols in proso millet, could also be detected as esters with C16 and C18 fatty acids. These esters represented together approximately 20 % of total steryl/stanyl fatty acid esters. Furthermore, 4,4'-dimethylsteryl esters, i.e. cycloartanyl and cycloartenyl-18:2, made up 5.9-16.3 % of total steryl fatty acid esters; stanyl esters could not be detected. To the author's knowledge, it is not only the first time that individual steryl/stanyl fatty acid esters were analyzed in proso millet, but also that intact cholesteryl esters were detected in cereal grains.

In agreement with the results obtained for proso millet, sitosteryl esters were also predominant in the seeds of teff, and accounted for >55.3 % of total fatty acid esters. Cholesteryl esters could not be identified, but Δ^7 sitosteryl-18:2 and 18:0/18:1, which were not detectable in proso millet, made up 8.0-15.1 % of total steryl fatty acid esters. 4,4'-Dimethylsteryl esters represented between 2.8 and 15.7 %, which is comparable to the proportions quantified in proso millet; however, no cycloartanyl esters were detected. Again, no stanyl esters could be identified.

In two of the three investigated sorghum samples, sitosteryl esters were dominating; only sample no. 1 revealed higher proportions of campesteryl esters. Additonally, Δ^7 sitosteryl esters and 4,4'-dimethylsteryl esters made up 4.8-5.9 % and 4.9-8.7 % of total esters, respectively. Overall, linoleic acid esters were more abundant, followed by oleic/stearic acid esters. Steryl esters of C16-fatty acids were minor constituents with relative amounts of 3.5-6.9 %.

Sitosteryl esters were also most abundant in foxtail millet and finger millet. However, the distribution patterns of the steryl esters in foxtail millet and finger millet differed from those determined in proso millet, teff, and sorghum. In finger millet, not steryl/stanyl linoleic acid esters, but steryl/stanyl oleic/stearic acid esters were predominant. This is in agreement with previously reported results obtained for the determination of the fatty acid compositions of steryl esters in finger millet (Mahadevappa and Raina, 1978a; Sridhar and Lakshminarayana, 1994). Sitostanyl-18:2 and campestanyl-18:2 could only be detected in foxtail millet with a relative amount of 14.6 % and 3.4 % of total fatty acid esters,

respectively. Comparably high amounts of stanols were also detected within the fraction of free sterols/stanols. The fact that in foxtail millet both sitostanol and campestanol were not only accumulated in free form, but also as esters has been observed in earlier studies (Narumi and Takatsuto, 1999; Takatsuto *et al.*, 1999).

The proportions of the coeluting stearic/oleic and palmitic/palmitoleic acid esters were determined after methanolysis of the respective steryl/stanyl esters fraction isolated via SPE in two samples of each type of grain (proso millet, teff, and sorghum) as well as in the lipids of foxtail millet and finger millet. Palmitoleic acid could only be detected in one proso millet and sorghum sample; the relative amounts were 16- to 50-fold lower than those of palmitic acid. Furthermore, the proportions of oleic acid were throughout more abundant (3- to 19-fold higher) than those of stearic acid (Appendix Table 37).

Total contents of steryl/stanyl fatty acid esters were distinctly different between the proso millet samples. The amounts were in the range of 214.8 and 751.0 µg/g dry matter flour and between 1.05 and 1.87 % in total extracted lipids.

The total amounts determined in the six teff samples averaged 729.6 ± 91.2 µg/g dry matter flour and 2.50 ± 0.37 % of extracted oil. Thus, the contents were on average 1.6- and 1.8-fold higher than those quantified in the proso millet flours and oils, respectively. Considering the contents based on dry matter flour, a 4-fold margin was observed between the highest level of teff (no. 5) and the lowest levels of proso millet (no. 6).

The sorghum kernels contained steryl fatty acid esters in the range of 465.6-573.8 µg/g dry matter flour; in total extracted lipids, the amounts ranged from 1.44 to 1.61 %. Hence, the levels were throughout lower than those determined in teff, but in the same order of magnitude as those analyzed in the proso millet samples. The total amounts of steryl fatty acid esters, which have previously been reported for two sorghum hybrids were lower (0.61 and 0.63 % of oil) compared to the levels obtained in the present study.

The extracted foxtail millet and finger millet oils revealed total steryl/stanyl fatty acid ester contents similar to those quantified in the lipid extracts of teff. However, due to the relative low lipid content of finger millet (Table 9), the amount calculated on the basis of dry matter flour was clearly lower. Mahadeveppa and Raina (1978b) reported for a single finger millet cultivar a steryl ester amount of 130 µg/g seed weight, which is lower than the amount determined in the present study.

Table 23. Contents and compositions of steryl/stanyl fatty acid esters in small millets and sorghum.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	sitosteryl-18:2 [%]	sitosteryl-18:0/18:1 [%]	sitosteryl-16:0/16:1 [%]	campesteryl-18:2 [%][d]	campesteryl-18:0/18:1 [%]	campesteryl-16:0/16:1 [%]
proso millet	1	751.0 ± 4.6	1867.3 ± 14.4	38.0 ± 0.1	9.4 ± 0.1	18.3 ± 0.2[b, c]	7.8 ± 0.2	8.0 ± 0.2	0.6 ± 0.1
	2	524.4 ± 11.9	1708.9 ± 57.5	42.9 ± 0.4	13.1 ± 0.7	15.7 ± 0.2[b, c]	8.0 ± 0.2	5.7 ± 0.8	0.6 ± 0.1
	3	345.4 ± 1.1	1096.5 ± 8.0	32.5 ± 2.9	12.3 ± 0.4	19.8 ± 2.7[b, c]	8.1 ± 0.8	7.6 ± 0.6	0.5 ± 0.1
	4	419.1 ± 16.2	1196.3 ± 35.2	35.0 ± 0.2	11.2 ± 0.1	18.1 ± 0.3[b, c]	7.4 ± 0.2	8.5 ± 0.2	0.3 ± 0.0
	5	559.3 ± 17.2	1255.7 ± 56.6	26.3 ± 0.6	9.8 ± 0.6	22.4 ± 0.6[b, c]	9.9 ± 0.2	8.8 ± 0.3	0.7 ± 0.0
	6	214.8 ± 8.3	1052.6 ± 38.3	48.7 ± 0.8	17.1 ± 0.4	10.7 ± 0.3[b, c]	8.4 ± 0.4	3.9 ± 0.2	0.9 ± 0.1
teff	1	759.8 ± 50.2	3061.6 ± 45.7	34.4 ± 1.3	12.2 ± 0.8	2.5 ± 0.3	13.1 ± 0.6	4.3 ± 0.2	1.4 ± 0.1
	2	756.4 ± 52.1	2224.2 ± 167.3	34.2 ± 2.1	16.9 ± 0.9	2.4 ± 0.2	11.1 ± 0.1	3.9 ± 0.2	1.1 ± 0.0
	3	666.9 ± 4.3	2179.4 ± 97.6	31.5 ± 0.4	20.1 ± 0.2	3.3 ± 0.2	11.5 ± 0.1	3.7 ± 0.1	1.0 ± 0.0
	4	630.5 ± 24.5	2428.4 ± 57.4	28.1 ± 0.4	21.6 ± 0.2	4.6 ± 0.3	11.6 ± 0.4	3.2 ± 0.0	1.2 ± 0.0
	5	883.9 ± 81.0	2849.8 ± 268.9	28.9 ± 0.9	22.6 ± 1.1	7.8 ± 0.7	13.5 ± 0.9	5.1 ± 1.0	2.4 ± 0.2
	6	680.2 ± 46.4	2238.1 ± 136.0	27.1 ± 0.4	16.1 ± 0.7	4.6 ± 0.4	7.8 ± 0.2	3.5 ± 0.5	0.4 ± 0.0
sorghum	1	573.8 ± 14.4	1609.0 ± 45.4	22.3 ± 0.4	13.8 ± 0.4	2.8 ± 0.1	26.5 ± 0.5	16.4 ± 0.6	0.7 ± 0.0
	2	530.3 ± 11.1	1444.0 ± 55.4	26.3 ± 0.4	15.4 ± 0.3	2.3 ± 0.1	31.2 ± 0.4	4.6 ± 0.2	0.8 ± 0.1
	3	465.6 ± 0.8	1571.2 ± 40.4	29.5 ± 0.0	20.6 ± 0.0	3.0 ± 0.1	11.2 ± 0.2	7.0 ± 0.2	2.0 ± 0.1
foxtail millet		775.1 ± 12.4	2499.3 ± 87.8	31.0 ± 1.9	13.1 ± 1.8	1.8 ± 0.0	10.2 ± 0.4	6.2 ± 0.8	0.4 ± 0.0
finger millet		335.1 ± 22.6	2333.0 ± 189.3	21.9 ± 1.9	34.8 ± 1.1	11.6 ± 0.8	6.6 ± 0.6	9.0 ± 0.8	2.5 ± 0.2

Table 23. continued.

	no.	stigmasteryl-18:2 [%]	stigmasteryl-16:0/16:1 [%]	cholesteryl-18:0/18:1 [%][g]	cholesteryl-16:0/16:1 [%][h]	Δ7 sitosteryl 18:2 [%][h]	Δ7 sitosteryl 18:0/18:1 [%][h]	cycloartenyl-18:2 [%][h]	cycloartenyl-18:0/18:1 [%][h]
proso millet	1	2.2 ± 0.1	0.1 ± 0.0	2.3 ± 0.1	0.7 ± 0.0	-	-	9.1 ± 0.4	-
	2	2.0 ± 0.2	0.3 ± 0.1	0.9 ± 0.2	0.7 ± 0.1	-	-	8.8 ± 0.4	-
	3	2.0 ± 0.2	0.2 ± 0.0	1.4 ± 0.2	0.7 ± 0.2	-	-	10.4 ± 0.8	-
	4	1.7 ± 0.2	<LOQ[e]	1.3 ± 0.2	0.5 ± 0.0	-	-	11.4 ± 0.3	-
	5	2.5 ± 0.1	0.3 ± 0.0	2.2 ± 0.2	1.1 ± 0.1	-	-	12.1 ± 0.3	-
	6	2.7 ± 0.3	0.3 ± 0.0	0.6 ± 0.0	0.7 ± 0.2	-	-	4.7 ± 0.1	-
teff	1	1.9 ± 0.1	-[f]	-	-	11.1 ± 1.1	3.9 ± 0.4	5.1 ± 0.3	1.8 ± 0.2
	2	1.8 ± 0.3	-	-	-	9.9 ± 0.4	3.0 ± 0.4	6.7 ± 0.4	2.3 ± 0.4
	3	1.8 ± 0.0	-	-	-	9.9 ± 0.4	3.5 ± 0.0	4.9 ± 0.2	1.8 ± 0.2
	4	1.8 ± 0.0	-	-	-	10.5 ± 0.5	2.3 ± 0.1	7.2 ± 0.1	1.7 ± 0.2
	5	2.7 ± 0.2	-	-	-	5.7 ± 0.2	2.3 ± 0.1	1.8 ± 0.3	1.0 ± 0.2
	6	2.0 ± 0.2	0.4 - 0.1	-	-	11.3 ± 0.2	3.8 ± 0.2	11.7 ± 0.1	4.0 ± 0.1
sorghum	1	2.6 ± 0.2	0.4 ± 0.0	-	-	3.4 ± 0.1	1.4 ± 0.1	2.5 ± 0.2	1.0 ± 0.2
	2	2.3 ± 0.2	0.4 ± 0.0	-	-	4.4 ± 0.1	1.5 ± 0.1	3.1 ± 0.1	1.4 ± 0.2
	3	3.8 ± 0.2	1.9 ± 0.0	-	-	3.9 ± 0.1	1.9 ± 0.0	3.6 ± 0.1	1.9 ± 0.1
foxtail millet		3.6 ± 0.2	0.2 ± 0.0	-	-	5.5 ± 0.3	1.8 ± 0.2	5.6 ± 0.1	1.0 ± 0.0
finger millet		1.0 ± 0.0	0.9 ± 0.0	-	-	2.6 ± 0.1	<LOQ	1.4 ± 0.1	1.7 ± 0.2

Table 23. continued.

	no.	24-meth.-cycl.-18:2 [%][h, i]	24-meth.-cycl.-18:0/18:1 [%][h, i]	cycloartanyl-18:2 [%][h]	sitosteryl-18:3 [%][h]	sitostanyl-18:2 [%]	campestanyl-18:2 [%][h]
proso millet	1	-	-	3.4 ± 0.2	-	-	-
	2	-	-	2.6 ± 0.1	-	-	-
	3	-	-	4.6 ± 0.0	-	-	-
	4	-	-	4.6 ± 0.0	-	-	-
	5	-	-	4.2 ± 0.2	-	-	-
	6	-	-	1.2 ± 0.1	-	-	-
teff	1	-	-	-	8.2 ± 0.7	-	-
	2	-	-	-	6.6 ± 0.5	-	-
	3	-	-	-	6.8 ± 0.0	-	-
	4	-	-	-	6.1 ± 0.3	-	-
	5	-	-	-	6.3 ± 0.2	-	-
	6	-	-	-	7.5 ± 0.1	-	-
sorghum	1	1.4 ± 0.2	-	-	3.8 ± 0.1	-	-
	2	1.1 ± 0.1	-	-	4.5 ± 0.1	-	-
	3	3.2 ± 0.1	-	-	4.8 ± 0.1	-	-
foxtail millet		1.7 ± 0.1	-	-	-	14.6 ± 0.3	3.4 ± 0.1
finger millet		1.3 ± 0.1	2.2 ± 0.3	-	2.6 ± 0.3	-	-

[a] Based on dry matter (dm) of ground kernels. [b] Coelution with cholesteryl-18:2. [c] Compound quantified via calibration of campesteryl-18:2. [d] Coelution with stigmasteryl-18:0/18:1. [e] Content below limit of quantification (Table 14). [f] (-) Content below limit of detection (Table 14). [g] Compound quantified via calibration of campesteryl-18:0/18:1. [h] Compound calculated with Rf = 1. [i] 24-Methylene cycloartanyl.

Steryl/Stanyl Ferulic Acid Esters

The analysis of *trans*-steryl/stanyl ferulic acid esters in small millets and sorghum revealed unexpected results (Table 24). Steryl/stanyl ferulic acid esters, which are reported to be unique compounds in a wide range of cereals (Piironen *et al.*, 2000; Moreau *et al.*, 2002), could not be detected in the seeds of proso millet and finger millet. To the author's knowledge, no qualitative and quantitative data on steryl ferulates in small millets have been reported to date. A previous study on whole corn kernels has shown that steryl/stanyl ferulates are mainly localized in the aleurone layer, a single cell layer of the outer pericarp (Moreau *et al.*, 2000). However, the proso millet samples analyzed in the present study were without husk. To investigate if steryl/stanyl ferulates were removed during peeling of the seeds, the proso millet samples no. 5 and no. 6 were also analyzed as whole grains with husk. As again no steryl/stanyl ferulates could be detected, it can be assumed that these esters were actually not present in proso millet.

The *trans*-steryl/stanyl ferulic acid esters in teff consisted mainly of *trans*-sitosteryl ferulate (90.0-93.7 %), followed by *trans*-campesteryl ferulate (5.0-9.3 %), and small amounts of *trans*-stigmasteryl ferulate (0.7-1.3 %). The saturated counterparts, i.e. *trans*-sitostanyl and *trans*-campestanyl ferulate, could not be detected. *Trans*-steryl ferulates were also predominant in the kernels of sorghum. Additionally, traces of *trans*-stanyl esters were detected; the amounts, however, were lower than the limits of quantification. In contrast to the results obtained for teff, *trans*-campesteryl ferulate was more abundant than *trans*-sitosteryl ferulate (57.2-67.1 % versus 32.9-42.8 %); additionally, no *trans*-stigmasteryl ferulate could be identified. In foxtail millet, four individual *trans*-steryl/stanyl ferulic acid esters were analyzed. In contrast to the distributions of *trans*-steryl/stanyl ferulic acid esters in teff and sorghum, *trans*-stanyl ferulic acid esters made up the majority (95.5 %) in foxtail millet, with *trans*-sitostanyl ferulate as most abundant ester.

The six investigated teff samples exhibited total contents of *trans*-steryl/stanyl ferulic acid esters of 106.9-226.3 µg/g dry matter flour; the amount determined in the foxtail millet sample was in the same order of magnitude (132.0 µg/g). Comparably low amounts were analyzed in sorghum with 12.9, 13.6, and 20.4 µg/g dry matter flour. Based on total extracted lipids, teff and foxtail millet contained 0.35-0.72 % *trans*-steryl/stanyl ferulic acid esters, sorghum on average 0.05 ± 0.01 %. Previously, total amounts of steryl ferulates have been determined in two sorghum hybrids via NP-HPLC (Singh *et al.*, 2003). The reported levels of 0.03 % steryl ferulic acid esters in *n*-hexane-extracted sorghum oil are comparable to the contents determined in the present study.

Table 24. Contents and compositions of *trans*-steryl/stanyl ferulic acid esters in small millets and sorghum.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	*trans*-sitosteryl ferulate [%]	*trans*-campesteryl ferulate [%]	*trans*-stigmasteryl ferulate [%]	*trans*-sitostanyl ferulate [%]	*trans*-campestanyl ferulate [%]
proso millet	1	-[b]	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-
teff	1	190.6 ± 26.3	681.6 ± 46.6	91.3 ± 0.4	7.8 ± 0.2	0.9 ± 0.1	-	-
	2	159.4 ± 2.7	494.7 ± 15.8	91.3 ± 0.2	7.8 ± 0.2	0.9 ± 0.1	-	-
	3	170.8 ± 9.0	537.7 ± 36.6	91.3 ± 0.2	7.8 ± 0.1	0.9 ± 0.1	-	-
	4	106.9 ± 8.8	395.8 ± 16.5	93.6 ± 0.1	5.7 ± 0.1	0.7 ± 0.1	-	-
	5	226.3 ± 12.5	723.9 ± 29.6	90.0 ± 0.0	9.3 ± 0.2	0.7 ± 0.2	-	-
	6	108.3 ± 10.9	351.1 ± 36.3	93.7 ± 0.2	5.0 ± 0.3	1.3 ± 0.3	-	-
sorghum	1	20.4 ± 0.9	59.9 ± 2.3	42.8 ± 1.6	57.2 ± 1.6	-	<LOQ[c]	<LOQ
	2	13.6 ± 0.7	42.4 ± 1.2	41.3 ± 0.9	58.7 ± 0.9	-	<LOQ	<LOQ
	3	12.9 ± 0.5	46.4 ± 3.2	32.9 ± 0.4	67.1 ± 0.4	-	<LOQ	<LOQ
foxtail millet		132.0 ± 6.2	359.5 ± 26.0	4.0 ± 0.7	0.6 ± 0.0	-	82.0 ± 1.0	13.5 ± 0.3
finger millet		-	-	-	-	-	-	-

[a] Based on dry matter (dm) of ground kernels. [b] (-) Content below limit of detection (Table 14). [c] Content below limit of quantification (Table 14).

4.4.3 Distribution Patterns of Free and Esterified Sterols/Stanols

Compared to free sterols/stanols, fewer kinds of sterols were detected as esters with fatty acids. In proso millet, a total of six individual sterols were detected as esters with fatty acids. Thereof, sitosterol, campesterol, and cholesterol were predominant, which is in accordance with the distribution patterns of free sterols/stanols. The most obvious differences were that the proportions of cholesterol and cycloartanol were throughout higher in the fractions of steryl/stanyl fatty acid esters, whereas the proportion of stigmasterol was higher within free sterols/stanols. In teff, sorghum, and finger millet sitosterol, campesterol, stigmasterol, Δ^7 sitosterol, cycloartenol, and in finger millet additionally 24-methylene cycloartanol were present as fatty acid esters. However, sitosterol and campesterol were dominating in free as well as in esterified form. Cycloartenol was particularly found to a higher degree as fatty acid ester; stigmasterol, in turn, to a lower amount. In the seeds of foxtail millet, almost all sterols/stanols which occurred in free form were also detected as ester with fatty acids. However, the proportions of stanols were higher within the fraction of steryl/stanyl fatty acid esters; hence, the amounts of esterified sterols lower. Furthermore, the amounts of esterified cycloartenol and 24-methylene cycloartanol were slightly higher.

Within the fraction of ferulic acid esters in teff, only sitosterol, campesterol, and stigmasterol could be detected. The percentage sterol/stanol compositions of ferulic acid esters, which were detected in the three investigated sorghum samples, revealed some difference. Firstly, only esters of sitosterol, campestanol, and traces of sitostanyl and campestanyl esters were present. Secondly, campesterol was the predominant esterified sterol, which is in contrast to the compositions of free sterols/stanols, where sitosterol was most abundant. The distribution patterns of sterols/stanols in the fraction of ferulic acid esters in foxtail millet differed from those of free sterols/stanols and steryl/stanyl fatty acid esters. The majority of ferulic acid was esterified to stanols (96 %). The observation that stanyl ferulic acid esters were more abundant than steryl ferulic acid esters is in agreement with the results observed for other cereals like corn, rye, wheat, and spelt (cf. 4.2.1, 4.3.1, and 4.5.1). The reason why phenolic acids in these types of cereals are preferentially esterified to stanols is not known.

4.4.4 Distribution Patterns of Total Fatty Acids and of Fatty Acids Esterified to Sterols/Stanols

The compositions of fatty acids determined in the total lipid extracts of the small millets and sorghum kernels are listed in Appendix Table 40. The analysis revealed large variations between the various types of grain. Linoleic acid was the predominant fatty acid in the seeds of proso millet, teff, sorghum, and foxtail millet with amounts of 59-66 %, 41-48 %, 47-52 %

and 72 % of total fatty acids, respectively, followed by oleic acid (21-24 %, 27-31 %, 32-37 %, and 16 %, respectively) and palmitic acid (7-12 %, 11-17 %, 11-12 %, and 6 %, respectively). The compositions of fatty acids determined in the proso millets and the kernels of sorghum are in accordance with earlier results (Rooney, 1978; Lorenz and Hwang, 1986; Mehmood *et al.*, 2008). In contrast to the present data obtained for the teff seeds, a previous study described oleic acid as predominant fatty acid in teff, followed by linoleic acid (El-Alfy *et al.*, 2011). The results are, however comparable to the fatty acid composition of teff reported in the National Nutrient Database for Standard Reference of the United States Department of Agriculture (USDA, 2013). The fatty acid composition of the foxtail millet sample is in line with data obtained for several glutinous and nonglutinous varieties; only the amount of stearic acid was slightly lower (Taira, 1984; Taira *et al.*, 1986). The fatty acids of finger millet consisted mainly of oleic acid, accounting for 44 % of total fatty acids. This agrees with previously published results (Mahadevappa and Raina, 1978b; Sridhar and Lakshminarayana, 1994).

Despite the differences in the total fatty acid compositions, it can be concluded for all investigated small millets as well as for sorghum that linoleic acid was found to a higher degree, oleic/stearic acid and palmitic/palmitoleic acid, in turn, to a lower degree esterified to sterols/stanols. Linolenic acid, which admittedly was present in all total lipids, could, however, only be detected as ester with sitosterol in teff and sorghum. The esterification degree of linolenic acid with sterols was in all of these samples higher than in the total lipids.

4.4.5 Proportions of Free Sterols/Stanols and Steryl/Stanyl Esters

The sums of the total contents of free sterols/stanols, intact steryl/stanyl fatty acid esters, and intact *trans*-steryl/stanyl ferulic acid esters were highest in teff with 0.97-1.46 mg/g dry matter flour and foxtail millet with 1.33 mg/g (Figure 28A). In total extracted lipids, teff sample no. 1 exhibited the highest total amounts of free sterols/stanols and steryl/stanyl esters (5.5 %, Figure 28B). This value was, for example, 2- to 3-times higher than the mean total sums determined in proso millet and sorghum.

The relative percentage distributions of the total amounts of free sterols/stanols, intact steryl/stanyl fatty acid esters, and intact *trans*-steryl/stanyl ferulic acid esters are illustrated in Figure 29. In all small millet and sorghum samples, the total contents of steryl/stanyl fatty acid esters were higher than those of free sterols/stanols; with a relative amount of >51.0 %. Steryl/stanyl ferulic acid ester could only be detected and quantified in teff, foxtail millet, and sorghum, but the amounts were the lowest of all three investigated sterol classes.

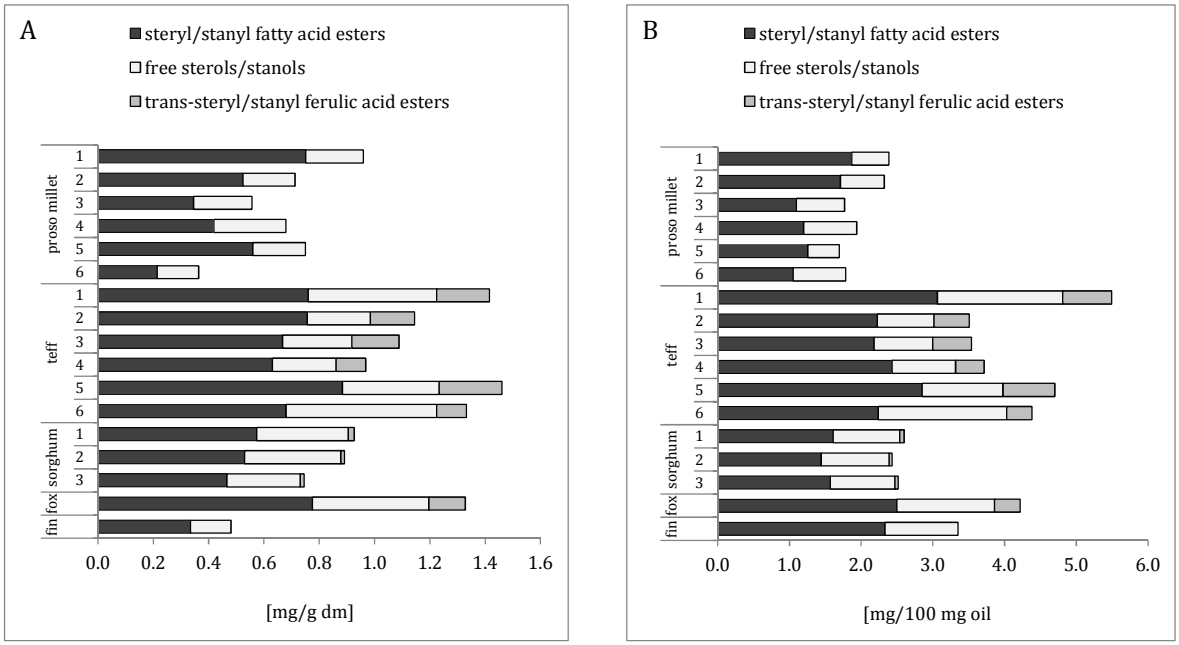

Figure 28. Sums of total contents of steryl/stanyl fatty acid esters, free sterols/stanols, and steryl/stanyl phenolic acid esters of the small millet and sorghum samples determined (**A**) in dry matter flour and (**B**) in extracted oil (The numberings of the samples correspond to those in Table 9.). fox: foxtail millet; fing: finger millet

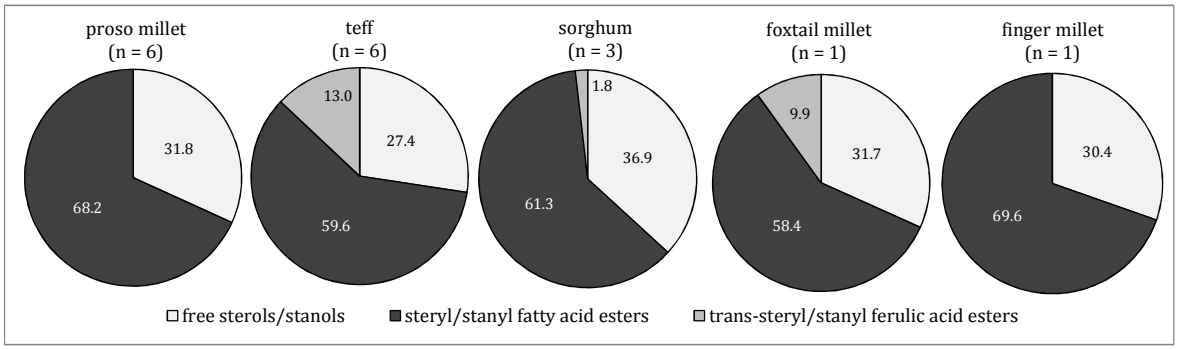

Figure 29. Relative percentage distributions of free sterols/stanols and intact steryl/stanyl esters in small millets and sorghum.

Two studies reported contradictory results, i.e. higher amounts of free sterols than of steryl esters in the nonpolar lipid fractions of proso millet, finger millet, and foxtail millet (Mahadevappa and Raina, 1978a; Sridhar and Lakshminarayana, 1994). Literature data concerning the proportions of free sterols and steryl fatty acid esters in sorghum are inconsistent. One study reported higher amounts of free sterols than of steryl fatty acid esters in the nonpolar lipid fraction of sorghum (Osagie, 1987). Another study observed partially reverse results as in one of the two investigated sorghum hybrids steryl fatty acid esters were predominant (Singh *et al.*, 2003).

4.5 Analysis of Free Sterols/Stanols and Steryl/Stanyl Esters in Rye, Wheat, and Spelt

Individual free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters were investigated in two commercially obtained pre-packaged samples of rye, wheat, and spelt kernels. These samples were worked up at the same time as the dent corn and flint corn cultivars were investigated and, thus, they were analyzed by means of the SPE-based approach (cf. 4.1.1). As rye, wheat and spelt revealed very similar patterns, the GC analyses of free sterols/stanols and steryl/stanyl esters are exemplarily shown for a rye sample in Figure 30.

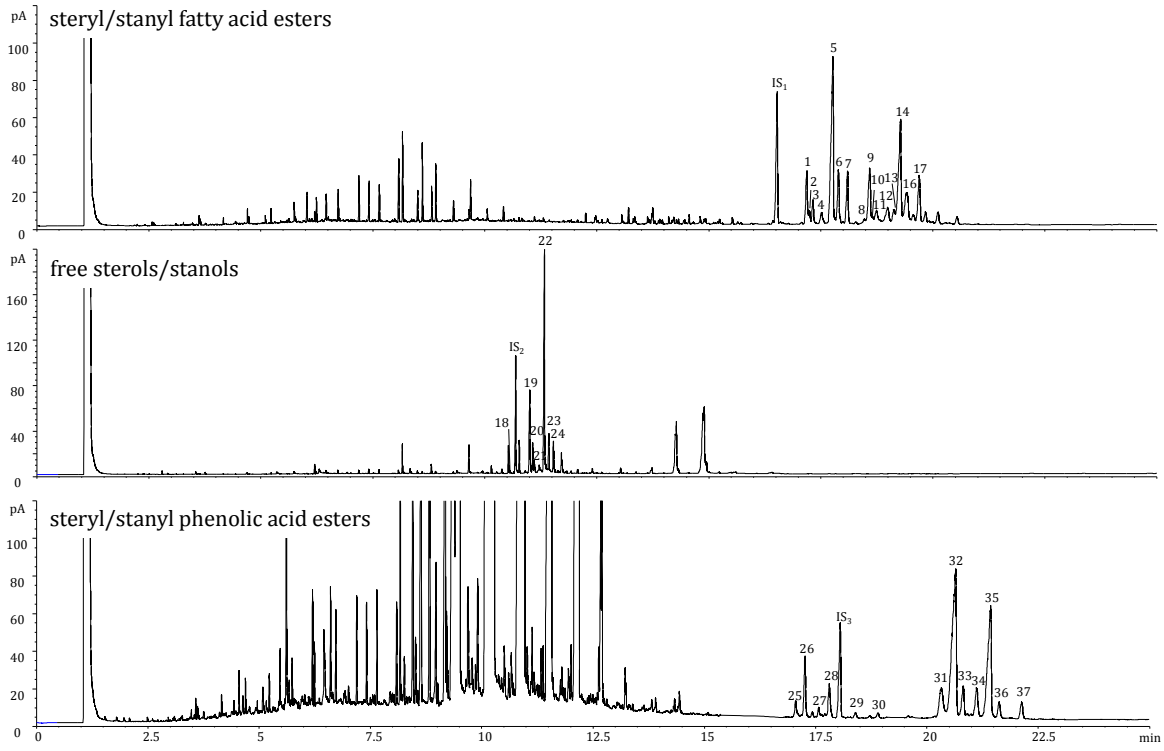

Figure 30. GC-FID analysis of the SPE fractions of steryl/stanyl fatty acid esters, free sterols/stanols, and steryl/stanyl phenolic acid esters extracted from rye.
Steryl/stanyl fatty acid esters: (1) campesteryl-16:0/16:1, (2) stigmasteryl-16:0/16:1, (3) campestanyl-16:0/16:1, (4) Δ^7 campesteryl-16:0/16:, (5) sitosteryl-16:0/16:1, (6) sitostanyl-16:0/16:1, (7) Δ^7 sitosteryl-16:0/16:1, (8) campesteryl-18:0/18:1, (9) stigmasteryl-18:0/18:1 + campesteryl-18:2, (10) stigmasteryl-18:2 + campestanyl-18:0/18:1, (11) campestanyl-18:2, (12) Δ^7 campesteryl-18:2, (13) sitosteryl-18:0/18:1, (14) sitosteryl-18:2, (15) sitostanyl-18:0/18:1, (16) sitostanyl-18:2, (17) Δ^7 sitosteryl-18:2, and (IS_1) cholesteryl-16:0; free sterols/stanols: (18) cholesterol, (19) campesterol, (20) stigmasterol, (21) campestanol, (22) sitosterol, (23) sitostanol, (24) Δ^7 sitosterol and unknown sterol, and (IS_2) 5*α*-cholestan-3*ß*-ol; steryl/stanyl phenolic acid esters: (25) *cis*-campesteryl ferulate, (26) *cis*-campestanyl ferulate, (27) *cis*-sitosteryl ferulate, (28) *cis*-sitostanyl ferulate, (29) *cis*-24-methylene cycloartanyl ferulatee, (30) *trans*-campestanyl *p*-coumarate, (31) *trans*-campesteryl ferulate, (32) *trans*-campestanyl ferulate, (33) *trans*-Δ^7 campesteryl ferulate, (34) *trans*-sitosteryl ferulate, (35) *trans*-sitostanyl ferulate, (36) *trans*-Δ^7 sitosteryl ferulate, (37) *trans*-24-methylene cycloartanyl ferulate, and (IS_3) *trans*-cholestanyl *p*-coumarate.

4.5.1 Compositions and Contents of Free Sterols/Stanols and Steryl/Stanyl Esters

Free Sterols/Stanols

The contents and compositions of free sterols/stanols determined in the rye, wheat, and spelt kernels are shown in Table 25. The profiles of free sterols/stanols detected in those cereals showed similarities, in particular those of wheat and spelt. Sitosterol was the dominating free sterol/stanol, accounting for >54 %. The proportions of campesterol and Δ^7 sitosterol were higher in rye, those of free stanols higher in wheat and spelt. Cholesterol represented 0.7-1.4 % of total free sterols/stanols; the amounts are comparable to those detected in corn (cf. 4.2.1). Total sterols have comprehensively been studied in these types of grain (Zangenberg *et al.*, 2004; Nurmi *et al.*, 2008; Nyström *et al.*, 2008a; Iafelice *et al.*, 2009), whereas data on free sterols/stanols are rare. Free sterols/stanols have been analyzed in whole flours of wheat and spelt after isolation of the free sterol/stanol fraction by TLC or CC (Takatsuto *et al.*, 1999; Pelillo *et al.*, 2003; Iafelice *et al.*, 2009). The observed distribution patterns generally agree with those determined in the present work.

The total contents of free sterols/stanols determined on the basis of dry matter flour were in the same order of magnitude for all samples (194.0-261.6 µg/g), except for spelt sample no. 1, which contained only 88.0 µg/g. The levels calculated for the extracted oils were highest in the rye oils with 1.5 and 1.8 %, respectively, and lowest in the spelt oils with 0.4 and 0.9 %, respectively. The two investigated samples of rye and wheat revealed higher contents of free sterols/stanols in the extracted oils than corn, sorghum, and most of the small millets (cf. 4.2.1, 4.3.1, and 4.4.2). Only the oil extracts of some teff samples as well as of the foxtail millet sample exhibited comparable levels. However, based on dry matter flour the contents of free sterols/stanols in rye, wheat, and spelt were lower than those determined in corn or sorghum. This can be explained by the low oil contents of the rye, wheat, and spelt kernels (1.5-2.5 %, Table 10). Previously reported contents of free sterols/stanols were in the range of 288-391 µg/g dry matter flour in wheat and in the range of 191-300 µg/g in spelt, and thus slightly higher than those determined in the present study (Pelillo *et al.*, 2003; Iafelice *et al.*, 2009). A higher total amount of free sterols/stanols has also been described for a single rye sample by Lampi *et al.* (2004).

Steryl/Stanyl Fatty Acid Esters

The profiles of steryl/stanyl fatty acid esters in rye, wheat, and spelt were comparable (Table 26), but differed distinctly from those detected in corn, small millets, or sorghum (cf. 4.2.1, 4.3.1, and 4.4.2). In the last mentioned cereals, steryl/stanyl esters of unsaturated C18-fatty

acids were predominant, accounting for >90 %. In contrast, in rye, wheat, and spelt steryl/stanyl esters of C16-fatty acids represented 42-52 % of total steryl/stanyl fatty acid esters. Sitosteryl-16:0/16:1 and sitosteryl-18:2 were the most abundant esters. The amounts of steryl/stanyl C16-fatty acid esters were slightly lower in rye compared to wheat and spelt; the proportions of Δ^7 sitosteryl and Δ^7 campesteryl esters were, in turn, higher. Previously, individual intact steryl fatty acid esters have been analyzed in whole flours of 5 wheat and 12 spelt cultivars (Caboni *et al.*, 2005). Thereby, comparably high amounts of C16-fatty acids have been reported. However, the GC resolution between steryl oleate and steryl linoleate was not sufficient and fatty acid esters of stanols and Δ^7 sterols have either not been detected or not considered in that study.

The distributions of steryl/stanyl esters of palmitic/palmitoleic acid and stearic/oleic acid were analyzed after transesterification of the respective steryl/stanyl fatty acid ester fractions. A predominance of palmitic acid and oleic acid esters was shown as the of ratios of palmitic/palmitoleic acid were in the range of 37:1 to 131:1 and those of oleic/stearic acid in the range of 4:1 to 8:1.

The total contents of steryl/stanyl fatty acid esters based on dry matter flour were lowest in wheat no. 2 (369.9 µg/g) and highest in spelt no. 1 (652.7 µg/g). In turn, both rye samples exhibited the highest amounts based on extracted oil with >3 %. Those were also above the levels determined in corn, small millets, and sorghum (cf. 4.2.1, 4.3.1, and 4.4.2)
The determined total contents of steryl/stanyl fatty acid esters in the whole flours of wheat are in line with earlier reported results; those of spelt and rye were somewhat higher (Zeringue and Feuge, 1980; Caboni *et al.*, 2005). Lampi *et al.* (2004) reported a total steryl/stanyl fatty acid ester amount for whole rye grains of 620 µg/g, which thus was little higher than those observed in the present study.

Steryl/Stanyl Phenolic Acid Esters

In total, up to 14 individual steryl/stanyl phenolic acid esters could be identified and quantified in the investigated rye, wheat, and spelt kernels (Table 27). The steryl/stanyl phenolic acid esters were mainly composed of ferulic acid esters, representing >98 %. In addition, small amounts of campestanyl *p*-coumarate and sitostanyl *p*-coumarate could be detected in all investigated kernels of rye, wheat, and spelt. Steryl/stanyl coumaric acid esters have been described as unique compounds in corn and its milling fraction corn bran (Seitz, 1989; Norton, 1995). Even though the contents of steryl/stanyl coumaric acid esters were very low in rye, wheat and spelt, this is the first time that these esters were detected in cereals other than corn. The contents of campesteryl/-stanyl ferulate in rye, wheat, and spelt were higher compared to those of sitosteryl/-stanyl ferulate. This is in accordance with data

from previous studies on rye and wheat (Seitz, 1989; Hakala *et al.*, 2002; Nurmi *et al.*, 2010). *Cis*-derivatives of steryl and stanyl ferulic acid esters made up between 4.9 % of total steryl/stanyl phenolic acid esters in wheat (no. 2) and 14.3 % in rye (no. 2). The steryl/stanyl ferulic acid esters of wheat and rye have been investigated in a few studies (Seitz, 1989; Hakala *et al.*, 2002; Werner *et al.*, 2002; Lampi *et al.*, 2004; Nyström *et al.*, 2007c). Recently, a further work provided very comprehensive data on the effect of cultivar and environment on the contents and compositions of steryl/stanyl ferulic acid esters (Nurmi *et al.*, 2010). However, most of the studies reported the presence of only four individual steryl/stanyl ferulates in wheat and rye, i.e. campestanyl, sitostanyl, campesteryl, and sitosteryl ferulate. Moreover, campestanyl ferulate, the main compound was coeluted with sitosteryl ferulate under the employed HPLC conditions. The present study thus provides valuable new data on the distribution patterns of steryl/stanyl phenolic acid esters in whole flours of rye, wheat, and spelt.

Within the investigated cereals, wheat contained the highest total contents of steryl/stanyl phenolic acid esters based on dry matter flour as well as extracted oil, which are in the same order of magnitude as those reported in the studies of Nurmi *et al.* (2010) and Seitz (1989). For rye, total contents have been reported in the range of 40-86 µg/g for steryl/stanyl ferulic acid esters, which are comparable to the values observed in the present study (Hakala *et al.*, 2002; Werner *et al.*, 2002; Lampi *et al.*, 2004; Nyström *et al.*, 2007c; Nurmi *et al.*, 2010). However, the steryl/stanyl ferulic acid ester content described by Seitz (1989) for as single rye variety (29 µg/g fresh weight) was lower. To the author's knowledge, steryl/stanyl ferulates have not been analyzed in spelt, hitherto.

Table 25. Contents and compositions of free sterols/stanols in rye, wheat, and spelt.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	sitosterol [%]	campesterol [%]	sitostanol [%]	Δ7 sitosterol[b] [%]	stigmasterol [%]	campestanol [%]	cholesterol [%]
rye	1	237.8 ± 6.8	1543.4 ± 48.9	53.8 ± 0.4	23.4 ± 0.3	7.6 ± 0.2	7.4 ± 0.2	5.5 ± 0.1	2.3 ± 0.1	-[c]
	2	261.6 ± 3.7	1790.4 ± 31.1	55.5 ± 0.2	20.7 ± 0.2	7.4 ± 0.2	7.0 ± 0.1	5.5 ± 0.0	3.2 ± 0.1	0.7 ± 0.1
wheat	1	194.0 ± 5.9	1255.6 ± 13.6	58.9 ± 0.3	16.9 ± 0.2	8.8 ± 0.1	5.1 ± 0.5	4.0 ± 0.0	5.6 ± 0.1	0.7 ± 0.0
	2	224.8 ± 4.7	1328.8 ± 18.7	60.9 ± 0.4	16.9 ± 0.1	10.3 ± 0.2	1.7 ± 0.1	3.0 ± 0.0	6.0 ± 0.3	1.2 ± 0.2
spelt	1	88.0 ± 4.5	406.6 ± 16.4	63.1 ± 0.5	15.2 ± 0.3	10.3 ± 0.3	1.6 ± 0.2	2.8 ± 0.1	5.7 ± 0.4	1.4 ± 0.1
	2	214.3 ± 2.4	866.3 ± 20.9	61.5 ± 0.3	17.2 ± 0.2	9.9 ± 0.3	1.7 ± 0.2	3.2 ± 0.0	5.4 ± 0.2	1.1 ± 0.1

[a] Based on dry matter (dm) of ground kernels. [b] Coelution with an unidentified sterol. [c] (-) Content below limit of detection (Table 13).

Table 26. Contents and compositions of steryl/stanyl fatty acid esters in rye, wheat, and spelt.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	sitosteryl-18:2 [%]	sitosteryl-18:0/18:1 [%]	sitosteryl 16:0/16:1 [%]	campesteryl-18:2 [%][b]	campesteryl-18:0/18:1 [%]	campesteryl-16:0/16:1 [%]	stigmasteryl-18:2 [%][c]
rye	1	589.7 ± 9.9	3827.0 ± 76.4	23.6 ± 0.6	3.2 ± 0.3	23.0 ± 0.4	9.4 ± 0.2	0.8 ± 0.2	5.7 ± 0.1	0.8 ± 0.0
	2	459.3 ± 6.2	3143.3 ± 36.0	18.8 ± 0.2	4.5 ± 0.1	23.1 ± 0.3	8.6 ± 0.0	0.6 ± 0.2	5.4 ± 0.1	0.8 ± 0.1
wheat	1	449.0 ± 4.7	2908.1 ± 87.4	21.9 ± 0.2	2.8 ± 0.1	31.8 ± 0.1	7.2 ± 0.1	0.4 ± 0.0	7.4 ± 0.0	1.0 ± 0.0
	2	369.9 ± 5.2	2186.8 ± 23.4	21.9 ± 0.2	4.9 ± 0.1	30.0 ± 0.1	7.5 ± 0.1	0.5 ± 0.1	7.4 ± 0.1	0.9 ± 0.1
spelt	1	652.7 ± 5.8	3017.1 ± 17.2	19.0 ± 0.1	6.2 ± 0.2	26.7 ± 0.2	5.7 ± 0.1	1.6 ± 0.2	6.1 ± 0.0	1.8 ± 0.1
	2	557.6 ± 2.6	2253.9 ± 36.9	20.5 ± 0.2	5.6 ± 0.1	31.4 ± 0.2	6.7 ± 0.1	1.1 ± 0.0	6.6 ± 0.1	1.2 ± 0.1

Table 26. continued.

	no.	stigmasteryl-16:0/16:1 [%]	sitostanyl-18:2 [%]	sitostanyl-18:0/18:1 [%]	sitostanyl-16:0/16:1 [%]	campestanyl-18:2 [%]	campestanyl-16:0/16:1 [%][f]	Δ7 sitosteryl-18:2 [%][f]	Δ7 sitosteryl-16:0/16:1 [%][f]	Δ7 campesteryl-18:2 [%][f]	Δ7 campesteryl-16:0/16:1 [%][f]
rye	1	0.7 ± 0.1	9.5 ± 0.5[d]	-[e]	5.6 ± 0.2	2.9 ± 0.1	2.7 ± 0.1	4.4 ± 0.3	3.2 ± 0.1	3.0 ± 0.1	1.5 ± 0.1
	2	1.4 ± 0.1	8.0 ± 0.1[d]	-	5.7 ± 0.0	2.1 ± 0.1	2.9 ± 0.1	6.7 ± 0.1	5.8 ± 0.0	3.6 ± 0.0	1.9 ± 0.1
wheat	1	0.4 ± 0.0	6.2 ± 0.0	1.4 ± 0.2	6.8 ± 0.0	2.9 ± 0.0	4.6 ± 0.0	2.1 ± 0.0	0.8 ± 0.0	1.7 ± 0.0	0.7 ± 0.0
	2	0.4 ± 0.0	7.1 ± 0.0	1.4 ± 0.0	6.0 ± 0.0	2.4 ± 0.1	3.8 ± 0.1	2.4 ± 0.0	0.9 ± 0.0	1.7 ± 0.1	0.9 ± 0.1
spelt	1	0.6 ± 0.0	7.6 ± 0.2	3.6 ± 0.2	9.7 ± 0.1	2.7 ± 0.2	4.0 ± 0.2	2.0 ± 0.0	0.7 ± 0.1	1.4 ± 0.1	0.7 ± 0.1[g]
	2	0.5 ± 0.0	6.7 ± 0.1	2.5 ± 0.2	6.0 ± 0.3	2.1 ± 0.0	3.7 ± 0.1	2.2 ± 0.0	0.8 ± 0.0	1.6 ± 0.1	0.7 ± 0.1[g]

[a] Based on dry matter (dm) of ground kernels. [b] Coelution with stigmasteryl-18:0/18:1. [c] Coelution with campestanyl-18:0/18:1. [d] Coelution with sitosteryl-18:3. [e] (-) Content below limit of detection (Table 13). [f] Compound calculated with Rf = 1. [g] Coelution with an unidentified steryl ester.

Table 27. Contents and compositions of steryl/stanyl phenolic acid esters in rye, wheat, and spelt.

	no.	∑ [µg/g dm[a]]	∑ [µg/100 mg oil]	*trans*-campestanyl ferulate [%]	*cis*-campestanyl ferulate [%]	*trans*-sitostanyl ferulate [%]	*cis*-sitostanyl ferulate [%]	*trans*-campesteryl ferulate [%]	*cis*-campesteryl ferulate [%]	*trans*-sitosteryl ferulate [%]
rye	1	64.8 ± 4.3	420.3 ± 28.1	41.2 ± 1.4	6.6 ± 0.5	29.5 ± 1.5	1.6 ± 0.2	6.1 ± 0.4	<LOQ[b]	4.2 ± 0.2
	2	92.0 ± 5.1	629.2 ± 29.0	37.6 ± 0.6	6.2 ± 0.4	25.7 ± 0.4	4.4 ± 0.1	7.1 ± 0.3	2.1 ± 0.1	5.1 ± 0.3
wheat	1	111.2 ± 4.0	720.2 ± 29.1	43.7 ± 0.2	4.6 ± 0.2	32.4 ± 0.4	1.6 ± 0.2	7.4 ± 0.1	<LOQ	4.4 ± 0.1
	2	124.2 ± 4.1	734.3 ± 31.0	42.7 ± 0.3	2.4 ± 0.2	32.1 ± 0.1	1.8 ± 0.0	9.3 ± 0.3	0.7 ± 0.0	5.1 ± 0.2
spelt	1	26.9 ± 4.8	124.3 ± 22.5	34.6 ± 3.3	5.5 ± 0.5	35.0 ± 0.8	5.8 ± 1.0	6.7 ± 0.8	1.0 ± 0.2	4.8 ± 0.6
	2	92.4 ± 5.9	373.2 ± 21.5	38.8 ± 1.7	5.1 ± 0.3	34.3 ± 1.3	5.0 ± 1.0	7.4 ± 0.4	1.6 ± 0.1	4.8 ± 0.3

Table 27. continued.

	no.	*cis*-sitosteryl ferulate [%]	*trans*-Δ⁷ campesteryl ferulate [%]	*trans*-Δ⁷ sitosteryl ferulate [%]	*trans*-24-meth.-cycl.[c] ferulate [%]	*cis*-24-meth.-cycl.[c] ferulate [%]	*trans*-campestanyl *p*-coumarate [%]	*trans*-campesteryl *p*-coumarate [%]	*trans*-sitostanyl *p*-coumarate [%]	*trans*-sitosteryl *p*-coumarate [%]
rye	1	<LOQ	5.6 ± 0.5	4.0 ± 0.5	-[d]	-	0.6 ± 0.0	-	0.5 ± 0.0	-
	2	0.9 ± 0.0	4.7 ± 0.1	2.3 ± 0.0	2.4 ± 0.3	0.7 ± 0.1	0.6 ± 0.0	-	0.3 ± 0.0	-
wheat	1	<LOQ	3.0 ± 0.2	2.1 ± 0.1	-	-	0.5 ± 0.0	-	0.4 ± 0.1	-
	2	<LOQ	1.7 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	<LOQ	1.4 ± 0.1	-	0.3 ± 0.0	-
spelt	1	0.8 ± 0.1	2.9 ± 0.4	3.0 ± 0.5	-	-	<LOQ	-	<LOQ	-
	2	<LOQ	1.6 ± 0.0	1.4 ± 0.1	<LOQ	<LOD	<LOQ	-	<LOQ	-

[a] Based on dry matter (dm) of ground kernels. [b] Content below limit of quantification (Table 13). [c] 24-Methylene cycloartanyl. [d] (-) Content below limit of detection (Table 13).

4.5.2 Distribution Patterns of Free and Esterified Sterols/Stanols

The major sterols/stanols which were detected in free form were also present as esters with fatty acids. In accordance with the results obtained for other cereals grains, e.g. corn (4.2.2 and 1.1.1), stanols were found to a higher extent esterified to fatty acids, whereas sterols, particularly stigmasterol, were found to a higher degree in free form. Within the fraction of steryl/stanyl phenolic acid esters, stanols were predominant.

4.5.3 Distribution Patterns of Total Fatty Acids and of Fatty Acids Esterified to Sterols/Stanols

The total fatty acid compositions determined in the lipids of rye, wheat, and spelt are presented in Appendix Table 38. Linoleic acid was most abundant (57-64 %), followed by oleic acid (14-22 %), and palmitic acid (13-15 %). Linolenic acid made up approximately 7 % in rye, 5 % in wheat, and 3 % in spelt. Similar fatty acid compositions have been reported elsewhere (Weihrauch and Matthews, 1977; Davis *et al.*, 1980; Zeringue and Feuge, 1980; Ryan *et al.*, 2007)

The distributions of fatty acids that were esterified to sterols/stanols revealed pronounced differences to that of the total lipids, as much higher proportions of palmitic/palmitoleic acid were detected (about 13-16 % versus 40-50 %). In turn, linoleic acid and oleic/stearic acid were found to be esterified to a lower degree to sterols/stanols. Steryl linolenic acid esters, i.e. sitosteryl linolenate could only be detected in rye; however, it coeluted with sitostanyl linoleate so that the proportions of these esters could not be determined.

4.5.4 Proportions of Free Sterols/Stanols and Steryl/Stanyl Esters

The sums of total contents of free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters ranged from 0.72 mg/g dry matter flour in wheat no. 2 to 0.89 mg/g in rye no. 1 (Figure 31A). The sums of the total amounts calculated on the basis of extracted oil are presented in Figure 31B and averaged 3.5 % in spelt, 4.6 % in wheat, and 5.7 % in rye; this confirms that among these common types of cereals, rye lipids are good sources of these bioactive molecules.

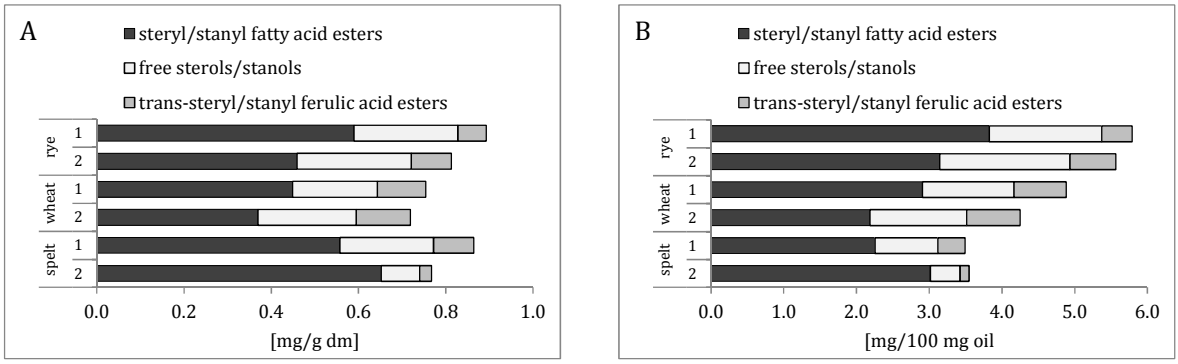

Figure 31. Sums of total contents of steryl/stanyl fatty acid esters, free sterols/stanols, and steryl/stanyl phenolic acid esters of the rye, wheat, and spelt samples determined (**A**) in dry matter flour and (**B**) in extracted oil (The numberings of the samples correspond to those in Table 10.).

The relative percentage distributions of the mean total contents of free sterols/stanols, intact steryl/stanyl fatty acid esters, and intact steryl/stanyl phenolic acid esters are shown in Figure 32. All three types of grain had in common that the majority of sterols/stanols was present as fatty acid esters, whereby the proportions were by far the highest in spelt. The rye and wheat kernels revealed equal parts of free sterols/stanols (about 30 %). Steryl/stanyl phenolic acid esters represented 3.5-17.3 % of the total amounts of the sterol classes, being highest in wheat and lowest in spelt.

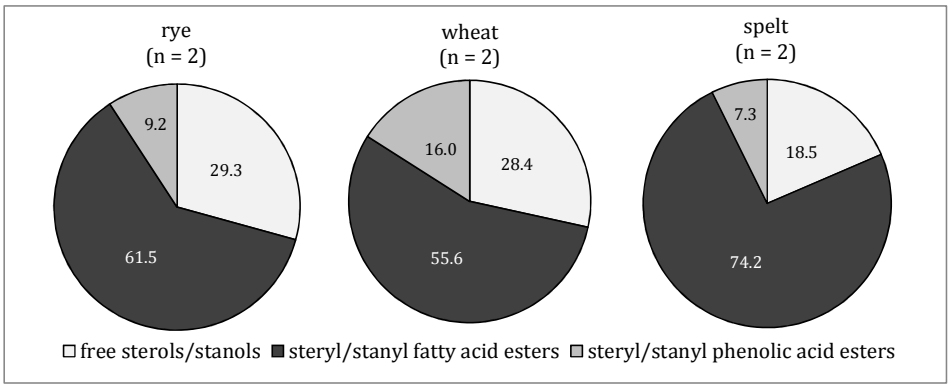

Figure 32. Relative percentage distributions of free sterols/stanols and intact steryl/stanyl esters in rye, wheat and spelt kernels.

The dominance of steryl/stanyl fatty acid esters in rye, wheat, and spelt kernels is in accordance with the results obtained for most of the investigated cereal grains (cf. 4.2.4. and 4.4.5). Previous studies also reported that steryl/stanyl fatty acid esters are the main sterol class in rye, wheat, and spelt (Lampi *et al.*, 2004; Caboni *et al.*, 2005; Iafelice *et al.*, 2009). However, in tetraploid wheats, i.e. durum and emmer wheat, free sterols/stanols have been shown to be dominant (Iafelice *et al.*, 2009).

4.6 Analysis of Free Sterols/Stanols, Steryl/Stanyl Esters, and Other Minor Lipids in Tree Nuts and Peanuts

Nuts are known to possess several health benefits for humans, which are mainly attributed to their positive lipid compositions characterized by high amounts of mono- and polyunsaturated fatty acids, phenolic compounds, tocopherols, and phytosterols (Griel and Kris-Etherton, 2006; Ros, 2010; Bolling *et al.*, 2011). In contrast to total phytosterols, comprehensive qualitative and quantitative information on free sterols/stanols and individual steryl/stanyl esters in tree nuts and peanuts is lacking (cf. 2.2.3). Therefore, the on-line LC-GC-based approach, established for the simultaneous analysis of free sterols/stanols and steryl/stanyl esters in cereal grains (cf. 4.1.2), was applied to the investigation of these compounds in ten different commercially important types of nuts, i.e. almonds, Brazil nuts, cashew nuts, hazelnuts, macadamias, peanuts, pine nuts, pistachios, and walnuts. For each nut type, material from three different providers was analyzed to consider the natural variability.

4.6.1 On-line LC-GC-Based Approach

The applied on-line LC-GC-based approach also offered the opportunity for the simultaneous analysis of tocopherols and squalene besides free sterols/stanols and steryl/stanyl esters. Due to silylation of the extracted nut lipids, steryl/stanyl fatty acid esters and squalene as well as the TMS-derivatives of free sterols/stanols and tocopherols eluted at the same time under the employed LC conditions and could thus be transferred together to GC. Additionally, all nut samples were tested for the presence of *trans*-steryl/stanyl ferulic acid esters, which could be transferred in a separate fraction to the GC (cf. 4.1.2). However, no esters of that type could be detected.

The suitability of the on-line LC-GC-based approach for the analysis of free sterols/stanols, steryl/stanyl esters, and tocopherols, in nuts was proven by determination of recoveries and repeatability. Recoveries were determined by spiking three different nut types (ground hazelnut, peanut, and walnut) with known amounts of selected reference compounds representative for each compound class. The recoveries were on average 98.6 ± 3.6 % for free sterols/stanols, 96.3 ± 5.4 % for steryl/stanyl fatty acid esters, and 104.9 ± 3.7 % for tocopherols; no impact of the matrix was observed. The repeatability of the approach, including lipid extraction, silylation, and on-line LC-GC analysis, was confirmed by working up a control sample (ground walnut) once on each day of analysis (in total, 10 replicates). The low relative standard deviations of the total contents of 3.0 % for free sterols/stanols, 8.7 % for steryl/stanyl fatty acid esters, and 3.1 % for tocopherols indicated a good repeatability.

The results demonstrated that the method provides reliable quantitative data on free sterols/stanols, steryl/stanyl fatty acid esters, and tocopherols in nuts. The on-line LC-GC analysis of the minor lipids is exemplarily shown for a walnut sample in Figure 33.

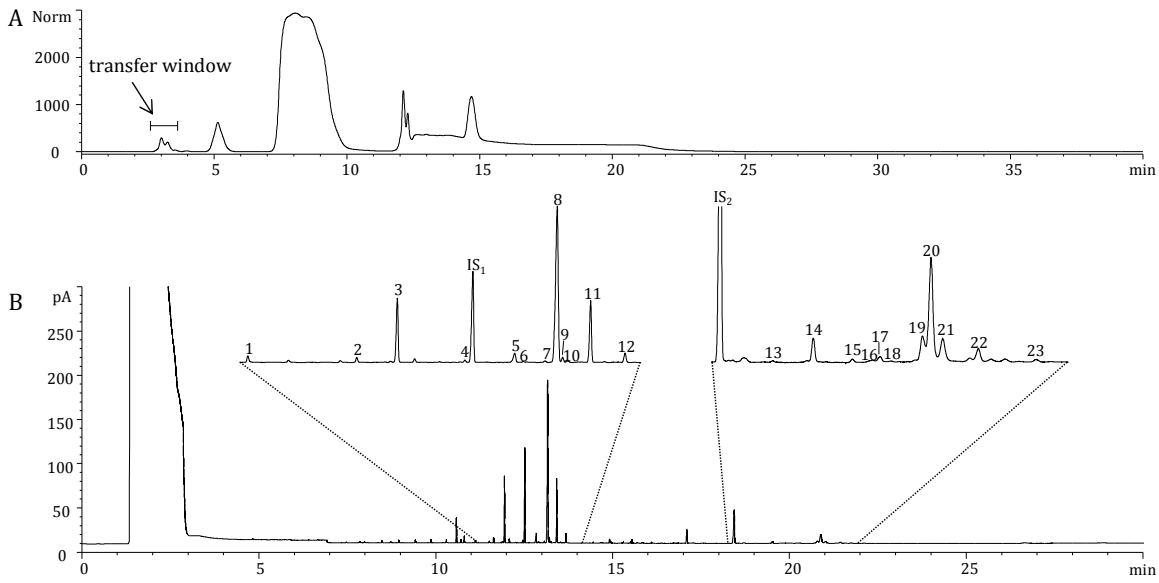

Figure 33. On-line LC-GC analysis of squalene, tocopherols, free sterols/stanols, and steryl/stanyl fatty acid esters in walnut. (**A**) LC-UV chromatogram at 205 nm and (**B**) GC-FID chromatogram of the transferred LC-fraction.
(1) squalene, (2) *δ*-tocopherol, (3) *γ*-tocopherol, (4) *α*-tocopherol, (5) campesterol, (6) stigmasterol, (7) clerosterol, (8) sitosterol, (9) Δ^5 avenasterol, (10) sitostanol, (11) cycloartenol, (12) 24-methylene cycloartanol + citrostadienol, (13) campesteryl-16:0/16:1, (14) sitosteryl-16:0/16:1, (15) cycloartenyl-16:0/16:1, (16) campesteryl-18:0/18:1, (17) campesteryl-18:2, (18) campesteryl-18:3, (19) sitosteryl-18:0/18:1, (20) sitosteryl-18:2, (21) sitosteryl-18:3, (22) cycloartenyl-18:2, (23) 24-methylene cycloartanyl-18:2, (IS_1) 5-*α*-cholestan-3*β*-ol, and (IS_2) cholesteryl-16:0.

4.6.2 Compositions and Contents of Free Sterols/Stanols and Steryl/Stanyl Esters

Free Sterols/Stanols

The compositions and contents of free sterols/stanols determined in the 30 investigated tree nut and peanut samples are presented in Table 28. Within a single kind of nut, only few differences were observed in the compositions of free sterols/stanols. However, the distribution patterns of the 10 different nut types revealed considerable variations.

Five sterols as well as one stanol were identified and quantified in all nut samples, i.e. sitosterol, campesterol, 24-methylene cycloartanol (coeluted with citrostadienol), Δ^5 avenasterol, clerosterol, and sitostanol. All nuts had in common that sitosterol was predominant, accounting for 59.1-87.5 % of total free sterols/stanols. This is in agreement with previously published data, where sitosterol has also been identified as the main free sterol in almonds, hazelnuts, peanuts, and walnuts as well as in refined peanut and walnut

oils (Worthington and Hitchcock, 1984; Choong *et al.*, 1999; Kalo and Kuuranne, 2001; Verleyen *et al.*, 2002a; Momchilova and Nikolova-Damyanova, 2007; Verardo *et al.*, 2009). Campesterol was the second most abundant free sterol in macadamias, pine nuts, hazelnut no. 1 and 2, almond no. 2, and cashew nut no. 1, representing 3.2-16.0 % of total free sterols/stanols. Cycloartenol was detected in 19 of the 30 analyzed nut samples. It was the second most abundant sterol in walnuts, accounting for approximately 20 % of total free sterols/stanols. This is in agreement with the profiles of total sterols/stanols reported in two studies of walnuts (Martínez *et al.*, 2006; Robbins *et al.*, 2011); other studies, however, did not mention cycloartenol (Amaral *et al.*, 2003; Crews *et al.*, 2005a; Momchilova and Nikolova-Damyanova, 2007; Verardo *et al.*, 2009; Bada *et al.*, 2010). Even though Δ^5avenasterol was present in all samples, the amounts were lower than 2 %, with the exception of pine nuts where it constituted up to 5.3-6.1 %. Several studies examining total sterols/stanols in pine nuts also reported high levels of Δ^5avenasterol (Phillips *et al.*, 2005; Nasri *et al.*, 2007; Robbins *et al.*, 2011). In turn, relatively high amounts of *α*- and *β*-amyrin were characteristic for Brazil nuts; both represented 18-24 % of total free sterols/stanols. High contents of *α*-amyrin in Brazil nuts have also been reported for the compositions of total sterols/stanols; *β*-amyrin, however, has not been mentioned (Robbins *et al.*, 2011). Free stanols, i.e. sitostanol and campestanol were minor components in all nuts, accounting for 0.8-4.0 % of total free sterols/stanols.

The highest total contents of free sterols/stanols based on fresh nuts were determined in pine nuts and pistachios, averaging 1.57 ± 0.10 mg/g and 1.61 ± 0.22 mg/g, respectively. The total amounts of the other nuts ranged from 0.47-1.26 mg/g, being lowest in hazelnuts. The free sterol/stanol contents calculated for the extracted oils were in the range of 0.10-0.42 %, being highest in pine nuts and pistachios and lowest in hazelnuts. The contents of 0.3 % in hazelnut oil, 0.6 % in almond oil, and 0.7 % in walnut oil as reported by a previous study were above these levels (Momchilova and Nikolova-Damyanova, 2007). The total content of free sterols/stanols, which has been reported for a single almond sample was comparable to the amounts determined in the present study; those for Brazil nuts, hazelnuts, peanuts, pecan nuts, and walnuts were somewhat higher, but in the same order of magnitude (Worthington and Hitchcock, 1984; Miraliakbari and Shahidi, 2008). The levels quantified for a single pine nut and pistachio sample were, in turn, 1.9- or 2.8-fold lower (Miraliakbari and Shahidi, 2008).

Table 28. Contents and compositions of free sterols/stanols in tree nuts and peanuts.

	no.	∑ [mg/g nut]	∑ [µg/100 mg oil]	sitosterol [%]	campesterol [%]	24-meth.-cycl.[a,b] [%]	Δ^5 avenasterol [%]	sitostanol [%]	clerosterol [%]
almond	1	1.21 ± 0.01	251.3 ± 2.8	83.5 ± 0.1	2.6 ± 0.2	5.1 ± 0.2	0.7 ± 0.2	2.5 ± 0.0	1.2 ± 0.1
	2	0.91 ± 0.02	243.3 ± 9.4	87.2 ± 0.1	3.2 ± 0.0	2.6 ± 0.2	0.6 ± 0.1	2.2 ± 0.1	1.1 ± 0.0
	3	1.16 ± 0.02	230.5 ± 3.2	87.2 ± 0.6	2.4 ± 0.2	2.9 ± 0.2	0.7 ± 0.1	2.7 ± 0.3	1.2 ± 0.0
Brazil nut	1	0.81 ± 0.03	129.5 ± 5.6	63.9 ± 0.4	2.0 ± 0.2	1.0 ± 0.1	1.3 ± 0.1	3.9 ± 0.2	0.9 ± 0.0
	2	0.96 ± 0.01	145.3 ± 8.6	59.1 ± 0.7	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	3.7 ± 0.0	1.1 ± 0.1
	3	0.90 ± 0.01	149.1 ± 1.5	59.6 ± 0.4	1.3 ± 0.0	1.3 ± 0.0	0.8 ± 0.0	4.0 ± 0.1	1.0 ± 0.0
cashew nut	1	0.64 ± 0.01	141.9 ± 3.2	78.2 ± 0.4	6.6 ± 0.2	4.6 ± 0.2	0.6 ± 0.0	1.1 ± 0.1	1.7 ± 0.1
	2	0.68 ± 0.04	156.2 ± 8.9	71.8 ± 1.5	6.4 ± 0.2	7.0 ± 1.0	0.8 ± 0.1	0.9 ± 0.0	1.7 ± 0.1
	3	0.64 ± 0.02	140.2 ± 3.5	76.5 ± 0.6	5.8 ± 0.1	4.6 ± 0.1	0.4 ± 0.0	1.3 ± 0.4	1.7 ± 0.1
hazelnut	1	0.47 ± 0.01	102.0 ± 0.6	82.8 ± 0.2	4.9 ± 0.0	4.2 ± 0.0	0.7 ± 0.1	2.8 ± 0.0	1.3 ± 0.1
	2	0.68 ± 0.01	124.7 ± 4.1	81.8 ± 0.3	5.8 ± 0.4	5.6 ± 0.1	0.8 ± 0.0	2.7 ± 0.4	1.5 ± 0.1
	3	0.71 ± 0.05	129.3 ± 3.5	82.1 ± 0.1	4.1 ± 0.0	4.5 ± 0.1	0.8 ± 0.0	3.0 ± 0.1	1.5 ± 0.1
macadamia	1	1.12 ± 0.05	205.8 ± 4.0	84.0 ± 1.0	7.9 ± 0.3	5.8 ± 0.2	0.3 ± 0.0	1.0 ± 0.0	0.9 ± 0.0
	2	1.17 ± 0.04	192.3 ± 0.4	82.6 ± 0.3	7.7 ± 0.0	7.6 ± 0.5	0.4 ± 0.1	0.8 ± 0.1	0.9 ± 0.0
	3	1.26 ± 0.06	216.1 ± 14.8	87.5 ± 0.4	6.6 ± 0.2	3.9 ± 0.1	0.3 ± 0.0	0.8 ± 0.1	1.0 ± 0.0
peanut	1	0.95 ± 0.01	204.8 ± 6.5	69.9 ± 0.4	10.3 ± 0.1	4.0 ± 0.1	0.8 ± 0.0	1.3 ± 0.1	0.9 ± 0.0
	2	0.73 ± 0.05	153.5 ± 2.0	65.6 ± 1.6	12.3 ± 0.4	2.7 ± 0.3	0.6 ± 0.1	1.0 ± 0.0	0.9 ± 0.0
	3	0.85 ± 0.00	181.8 ± 2.5	67.3 ± 0.5	11.1 ± 0.2	5.0 ± 0.5	1.0 ± 0.0	1.3 ± 0.2	1.1 ± 0.1
pecan nut	1	1.09 ± 0.02	162.6 ± 2.2	76.6 ± 0.6	3.3 ± 0.0	7.5 ± 0.4	0.5 ± 0.0	1.0 ± 0.0	0.9 ± 0.1
	2	1.26 ± 0.04	185.8 ± 4.1	77.8 ± 2.0	3.6 ± 0.1	6.2 ± 1.5	0.4 ± 0.0	1.2 ± 0.0	0.9 ± 0.0
	3	1.02 ± 0.06	149.3 ± 9.2	71.1 ± 1.1	3.2 ± 0.0	6.9 ± 0.2	0.6 ± 0.1	1.2 ± 0.1	0.9 ± 0.1
pine nut	1	1.56 ± 0.03	361.1 ± 2.8	75.8 ± 0.4	15.4 ± 0.2	0.8 ± 0.2	6.1 ± 0.6	1.1 ± 0.0	0.9 ± 0.1
	2	1.67 ± 0.02	362.5 ± 5.3	75.6 ± 0.1	16.0 ± 0.2	0.7 ± 0.0	6.0 ± 0.1	1.0 ± 0.0	0.7 ± 0.0
	3	1.47 ± 0.10	385.5 ± 19.1	77.2 ± 0.5	15.4 ± 0.7	0.2 ± 0.0	5.3 ± 0.2	1.0 ± 0.0	0.6 ± 0.0
pistachio	1	1.85 ± 0.02	418.0 ± 3.7	80.2 ± 0.4	4.1 ± 0.1	6.8 ± 0.2	1.9 ± 0.0	1.4 ± 0.0	1.2 ± 0.0
	2	1.43 ± 0.05	331.3 ± 2.6	82.2 ± 0.1	4.6 ± 0.1	5.2 ± 0.0	2.0 ± 0.0	1.4 ± 0.0	1.5 ± 0.0
	3	1.54 ± 0.03	338.6 ± 6.3	79.6 ± 0.1	4.7 ± 0.0	6.4 ± 0.1	2.0 ± 0.0	1.3 ± 0.0	1.5 ± 0.0
walnut	1	0.88 ± 0.03	145.5 ± 3.8	69.0 ± 0.6	4.0 ± 0.1	3.7 ± 0.1	1.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
	2	0.80 ± 0.04	139.8 ± 4.2	69.0 ± 0.1	3.8 ± 0.0	3.2 ± 0.3	1.7 ± 0.0	0.9 ± 0.0	0.6 ± 0.0
	3	1.17 ± 0.06	201.8 ± 4.5	70.1 ± 0.2	3.5 ± 0.1	2.7 ± 0.1	1.3 ± 0.0	0.8 ± 0.0	0.6 ± 0.0

Table 28. continued.

	no.	stigmasterol [%]	cycloartenol [%]	Δ^7 sitosterol [%]	β-amyrin [%]	α-amyrin [%]	cycloartanol [%]	cholesterol [%]	campestanol [%]
almond	1	0.9 ± 0.1	1.6 ± 0.1	2.0 ± 0.1	-	-	-	-	-
	2	1.2 ± 0.1	<LOQ	1.7 ± 0.2	-	-	-	-	-
	3	-[c]	<LOQ	2.9 ± 0.1	-	-	-	-	-
Brazil nut	1	5.1 ± 0.4	0.7 ± 0.0	3.1 ± 0.2	3.6 ± 0.1	14.4 ± 0.3	-	-	-
	2	4.8 ± 0.1	0.7 ± 0.0	3.1 ± 0.2	5.0 ± 0.2	18.5 ± 0.1	-	-	-
	3	4.5 ± 0.1	1.1 ± 0.0	2.7 ± 0.1	5.5 ± 0.5	18.1 ± 0.1	-	-	-
cashew nut	1	0.6 ± 0.0	4.8 ± 0.2	-	-	-	1.7 ± 0.0	-	-
	2	0.4 ± 0.0	8.3 ± 0.8	-	-	-	2.4 ± 0.5	0.4 ± 0.0	-
	3	0.3 ± 0.0	7.5 ± 0.1	-	-	-	1.6 ± 0.0	0.4 ± 0.0	-
hazelnut	1	1.2 ± 0.1	-	2.2 ± 0.0	-	-	-	-	-
	2	1.5 ± 0.2	-	0.5 ± 0.0	-	-	-	-	-
	3	1.1 ± 0.1	-	2.9 ± 0.1	-	-	-	-	-
macadamia	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
peanut	1	10.3 ± 0.2	1.4 ± 0.0	-	1.2 ± 0.0	-	-	-	-
	2	14.6 ± 1.0	0.8 ± 0.5	-	1.4 ± 0.1	-	-	-	-
	3	8.0 ± 0.3	3.4 ± 0.7	-	1.7 ± 0.1	-	-	-	-
pecan nut	1	1.0 ± 0.0	7.4 ± 0.1	1.2 ± 0.0	0.7 ± 0.0	-	-	-	-
	2	1.4 ± 0.1	6.9 ± 0.4	1.0 ± 0.0	0.7 ± 0.2	-	-	-	-
	3	1.4 ± 0.1	11.3 ± 0.4	1.2 ± 0.0	2.2 ± 0.3	-	-	-	-
pine nut	1	<LOQ[d]	-	-	-	-	-	-	-
	2	<LOQ	-	-	-	-	-	-	-
	3	0.2 ± 0.0	-	-	-	-	-	-	0.2 ± 0.0
pistachio	1	0.8 ± 0.1	1.9 ± 0.0	1.0 ± 0.0	-	0.8 ± 0.0	-	-	-
	2	0.5 ± 0.0	1.1 ± 0.0	0.9 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	-	-	-
	3	0.5 ± 0.0	1.5 ± 0.0	1.3 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	-	-	-
walnut	1	0.3 ± 0.0	19.8 ± 0.5	-	-	-	-	-	-
	2	0.3 ± 0.1	20.4 ± 0.5	-	-	-	-	-	-
	3	0.2 ± 0.0	20.7 ± 0.2	-	-	-	-	-	-

[a] 24-Methylene cycloartanol. [b] Coelution with citrostadienol. [c] (-) Content below limit of detection (5 ng/mL i.v.; determined on the basis of 20 µL i.v.). [d] Content below limit of quantification (15 ng/mL i.v.; determined on the basis of 20 µL i.v.).

Steryl/Stanyl Fatty Acid Esters

The percentage compositions of steryl fatty acid esters determined in tree nuts and peanuts are presented in Table 29. Up to 14 individual steryl fatty acid esters were identified; stanyl esters could not be detected. Six esters, namely sitosteryl-18:2, sitosteryl-18:0/18:1, campesteryl-18:2, campesteryl-18:0/18:1, sitosteryl-16:0/16:1, and campesteryl-16:0/16:1 were present in all samples. However, the compositions of the steryl fatty acid esters showed large variations not only between the different nuts but also within certain kinds of nuts, e.g. almonds, macadamias, pecan nuts, and peanuts. The main part of the fatty acids occurred as sitosteryl esters of at least 74 % (cashew no. 2), most often followed by esters of campesterol. 4,4'-Dimethylsteryl fatty acid esters were additionally detected in cashew nuts, pecan nuts, pine nuts, pistachios, and walnuts, where they represented 0.9-13.9 % of total steryl fatty acid esters. Regarding the fatty acid part, the majority of the sterols was esterified to C18-fatty acids, accounting for 83.0-99.6 %. Thereof, sitosteryl-18:2 was the predominant ester in almonds, Brazil nuts, cashew kernels (except for cashew no. 2), peanuts, pecan nuts, pine nuts, pistachios, and walnuts. The amounts of C16-fatty acids were particularly high in macadamias (12.1-17.0 %) and Brazil nuts (10.1-16.9 %). In contrast to all other kinds of nuts, the majority of sterols in hazelnuts and macadamias occurred as esters of oleic/stearic acid. Additionally, walnuts exhibited a distinct profile with sitosteryl-18:3 as second most abundant ester with an average relative amount of 22 % of total esters.
According to Momchilova and Nikolova-Damyanova (2007), sitosterol was the only sterol present in steryl esters of hazelnuts and walnuts. Furthermore, the fatty acid compositions of the steryl esters, particularly those of walnuts differed from those determined in the present study. Other studies reported sitosterol, campesterol, stigmasterol, and Δ^5 avenasterol as main esterified sterols in walnut and peanut oils (Worthington and Hitchcock, 1984; Kalo and Kuuranne, 2001; Verleyen *et al.*, 2002a). Owing to the lack of information on the composition of steryl fatty acid esters in nuts, a further comparison to literature data is not possible. Only one study investigated steryl esters in Scot's pine nuts (*Pinus sylvestris* L.). However, in the present work pine nuts of the type *Pinus pinea* L. were studied and due to the genetic differences a comparison is not meaningful (Fischer and Höll, 1991).

The distributions of esterified palmitic/palmitoleic acid and stearic/oleic acid were determined by GC-FID as fatty acid methyl esters after transesterification of the respective steryl/stanyl fatty acid ester fraction isolated via SPE. The ratios of palmitic/palmitoleic acid were 73:1, 94:1, and 118:1 in hazelnuts, pecan nuts, and Brazil nuts, respectively. In almonds, cashew nuts, peanuts, pine nuts, and pistachios no palmitoleic acid was detected. The macadamia nuts, in turn, exhibited remarkably high amounts of palmitoleic acid (ratio palmitic/palmitoleic acid 1:1). A predominance of oleic acid was observed in all nut samples.

The amounts of oleic acid were 2- to 19-fold above the amounts of stearic acid, being lowest in Brazil nuts and highest in pistachios (Appendix Table 37).

In agreement with the levels determined for free sterols/stanols, the analysis of pine nuts and pistachios revealed also the highest contents of steryl fatty acid esters, averaging 1.07 ± 0.18 mg/g and 1.26 ± 0.67 mg/g. The total contents of steryl fatty acid esters of the other nut types ranged from 0.06-0.48 mg/g, being lowest in Brazil nuts. Large variations were observed regarding the total contents not only between the different nut types, but also within a single kinds of nuts, e.g. the amounts of steryl fatty acid esters determined in pistachios ranged from 0.75-2.02 mg/g, those in almonds from 0.18-0.40 mg/g. The total contents calculated on the basis of extracted oil were between 10.2 and 443.1 µg/100 mg oil. The contents of steryl fatty acid esters of almonds, hazelnuts, peanuts, and pecan nuts are in good agreement with previously published data (Worthington and Hitchcock, 1984; Miraliakbari and Shahidi, 2008). The levels quantified in Brazil nuts and walnuts were slightly higher, those of pine nuts and pistachios lower (Miraliakbari and Shahidi, 2008). Momchilova and Nikolova-Damyanova (2007) could not detect steryl esters in almonds and the contents analyzed in hazelnuts and walnuts were 10- and 31-fold above the levels determined in the present study.

Table 29. Contents and compositions of steryl/stanyl fatty acid esters in tree nuts and peanuts.

	no.	∑ [mg/g nut]	∑ [µg/100 mg oil]	sitosteryl-18:2 [%]	sitosteryl-18:0/18:1 [%]	campesteryl-18:2 [%]	campesteryl-18:0/18:1 [%]	sitosteryl-16:0/16:1 [%]	campesteryl-16:0/16:1 [%]	cycloartenyl-18:2 [%][c]
almond	1	0.40 ± 0.02	82.5 ± 2.7	55.9 ± 0.5	34.9 ± 0.4	4.6 ± 0.1[a]	1.8 ± 0.0	2.9 ± 0.0	<LOQ	-[d]
	2	0.39 ± 0.01	105.1 ± 2.3	77.8 ± 0.4	11.6 ± 0.2	7.0 ± 0.1[a]	0.3 ± 0.0	0.4 ± 0.0	<LOQ	-
	3	0.19 ± 0.00	36.8 ± 1.4	63.9 ± 0.9	27.0 ± 1.4	5.6 ± 0.3[a]	1.3 ± 0.1	2.1 ± 0.2	<LOQ	-
Brazil nut	1	0.11 ± 0.01	18.3 ± 1.2	54.8 ± 1.6	25.1 ± 1.6	6.6 ± 0.2[a]	1.5 ± 0.1	10.1 ± 0.6	<LOQ	-
	2	0.16 ± 0.01	24.5 ± 2.3	42.8 ± 1.0	31.1 ± 0.3	8.4 ± 0.3[a]	3.0 ± 0.4	10.8 ± 0.9	1.5 ± 0.2	-
	3	0.06 ± 0.00	10.2 ± 0.5	47.1 ± 1.0	36.0 ± 1.3	<LOQ[b]	<LOQ	16.9 ± 1.1	<LOQ	-
cashew nut	1	0.31 ± 0.00	69.1 ± 1.7	41.6 ± 0.1	33.8 ± 0.1	6.9 ± 0.0	3.1 ± 0.0	6.3 ± 0.0	0.6 ± 0.0	3.5 ± 0.1
	2	0.18 ± 0.01	39.8 ± 1.6	32.0 ± 1.5	33.4 ± 0.8	8.7 ± 0.1	4.0 ± 0.3	8.6 ± 0.3	1.2 ± 0.0	2.8 ± 0.0
	3	0.22 ± 0.01	48.6 ± 1.0	50.4 ± 0.6	28.3 ± 0.4	4.4 ± 0.3	2.0 ± 0.2	5.0 ± 0.1	0.3 ± 0.0	3.6 ± 0.4
hazelnut	1	0.24 ± 0.01	51.3 ± 1.6	29.7 ± 1.0	55.6 ± 0.7	2.1 ± 0.3	3.8 ± 0.4	8.3 ± 0.3	0.4 ± 0.2	-
	2	0.27 ± 0.00	50.2 ± 2.3	33.1 ± 1.2	49.4 ± 1.8	3.5 ± 0.1	4.4 ± 0.3	8.6 ± 0.2	0.9 ± 0.1	-
	3	0.22 ± 0.01	39.0 ± 0.8	28.6 ± 0.6	58.6 ± 0.9	1.1 ± 0.0	2.2 ± 0.1	6.9 ± 0.1	0.2 ± 0.0	-
macadamia	1	0.20 ± 0.02	34.4 ± 2.5	23.5 ± 0.3	53.8 ± 0.3	1.1 ± 0.2	4.6 ± 0.1	15.4 ± 0.1	1.6 ± 0.2	-
	2	0.16 ± 0.01	27.0 ± 0.7	16.9 ± 0.5	62.2 ± 1.3	1.3 ± 0.2	5.2 ± 0.2	13.4 ± 0.5	1.0 ± 0.1	-
	3	0.16 ± 0.01	26.6 ± 1.1	24.0 ± 2.2	54.2 ± 2.6	3.5 ± 0.3	6.2 ± 0.3	10.7 ± 0.4	1.4 ± 0.0	-
peanut	1	0.48 ± 0.03	102.6 ± 5.2	68.2 ± 0.2	9.9 ± 0.1	11.5 ± 0.5[a]	2.1 ± 0.2	4.2 ± 0.1	0.9 ± 0.1	-
	2	0.17 ± 0.01	36.6 ± 2.5	54.9 ± 1.5	17.7 ± 0.8	17.0 ± 1.0[a]	3.5 ± 0.5	4.5 ± 0.2	1.4 ± 0.2	-
	3	0.19 ± 0.02	41.3 ± 4.3	40.1 ± 1.0	29.5 ± 1.0	13.6 ± 0.8[a]	7.5 ± 0.5	5.0 ± 0.5	2.1 ± 0.2	-
pecan nut	1	0.40 ± 0.01	60.2 ± 1.4	50.8 ± 0.5	30.3 ± 0.7	2.0 ± 0.1	1.4 ± 0.1	5.7 ± 0.1	<LOQ	2.2 ± 0.1
	2	0.16 ± 0.01	23.2 ± 0.9	63.8 ± 0.3	17.0 ± 0.4	2.5 ± 0.1	0.3 ± 0.0	1.8 ± 0.1	<LOQ	3.2 ± 0.1
	3	0.27 ± 0.01	40.4 ± 2.2	35.7 ± 4.3	32.3 ± 3.8	2.9 ± 0.2	2.5 ± 0.1	6.4 ± 0.1	0.5 ± 0.0	5.3 ± 0.5
pine nut	1	1.28 ± 0.02	296.1 ± 8.3	73.7 ± 0.5	8.4 ± 0.5	14.0 ± 0.0	1.8 ± 0.1	1.1 ± 0.0	0.3 ± 0.0	-
	2	0.92 ± 0.02	200.6 ± 5.2	72.0 ± 0.2	7.4 ± 0.2	14.3 ± 0.2	1.9 ± 0.2	1.1 ± 0.0	0.3 ± 0.0	-
	3	1.01 ± 0.09	264.6 ± 17.2	69.9 ± 0.5	7.9 ± 0.1	13.3 ± 0.2	1.6 ± 0.1	1.1 ± 0.0	0.2 ± 0.0	-
pistachio	1	0.75 ± 0.02	169.5 ± 4.4	73.1 ± 0.7	9.9 ± 0.6	5.7 ± 0.1	0.6 ± 0.0	1.6 ± 0.1	0.2 ± 0.0	4.3 ± 0.1
	2	1.01 ± 0.05	234.6 ± 1.8	77.8 ± 0.4	10.7 ± 0.4	3.7 ± 0.1	0.6 ± 0.1	1.5 ± 0.1	0.2 ± 0.0	2.4 ± 0.2
	3	2.02 ± 0.14	443.1 ± 29.6	68.1 ± 0.0	18.3 ± 0.2	5.1 ± 0.2	1.5 ± 0.1	3.5 ± 0.2	0.5 ± 0.1	1.3 ± 0.1
walnut	1	0.25 ± 0.01	41.9 ± 0.2	47.4 ± 1.9	10.6 ± 0.7	2.6 ± 0.2	1.2 ± 0.0	6.2 ± 0.1	0.3 ± 0.0	5.3 ± 0.1
	2	0.27 ± 0.01	48.1 ± 1.5	46.8 ± 0.2	13.7 ± 0.1	2.6 ± 0.0	1.1 ± 0.0	6.3 ± 0.0	0.1 ± 0.0	5.7 ± 0.5
	3	0.37 ± 0.01	63.9 ± 2.6	53.5 ± 1.5	11.5 ± 2.6	2.0 ± 0.1	0.7 ± 0.0	2.7 ± 0.2	<LOQ	3.7 ± 0.6

Table 29. continued

	no.	cycloartenyl-18:0/18:1 [%][c]	cycloartenyl-16:0/16:1[%][c]	24-meth.cycl.[e] 18:2 [%][c]	24-meth.cycl.[e] 18:0/18:1 [%][c]	sitosteryl-18:3 [%]	campesteryl-18:3 [%]	stigmasteryl-18:2 [%]	others [%][c]
almond	1	-	-	-	-	-	-	<LOQ	-
	2	-	-	-	-	-	-	2.9 ± 0.2	-
	3	-	-	-	-	-	-	<LOQ	-
Brazil nut	1	-	-	-	-	-	-	1.8 ± 0.1	-
	2	-	-	-	-	-	-	2.4 ± 0.1	-
	3	-	-	-	-	-	-	<LOQ	-
cashew nut	1	<LOQ	1.7 ± 0.0	1.2 ± 0.0	1.4 ± 0.1	-	-	-	-
	2	4.6 ± 0.1	1.5 ± 0.1	1.2 ± 0.3	1.9 ± 0.4	-	-	-	-
	3	3.5 ± 0.2	0.9 ± 0.0	0.7 ± 0.1	0.9 ± 0.1	-	-	-	-
hazelnut	1	-	-	-	-	-	-	-	<LOQ
	2	-	-	-	-	-	-	-	<LOQ
	3	-	-	-	-	-	-	-	2.4 ± 0.3
macadamia	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
peanut	1	-	-	-	-	2.1 ± 0.4	-	1.0 ± 0.1	-
	2	-	-	-	-	<LOQ	-	1.0 ± 0.1	-
	3	-	-	-	-	<LOQ	-	2.3 ± 0.4	-
pecan nut	1	2.5 ± 0.1	0.9 ± 0.0	-	-	-	-	-	4.1 ± 0.9
	2	3.5 ± 0.1	2.4 ± 0.1	-	-	-	-	-	5.5 ± 0.1
	3	5.6 ± 0.2	3.1 ± 0.1	-	-	-	-	-	5.9 ± 1.1
pine nut	1	-	-	<LOQ	-	-	-	-	0.7 ± 0.0
	2	-	-	0.9 ± 0.0	-	-	-	-	2.0 ± 0.1
	3	-	-	0.9 ± 0.1	-	-	-	-	5.1 ± 0.9
pistachio	1	-	-	4.6 ± 0.5	-	-	-	-	-
	2	-	-	3.0 ± 0.1	-	-	-	-	-
	3	-	-	1.7 ± 0.0	-	-	-	-	-
walnut	1	<LOQ	1.4 ± 0.1	1.4 ± 0.1	-	21.8 ± 0.6	1.7 ± 0.1	-	-
	2	<LOQ	1.2 ± 0.2	1.2 ± 0.1	-	19.8 ± 0.1	1.5 ± 0.0	-	-
	3	<LOQ	1.0 ± 0.1	2.7 ± 0.5	-	20.9 ± 0.2	1.3 ± 0.1	-	-

[a] Coelution with stigmasteryl-18:0/18:1. [b] Content below limit of quantification (40 ng/mL i.v.; determined on the basis of 20 µL i.v.). [c] Compound calculated with Rf = 1. [d] Content below limit of detection (14 ng/mL i.v.; determined on the basis of 20 µL i.v.). [e] 24-Methylene cycloartanyl.

4.6.3 Distribution Patterns of Free and Esterified Sterols/Stanols

Those sterols that were predominant within free sterols/stanols could most often also be detected as ester with fatty acids. However, the sterol distribution between both fractions showed slight differences. For example, sitosterol and campesterol were usually found to a higher degree within the fraction of steryl/stanyl fatty acid esters, whereas the proportion of stigmasterol (if it was present) was higher in the fraction of free sterols/stanols. These observations are in agreement with data reported for peanuts and refined peanut oils (Worthington and Hitchcock, 1984; Verleyen *et al.*, 2002a). Cycloartenol, the second most abundant free sterol/stanol of walnuts, was detected to a lower degree esterified to fatty acids. Δ^5 Avenasterol, which was present in free from in all nuts, could, however, not be identified as ester with fatty acids. As Δ^5 avenasterol is not commercially available, no fatty acid esters with this sterol could be synthesized and the chromatographic behavior of Δ^5 avenasteryl fatty acid esters under the employed GC conditions is not known. However, mass spectrometric analyses did not provide an indication of the presence of these esters.

4.6.4 Distribution Patterns of Total Fatty Acids and of Fatty Acids Esterified to Sterols/Stanols

The total fatty acid profiles of the 30 investigated tree nut and peanut samples are presented in Appendix Table 41. In all nut oils, except for macadamia oil, linoleic and oleic acid made up the majority of the fatty acids, representing together 74-93 %. Linoleic acid was the major fatty acid in Brazil nuts, pine nuts, and walnuts; the other nuts contained mainly oleic acid. The proportion of linolenic acid was lower than 1.4 %, except for walnuts, where this fatty acid made up 13-14 %. Macadamia was the only kind of nut, in which palmitoleic acid was the second most abundant fatty acid with an average amount of 20.5 ± 1.7 %. The levels of saturated fatty acids in almonds, hazelnuts, pecan nuts, pine nuts, pistachios, and walnuts were below 12 %. Brazil nuts, cashew nuts, and macadamias, in turn, exhibited relatively high amounts of saturated fatty acids, averaging 25, 20, and 16 %, respectively. This is one reason why the United States Food and Drug Administration excluded these three types of nuts from their qualified health claim on nuts and coronary heart disease (FDA, 2003). The fatty acid compositions of the tree nuts and peanuts determined in the present study agree very well with earlier reported results (García-López *et al.*, 1996; Kaijser *et al.*, 2000; Amaral *et al.*, 2003; Nergiz and Dönmez, 2004; Crews *et al.*, 2005b; Crews *et al.*, 2005a; Amaral *et al.*, 2006; Venkatachalam and Sathe, 2006; Evaristo *et al.*, 2010; Shin *et al.*, 2010b; Robbins *et al.*, 2011). However, the distributions of fatty acids esterified to sterols differed considerably from those of the total lipids. The steryl fatty acid esters of all nuts contained higher proportions of

linoleic acid and lower proportions of oleic/stearic and palmitic/palmitoleic acid than the corresponding total lipids. Linolenic acid, which was present as steryl esters in walnuts and in peanut no. 1, was found to a higher proportion esterified to sterols than in total lipids. These findings are in agreement with the fatty acid distributions obtained for two peanut cultivars (Worthington and Hitchcock, 1984). Other studies found partially contradictory results (Momchilova and Nikolova-Damyanova, 2007; Speer and Zahm, 2011). However, it should be considered that these studies compared the fatty acid compositions of steryl esters not to those of total extracted lipids, but only to those of triacylglycerols.

4.6.5 Proportions of Free Sterols/Stanols and Steryl/Stanyl Esters

The sums of the total contents of free sterols/stanols and steryl/stanyl fatty acid esters ranged in most of the nuts from 0.71 to 1.61 mg/g. Only pine nuts and pistachios revealed levels higher than 2 mg/g (Figure 34A). The amounts calculated for the extracted oils were in the range of 0.15-0.78 mg/100 mg, being also by far the highest in pine nuts and pistachios (Figure 34B). Thus, pine nuts and pistachios can be considered as the best sources of free sterols/stanols and steryl/stanyl esters among the nut types investigated in this study.

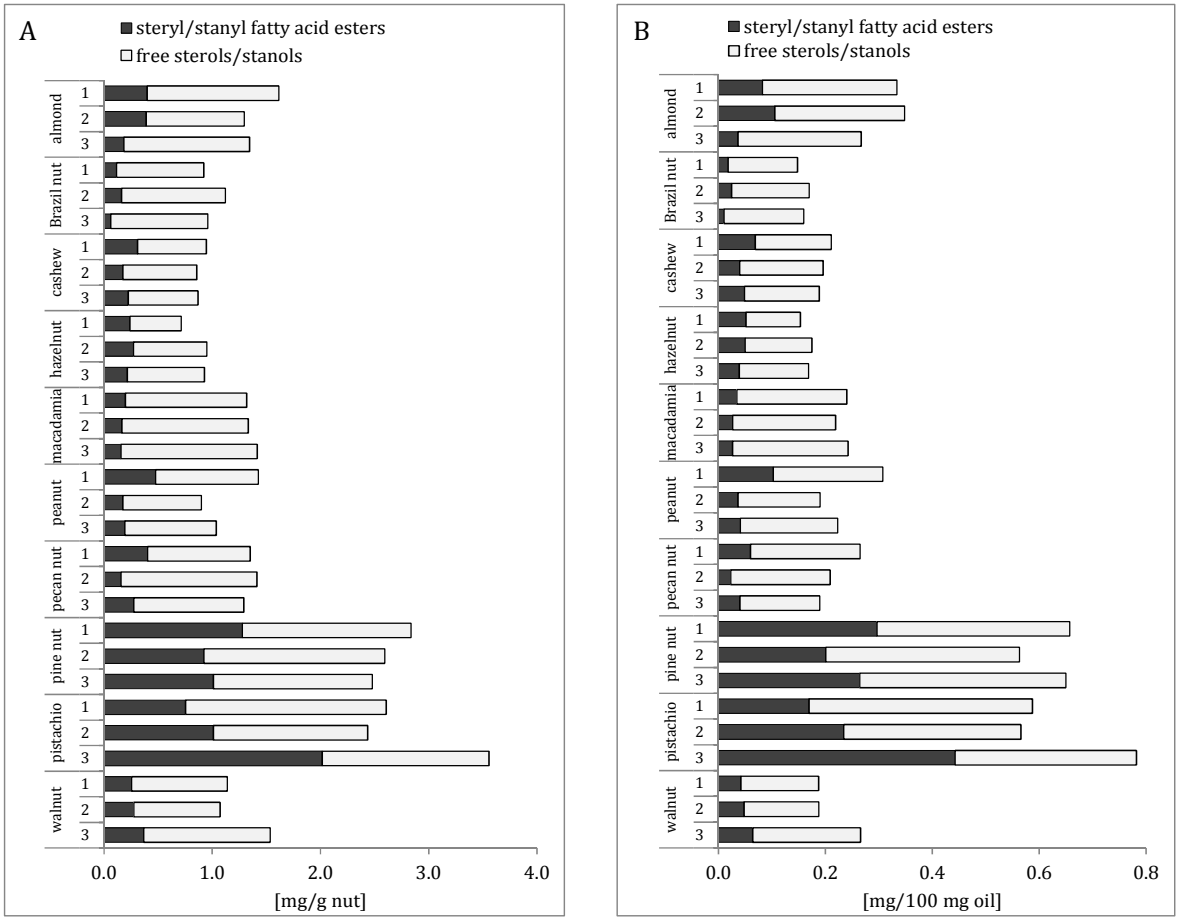

Figure 34. Sums of total contents of steryl/stanyl fatty acid esters and free sterols/stanols in tree nuts and peanuts determined (**A**) in fresh nuts and (**B**) in extracted oil (The numberings of the samples correspond to those in Table 11.).

All tree nut and peanut samples revealed higher amounts of free sterols/stanols than of steryl fatty acid esters, except for pistachio no. 3. This sample was the only one in which a reversed distribution was observed. The mean relative percentage distributions of each kind of nut regarding the sum of total free sterols/stanols and intact steryl/stanyl fatty acid esters are illustrated in Figure 35. The dominance of free sterols/stanols in nuts has already been reported for almonds, Brazil nuts, hazelnuts, peanuts, pecan nuts, pine nuts, pistachios, and walnuts (Kashani and Valadon, 1983; Worthington and Hitchcock, 1984; Miraliakbari and Shahidi, 2008; Speer and Zahm, 2011). In contrast, another study reported that the amounts of steryl esters in hazelnuts and walnuts were higher than those of free sterols/stanols (Momchilova and Nikolova-Damyanova, 2007).

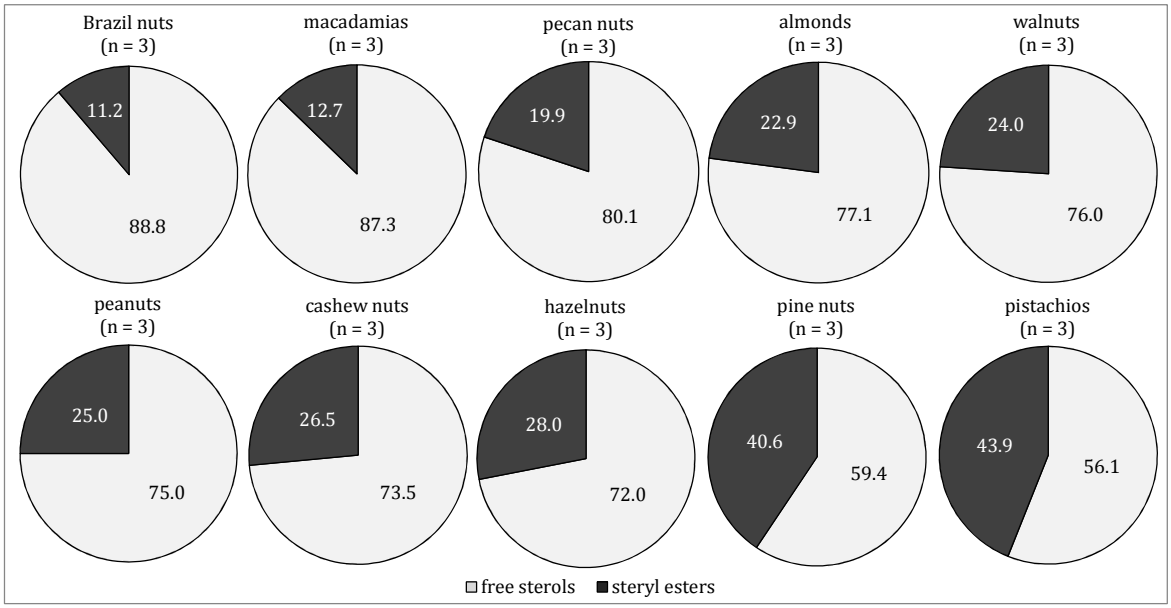

Figure 35. Percentage distributions of free sterols/stanols and intact steryl/stanyl esters in tree nuts and peanuts.

4.6.6 Tocopherols and Squalene

The total contents of tocopherols ranged from 2.5 to 405.0 µg/g nut, being lowest in peanut no. 1 and highest in pine nut no. 2 (Table 30). *γ*-Tocopherol was the predominant tocopherol in Brazil nuts, cashew nuts, pecan nuts, pine nuts, pistachios, and walnuts, whereas *α*-tocopherol was most abundant in almonds and hazelnuts. *δ*-Tocopherol was present in all samples, except for hazelnuts and macadamias. The results obtained for the distributions of tocopherols in almonds, Brazil nuts, cashew nuts, hazelnuts, pecan nuts, pine nuts, pistachios, and walnuts are comparable to those described in literature; total contents are also in the same order of magnitude (Gallina Toschi *et al.*, 1993; Savage *et al.*, 1997; Maguire *et al.*, 2004; Crews *et al.*, 2005b; Crews *et al.*, 2005a; Alasalvar *et al.*, 2006; Kornsteiner *et al.*, 2006; Ryan *et al.*, 2006; Miraliakbari and Shahidi, 2008; Alasalvar *et al.*, 2009; Bada *et al.*, 2010; Robbins *et al.*, 2011). In contrast to all other tree nuts, tocopherols could not be detected in

macadamia nuts; this is in agreement with data reported by Kornsteiner *et al.* (2006). Other studies reported contradictory results. Robbins *et al.* (2011) described *γ*-tocopherol contents of 0.1 ± 0.2 µg/g for three independent macadamia samples, but they could not detect *α*-, *β*-, and *δ*-tocopherol. A further study identified *α*- and *δ*-tocopherol in macadamias, but total amounts were lower than 5.6 µg/g oil (Kaijser *et al.*, 2000). Maguire *et al.* (2004), in turn, determined levels of about 185 µg/g oil. The three investigated peanut samples revealed large differences in their tocopherol contents and compositions. A 36-fold margin was observed between the highest and lowest tocopherol level. *γ*-Tocopherol was predominant in peanut no. 2 and no. 3 with 84 % and 65 %, respectively, followed by *δ*- and *α*-tocopherol. In contrast, peanut no. 1 consisted mainly of *γ*-tocopherol (40 %); *α*- and *γ*-tocopherol both made up 30 %. Considering the fact, that the peanuts investigated in the present study were roasted, comparison to literature is difficult as roasting conditions as well as storage of roasted peanuts have been shown to influence tocopherol contents and compositions (Chun *et al.*, 2005; Eitenmiller *et al.*, 2011). Literature data concerning tocopherols in raw peanuts are also inconsistent. Whereas *α*- and *γ*- tocopherol both made up approximately 48 % in a total of 151 raw peanut samples (Shin *et al.*, 2009), other studies detected *α*-tocopherol as predominant tocopherol in peanuts (Jonnala *et al.*, 2006b; Jonnala *et al.*, 2006a).

The contents of squalene exhibited large differences between the various kinds of nuts (Table 30). The lowest values were determined in walnuts and pine nuts, being <18.8 µg/g. Squalene levels in almonds, cashew kernels, hazelnuts, macadamias, peanuts, pecan nuts, and pistachios ranged from 33.5 to 220.7 µg/g. Brazil nuts contained by far the highest squalene contents, averaging 1112.3 ± 54.1 µg/g. The observed values are in line to those reported elsewhere (Bada *et al.*, 2004; Maguire *et al.*, 2004; Ryan *et al.*, 2006).

Table 30. Contents and compositions of tocopherols and contents of squalene in tree nuts and peanuts.

	no.	∑ tocopherols [µg/g nut]	∑ tocopherols [µg/100 mg oil]	α-tocopherol [%]	γ-tocopherol [%]	δ-tocopherol [%]	squalene [µg/g nut]	squalene [µg/100 mg oil]
almond	1	111.5 ± 0.5	23.1 ± 0.4	95.4 ± 0.1	4.6 ± 0.1	<LOQ[b]	99.0 ± 5.1	20.5 ± 1.2
	2	307.8 ± 48.9	82.8 ± 13.9	95.6 ± 0.2	4.4 ± 0.2	<LOQ	40.3 ± 5.7	10.8 ± 1.6
	3	214.5 ± 20.7	42.8 ± 5.4	93.2 ± 1.1	4.8 ± 0.8	2.0 ± 0.3	44.3 ± 3.6	8.8 ± 0.5
Brazil nut	1	137.1 ± 5.1	22.0 ± 0.8	25.2 ± 1.0	73.2 ± 1.3	1.6 ± 0.3	1077.9 ± 44.0	172.7 ± 8.4
	2	181.8 ± 3.6	27.5 ± 1.8	35.3 ± 0.9	63.5 ± 0.9	1.1 ± 0.1	1084.3 ± 20.4	164.0 ± 7.2
	3	174.3 ± 4.6	28.9 ± 0.2	25.9 ± 0.2	72.9 ± 0.2	1.2 ± 0.0	1174.6 ± 24.0	195.1 ± 2.3
cashew nut	1	71.2 ± 1.7	15.9 ± 0.5	5.3 ± 0.2	88.8 ± 0.1	5.9 ± 0.1	33.5 ± 1.7	7.5 ± 0.4
	2	71.2 ± 16.3	16.3 ± 3.7	7.6 ± 0.7	88.1 ± 0.8	4.3 ± 0.1	97.4 ± 20.9	22.2 ± 4.8
	3	66.0 ± 1.4	14.3 ± 0.2	5.5 ± 0.2	88.8 ± 0.4	5.7 ± 0.3	38.9 ± 1.4	8.5 ± 0.4
hazelnut	1	52.7 ± 5.8	11.3 ± 1.1	90.8 ± 2.2	9.2 ± 2.2	-	112.8 ± 4.1	24.3 ± 0.1
	2	199.1 ± 18.6	36.8 ± 4.6	93.1 ± 0.9	6.9 ± 0.9	-	127.7 ± 5.5	23.5 ± 0.2
	3	244.0 ± 16.2	44.2 ± 0.7	95.4 ± 0.0	4.6 ± 0.0	-	149.2 ± 12.4	27.0 ± 1.0
macadamia	1	-[a]	-	-	-	-	116.8 ± 2.2	21.5 ± 0.4
	2	-	-	-	-	-	139.5 ± 6.0	22.9 ± 0.4
	3	-	-	-	-	-	219.7 ± 36.6	37.8 ± 7.0
peanut	1	2.5 ± 0.2	0.7 ± 0.0	30.6 ± 2.3	29.6 ± 1.2	39.8 ± 2.1	111.2 ± 8.7	32.0 ± 2.5
	2	46.4 ± 6.8	9.6 ± 0.9	3.8 ± 0.1	83.6 ± 1.6	12.7 ± 1.7	75.3 ± 13.1	16.0 ± 3.2
	3	89.7 ± 16.4	25.7 ± 3.8	28.7 ± 1.8	64.7 ± 2.0	6.6 ± 0.6	146.2 ± 21.6	41.9 ± 5.1
pecan nut	1	191.2 ± 3.5	28.6 ± 0.6	3.9 ± 0.3	95.5 ± 0.3	0.6 ± 0.0	184.0 ± 3.5	27.5 ± 0.7
	2	272.5 ± 4.2	40.3 ± 0.7	3.3 ± 0.0	96.3 ± 0.1	0.5 ± 0.1	123.3 ± 3.2	18.3 ± 0.5
	3	249.6 ± 18.6	36.7 ± 3.0	11.0 ± 0.7	88.3 ± 0.6	0.6 ± 0.1	220.7 ± 13.8	32.4 ± 2.5
pine nut	1	286.1 ± 5.9	66.3 ± 0.5	11.0 ± 0.4	88.0 ± 0.5	1.0 ± 0.1	12.0 ± 0.4	2.8 ± 0.1
	2	405.0 ± 17.3	88.0 ± 3.9	10.9 ± 0.2	88.3 ± 0.2	0.8 ± 0.0	18.8 ± 0.9	4.1 ± 0.2
	3	292.8 ± 11.3	76.9 ± 4.0	7.9 ± 0.6	91.5 ± 0.6	0.7 ± 0.0	18.0 ± 1.7	4.7 ± 0.4
pistachio	1	257.3 ± 6.7	58.0 ± 1.4	4.5 ± 0.3	93.2 ± 0.5	2.2 ± 0.2	58.4 ± 3.5	13.2 ± 0.8
	2	269.3 ± 5.0	62.6 ± 1.7	0.9 ± 0.0	96.7 ± 0.1	2.4 ± 0.1	65.7 ± 3.4	15.3 ± 0.2
	3	228.1 ± 6.5	50.1 ± 1.3	1.8 ± 0.2	95.7 ± 0.3	2.5 ± 0.1	81.9 ± 3.6	18.0 ± 0.8
walnut	1	272.2 ± 11.2	44.8 ± 1.8	2.9 ± 0.3	88.4 ± 0.3	8.7 ± 0.4	9.2 ± 0.3	1.5 ± 0.1
	2	224.8 ± 25.3	39.3 ± 2.7	3.7 ± 0.3	89.6 ± 0.2	6.7 ± 0.3	13.3 ± 4.5	2.3 ± 0.8
	3	293.6 ± 16.1	50.8 ± 3.0	2.9 ± 1.1	85.4 ± 0.6	11.7 ± 0.6	6.6 ± 0.1	1.2 ± 0.1

[a] (-) Content below limit of detection (6 ng/mL i.v.; determined on the basis of 20 µL i.v.). [b] Content below limit of quantification (16 ng/mL i.v.; determined on the basis of 20 µL i.v.).

4.7 Analysis of Free Sterols/Stanols, Steryl/Stanyl Esters and Other Minor Lipids in Edible Plant Oils

Edible plant oils are an inherent part of human nutrition and good sources of phytosterols, contributing up to 40 % of the total daily phytosterol intake (cf. 2.2.1). Even though there is some information available regarding the contents and compositions of free and esterified sterols/stanols in vegetable oils, comparable data on the distributions of intact steryl/stanyl fatty acid esters are still rare (cf. 2.2.4). The analysis of the profiles of individual steryl/stanyl fatty acid esters along with those of free sterols/stanols and other minor lipids provides more detailed information which could be useful for the characterization and authentication of vegetable oils. The on-line LC-GC-based approach was applied in order to establish a fast and robust analysis of free sterols/stanols, steryl/stanyl fatty acid esters, and other minor lipids in edible plant oils. In total, 39 commercially obtained vegetable oils (15 rapeseed oils, 10 sunflower oils, 7 olive oils, 2 corn germ oils, 1 grape seed oil, 1 linseed oil, 1 safflower oil, 1 sesame seed oil, and 1 soybean oil) were analyzed.

4.7.1 On-line LC-GC-Based Approach

The sample preparation for the analysis of free sterols/stanols, steryl/stanyl esters, and other minor lipids in edible plant oils only required a silylation of the oils. After silylation, the oils were directly subjected to on-line LC-GC analysis. Free sterols/stanols, steryl/stanyl fatty acid esters, tocopherols, squalene, and free fatty acids could be transferred in a single fraction to the GC. Free fatty acids were quantified in the vegetable oils as their amounts are an important criterion to differentiate native and refined oils. The simultaneous analysis of free fatty acids, tocopherols, free sterols/stanols, and steryl/stanyl fatty acid esters by on-line LC-GC is exemplarily shown for a rapeseed oil in Figure 36. The oil samples were also screened for the presence of *trans*-steryl/stanyl ferulic acid esters via a second transfer.

Prior to the investigations, the on-line LC-GC-based approach was re-validated for the matrix oil in terms of recovery and repeatability. Recoveries were determined by spiking two different amounts of selected reference compounds to a rapeseed, sunflower, and corn germ oil. The mean recoveries of all individual values determined for each substance class were 99.5 ± 2.6 % for free sterols/stanols, 94.2 ± 3.0 % for steryl/stanyl fatty acid esters, 100.5 ± 2.8 % for tocopherols, and 102.9 ± 5.1 % for free fatty acids. Repeatability was determined by 10-fold working up of 3 oil samples at two different days, respectively. The low relative standard deviations of the total contents determined in rapeseed oil, sunflower oil, and corn germ oil indicated a good repeatability of the applied approach: 2.5, 3.1, and 5.3 % for free sterols/stanols, respectively; 2.5, 5.4, and 2.6 % for steryl/stanyl fatty acid

esters respectively; 3.0, 1.8, and 6.7 % for tocopherols, respectively; 9.5, 7.0, and 7.0 % for squalene, respectively; 7.8, 7.4, and 10.7 % for free fatty acids; respectively.

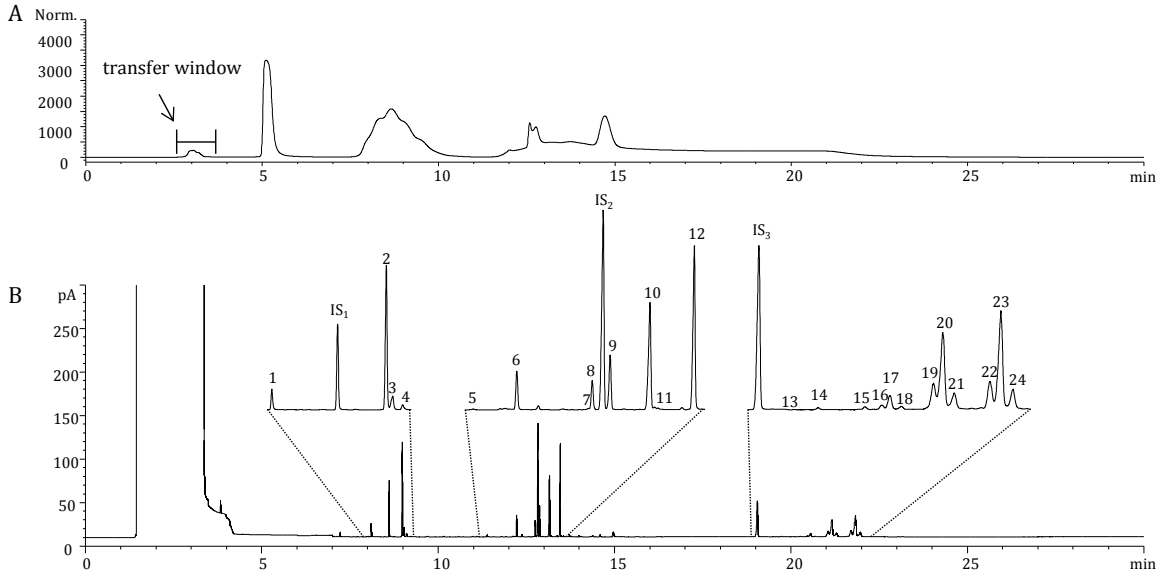

Figure 36. On-line LC-GC analysis of free fatty acids, tocopherols, free sterols, and steryl fatty acid esters in rapeseed oil. (**A**) LC-UV chromatogram at 205 nm and (**B**) GC-FID chromatogram of the transferred LC fraction.
(1) palmitic acid, (2) linoleic + oleic acid, (3) linolenic acid, (4) stearic acid, (5) δ-tocopherol, (6) γ-tocopherol, (7) cholesterol, (8) α-tocopherol, (9) brassicasterol, (10) campesterol, (11) stigmasterol, (12) sitosterol, (13) brassicasteryl-16:0/16:1, (14) campesteryl-16:0/16:1, (15) sitosteryl-16:0/16:1, (16) brassicasteryl-18:0/18:1, (17) brassicasteryl-18:2, (18) brassicasteryl-18:3, (19) campesteryl-18:0/18:1, (20) campesteryl-18:2, (21) campesteryl-18:3, (22) sitosteryl-18:0/18:1, (23) sitosteryl-18:2, (24) sitosteryl-18:3, (IS_1) heptadecanoic acid, (IS_2) 5α-cholestan-3β-ol, and (IS_3) cholesteryl-16:0.

4.7.2 Compositions and Contents of Free Sterols/Stanols and Steryl/Stanyl Esters

Free Sterols/Stanols

Even though sitosterol was consistently predominant in all samples, considerable variances were detected between the different oil types. However, the distribution patterns of free sterols/stanols within a certain type of oil were almost comparable. The determined total contents and compositions of free sterols/stanols in the rapeseed, sunflower, and olive oils are presented in Table 31.

Sitosterol accounted for 47.1-51.9 % in rapeseed oil, followed by campesterol (29.9-35.1 %), and brassicasterol (14.3-16.7 %); the last-mentioned sterol could only be detected in rapeseed oil. The observed profiles agree with earlier reported data (Kalo and Kuuranne, 2001; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a).

In sunflower oils, sitosterol represented 57.9-64.2 % of total free sterols/stanols, followed by stigmasterol (9.6-10.8 %), and campesterol (8.2-9.7 %). Additionally, comparatively high percentages of Δ^7 sitosterol and 4,4'-dimethylsterols were detected. Those made up at least

4.5 % and 8.0 % of total free sterols/stanols, respectively. The compositions of free sterols/stanols detected in the sunflower oils generally agree with previously described results, but Δ^7 sitosterol and 4,4'-dimethylsterols have not been analyzed (Phillips *et al.*, 2002; Verleyen *et al.*, 2002a). Kalo and Kuuranne (2001) detected 5.4 % Δ^7 sitosterol within the fraction of free sterols of a single sunflower oil sample, but 4,4'-dimethylsterols have also not been included in the analysis.

The 7 investigated olive oils were mainly composed of sitosterol (56.3-72.9 %); their high levels of 24-methylene cycloartanol and cycloartenol, which represented together 15.2-34.3 % of total free sterols/stanols are remarkable. Most studies on free sterols/stanols in olive oils did not report amounts for 4,4'-dimethylsterols (Choong *et al.*, 1999; Pasqualone and Catalano, 2000; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a; Cunha *et al.*, 2006). However, Grob *et al*. (1990) detected cycloartenol as second most abundant free sterol in several olive oils. Another study analyzed the composition of free 4,4'-dimethylsterols in two olive oils, and detected 24-methylene cycloartanol and cycloartenol as major constituents (Azadmard-Damirchi and Dutta, 2007).

The contents and compositions of free sterols/stanols quantified in the corn germ, grape seed, linseed, safflower, sesame seed, and soybean oils are summarized in Table 32. Both corn germ oils exhibited comparably high percentages of campesterol (~24 %). The amount of cycloartenol was particularly high in linseed oil (28.5 %); safflower oil, in turn, showed high levels of Δ^7 sitosterol (17.4 %) and Δ^7 campesterol (4.3 %). The sesame seed oil consisted mainly of sitosterol (70.3 %), followed by campesterol, and stigmasterol. The soybean oil was remarkable regarding its high amount of stigmasterol, representing 18.2 % of total free sterols/stanols. The profiles of the corn germ, grape seed, safflower, sesame seed, and soybean oils are almost similar to previously published data, but comparison of results is partially different as most studies have not reported amounts of 4,4'-dimethylsterols (Choong *et al.*, 1999; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a; Dulf *et al.*, 2010).

Total contents of free sterols/stanols averaged 271.5 ± 28.2 µg/100 mg in rapeseed oil, 241.8 ± 33.6 µg/100 mg in sunflower oils, and 152.3 ± 17.9 µg/100 mg in olive oils. The other oils exhibited total sterol/stanol levels in the range of 141.4-363.4 µg/100 mg, being the highest in corn germ oil and the lowest in grape seed oil. Total amounts of free sterols/stanols quantified in most oils are in the same order of magnitude as earlier reported (Johansson and Appelqvist, 1978; Worthington and Hitchcock, 1984; Choong *et al.*, 1999; Lechner *et al.*, 1999; Pasqualone and Catalano, 2000; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a). Only Choong *et al*. (1999) and Grob *et al*. (1990) detected slightly lower free sterol/stanol levels in olive oils. Furthermore, the content of free sterols/stanols quantified in the sesame seed oil was lower than amounts reported by Choong *et al*. (1999).

Table 31. Contents and compositions of free sterols/stanols in rapeseed, sunflower, and olive oils.

	no.	∑ [µg/100 mg oil]	sitosterol [%]	campesterol [%]	brassicasterol [%]	stigmasterol [%]	cholesterol [%]	Δ^7 sitosterol [%]	sitostanol [%]
rapeseed oil	1	271.1 ± 7.7	50.6 ± 0.1	33.8 ± 0.1	14.3 ± 0.1	1.0 ± 0.1	0.4 ± 0.0	-	-
	2	231.4 ± 1.9	50.1 ± 0.1	33.6 ± 0.1	14.7 ± 0.1	1.3 ± 0.1	0.3 ± 0.0	-	-
	3	249.7 ± 6.7	50.2 ± 0.0	33.6 ± 0.0	14.9 ± 0.0	1.0 ± 0.0	0.3 ± 0.0	-	-
	4	234.7 ± 1.5	50.6 ± 0.0	33.8 ± 0.1	14.3 ± 0.1	1.0 ± 0.1	0.3 ± 0.0	-	-
	5	249.4 ± 3.6	50.0 ± 0.1	33.8 ± 0.0	14.7 ± 0.0	1.1 ± 0.1	0.3 ± 0.0	-	-
	6	266.5 ± 3.4	50.4 ± 0.1	32.2 ± 0.2	16.1 ± 0.1	0.9 ± 0.1	0.3 ± 0.0	-	-
	7	284.3 ± 4.2	49.0 ± 0.0	33.0 ± 0.1	16.7 ± 0.1	1.0 ± 0.1	0.4 ± 0.0	-	-
	8	259.9 ± 4.7	49.2 ± 0.1	34.6 ± 0.1	14.8 ± 0.1	1.0 ± 0.1	0.4 ± 0.0	-	-
	9	275.8 ± 1.4	50.0 ± 0.1	32.8 ± 0.0	16.0 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	-	-
	10	275.4 ± 10.0	49.7 ± 0.1	34.0 ± 0.2	14.6 ± 0.0	1.4 ± 0.1	0.3 ± 0.0	-	-
	11	262.6 ± 1.9	48.2 ± 0.1	34.7 ± 0.1	16.2 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	-	-
	12	325.0 ± 2.9	48.3 ± 0.1	35.1 ± 0.0	15.6 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	-	-
	13	285.4 ± 1.6	51.9 ± 0.1	29.9 ± 0.0	16.5 ± 0.1	1.5 ± 0.1	0.4 ± 0.0	-	-
	14	268.4 ± 10.8	48.1 ± 0.3	34.7 ± 0.3	15.7 ± 0.1	1.3 ± 0.0	0.3 ± 0.0	-	-
	15	332.3 ± 2.0	47.1 ± 0.0	34.2 ± 0.1	16.7 ± 0.1	1.5 ± 0.1	0.4 ± 0.0	-	-
sunflower oil	1	197.2 ± 2.4	61.5 ± 0.5	9.3 ± 0.1	-[a]	9.6 ± 0.1	-	4.5 ± 0.3	2.1 ± 0.1
	2	233.7 ± 1.4	59.5 ± 0.1	9.0 ± 0.0	-	10.7 ± 0.1	-	6.5 ± 0.1	1.0 ± 0.1
	3	230.4 ± 5.3	61.7 ± 0.2	9.7 ± 0.0	-	10.8 ± 0.1	-	4.9 ± 0.2	1.5 ± 0.0
	4	173.6 ± 5.3	64.2 ± 0.2	9.6 ± 0.1	-	10.1 ± 0.2	-	4.5 ± 0.1	2.7 ± 0.1
	5	247.8 ± 2.3	58.2 ± 0.2	8.7 ± 0.1	-	10.4 ± 0.1	-	7.1 ± 0.1	1.2 ± 0.0
	6	267.2 ± 1.9	58.5 ± 0.5	8.2 ± 0.1	-	10.4 ± 0.1	-	7.8 ± 0.0	1.3 ± 0.0
	7	258.7 ± 5.5	59.0 ± 0.4	9.3 ± 0.1	-	10.2 ± 0.0	-	7.0 ± 0.1	1.2 ± 0.1
	8	268.4 ± 8.9	58.5 ± 0.2	9.3 ± 0.1	-	10.0 ± 0.1	-	6.8 ± 0.1	1.3 ± 0.0
	9	269.3 ± 1.1	58.6 ± 0.1	8.3 ± 0.1	-	9.6 ± 0.2	-	6.6 ± 0.0	1.6 ± 0.0
	10	271.5 ± 2.0	57.9 ± 0.3	8.4 ± 0.0	-	10.1 ± 0.0	-	7.5 ± 0.0	1.3 ± 0.1
olive oil	1	169.8 ± 2.2	64.3 ± 0.1	2.7 ± 0.0	-	0.6 ± 0.0	-	0.6 ± 0.0	0.6 ± 0.0
	2	149.5 ± 1.0	61.9 ± 0.2	2.7 ± 0.1	-	0.8 ± 0.0	-	0.7 ± 0.0	0.8 ± 0.0
	3	154.9 ± 1.4	64.1 ± 0.1	2.9 ± 0.1	-	0.8 ± 0.0	-	0.9 ± 0.0	1.0 ± 0.1
	4	135.3 ± 5.1	72.9 ± 0.3	3.8 ± 0.1	-	0.9 ± 0.1	-	0.8 ± 0.1	0.9 ± 0.1
	5	125.3 ± 4.7	64.8 ± 1.2	3.4 ± 0.0	-	1.0 ± 0.0	-	0.8 ± 0.1	1.0 ± 0.1
	6	155.0 ± 3.3	56.3 ± 0.1	2.4 ± 0.0	-	0.9 ± 0.1	-	0.6 ± 0.0	0.9 ± 0.0
	7	176.1 ± 0.7	59.2 ± 0.0	2.3 ± 0.0	-	0.7 ± 0.0	-	0.6 ± 0.0	0.7 ± 0.0

Table 31. continued.

	no.	24-meth.cycl.[b] [%]	cycloartenol [%]	Δ5 avenasterol [%]	clerosterol [%]	β-amyrin [%]	others [%]
rapeseed oil	1	-	-	-	-	-	-
	2	-	-	-	-	-	-
	3	-	-	-	-	-	-
	4	-	-	-	-	-	-
	5	-	-	-	-	-	-
	6	-	-	-	-	-	-
	7	-	-	-	-	-	-
	8	-	-	-	-	-	-
	9	-	-	-	-	-	-
	10	-	-	-	-	-	-
	11	-	-	-	-	-	-
	12	-	-	-	-	-	-
	13	-	-	-	-	-	-
	14	-	-	-	-	-	-
	15	-	-	-	-	-	-
sunflower oil	1	5.8 ± 0.2	3.7 ± 0.2	-	-	-	3.5 ± 0.1
	2	8.6 ± 0.1	4.4 ± 0.0	-	-	-	0.2 ± 0.0
	3	6.7 ± 0.4	3.8 ± 0.1	-	-	-	1.0 ± 0.0
	4	4.5 ± 0.1	3.5 ± 0.0	-	-	-	0.8 ± 0.1
	5	9.3 ± 0.1	4.8 ± 0.1	-	-	-	0.3 ± 0.0
	6	9.5 ± 0.4	4.0 ± 0.1	-	-	-	0.5 ± 0.2
	7	9.3 ± 0.3	3.9 ± 0.1	-	-	-	0.2 ± 0.0
	8	10.1 ± 0.0	3.7 ± 0.1	-	-	-	0.4 ± 0.0
	9	11.1 ± 0.1	3.8 ± 0.0	-	-	-	0.4 ± 0.1
	10	9.9 ± 0.0	4.7 ± 0.2	-	-	-	0.3 ± 0.0
olive oil	1	23.9 ± 0.2	3.3 ± 0.1	0.9 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	1.7 ± 0.0
	2	19.0 ± 0.1	9.0 ± 0.1	1.2 ± 0.0	0.8 ± 0.0	1.2 ± 0.0	1.7 ± 0.0
	3	19.8 ± 0.1	4.2 ± 0.0	1.2 ± 0.0	0.9 ± 0.0	1.5 ± 0.1	2.5 ± 0.1
	4	12.6 ± 0.1	2.6 ± 0.0	1.4 ± 0.0	1.0 ± 0.0	1.6 ± 0.1	1.5 ± 0.0
	5	18.4 ± 1.2	4.9 ± 0.1	1.7 ± 0.1	0.9 ± 0.0	1.4 ± 0.1	1.7 ± 0.1
	6	16.6 ± 0.0	17.7 ± 0.0	1.2 ± 0.0	0.8 ± 0.0	1.3 ± 0.0	1.3 ± 0.0
	7	29.2 ± 0.1	2.8 ± 0.1	1.2 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	1.8 ± 0.0

[a] (-) Content below limit of detection (Table 14 and 0.01 µg/mL i.v. (calculated on the basis of 10 µL i.v). [b] 24-Methylene cycloartanol.

Table 32. Contents and compositions of free sterols/stanols in corn germ, grape seed, linseed, safflower, sesame seed, and soybean oils.

	no.	∑ [µg/100 mg oil]	sitosterol [%]	campesterol [%]	stigmasterol [%]	cholesterol [%]	Δ7 sitosterol [%]	sitostanol [%]	24-meth.cycl.[b] [%]
corn germ oil	1	363.4 ± 3.1	63.3 ± 0.2	24.5 ± 0.1	7.5 ± 0.2	-[a]	1.2 ± 0.0	2.5 ± 0.0	-
	2	278.4 ± 9.2	64.1 ± 1.2	24.2 ± 1.1	7.4 ± 0.1	-	-	3.2 ± 0.1	-
grape seed oil		141.4 ± 1.2	48.5 ± 0.3	14.7 ± 0.1	8.4 ± 0.1	-	3.0 ± 0.1	4.2 ± 0.1	4.3 ± 0.1
linseed oil		326.8 ± 8.9	34.8 ± 0.1	18.3 ± 0.0	8.1 ± 0.0	0.3 ± 0.0	-	1.2 ± 0.0	0.5 ± 0.0
safflower oil		218.4 ± 5.3	37.0 ± 0.5	9.3 ± 0.0	6.5 ± 0.1	0.3 ± 0.0	17.4 ± 0.1	4.6 ± 0.2	1.7 ± 0.1
sesame seed oil		341.8 ± 7.7	70.3 ± 0.1	14.0 ± 0.1	7.7 ± 0.0	-	1.2 ± 0.0	1.9 ± 0.0	2.0 ± 0.0[c]
soybean oil		168.2 ± 2.0	44.6 ± 0.5	17.7 ± 0.2	18.2 ± 0.1	-	2.8 ± 0.1	3.3 ± 0.1	3.4 ± 0.2

Table 32. continued.

	no.	cycloartenol [%]	Δ5 avenasterol [%]	clerosterol [%]	β-amyrin [%]	campestanol [%]	Δ7 campesterol [%]	others [%]
corn germ oil	1	-	-	0.9 ± 0.0	-	-	-	-
	2	-	-	1.1 ± 0.1	-	-	-	-
grape seed oil		2.8 ± 0.0	1.2 ± 0.0	2.2 ± 0.1	7.2 ± 0.1	-	-	3.6 ± 0.1
linseed oil		28.5 ± 0.2	3.2 ± 0.0	0.9 ± 0.0	1.6 ± 0.1	-	-	2.5 ± 0.1
safflower oil		1.9 ± 0.1	3.9 ± 0.1	-	3.8 ± 0.2	1.2 ± 0.1	4.3 ± 0.2	8.1 ± 0.2
sesame seed oil		0.7 ± 0.0	-	1.3 ± 0.0	-	-	-	0.9 ± 0.0
soybean oil		1.4 ± 0.0	1.0 ± 0.1	-	2.6 ± 0.1[d]	1.6 ± 0.1	-	3.5 ± 0.2

[a] (-) Content below limit of detection (Table 14 and 0.01 µg/mL i.v. (calculated on the basis of 10 µL i.v). [b] 24-Methylene cycloartanol. [c] Coelution with citrostadienol. [d] Coelution with cycloartanol.

Steryl/Stanyl Fatty Acid Esters

Up to 28 individual steryl/stanyl fatty acid esters were identified in the different edible plant oils, but only 4 of them were detected in all oil samples; i.e. sitosteryl-18:2, sitosteryl-18:0/18:1, campesteryl-18:0/18:1, and sitosteryl-16:0/16:1 (Table 33 and Table 34). Each kind of oil showed a very distinct distribution pattern, whereas only little variation was observed within a certain type of oil.

In all oils, the majority of the steryl/stanyl fatty acid esters occurred as sitosteryl esters of at least 39 % (sunflower oil no. 6). Thereof, sitosteryl-18:2 was the predominant ester in rapeseed, sunflower, corn germ, grape seed, linseed, sesame seed, and soybean oils. In contrast, in the olive oils and safflower oil not sitosteryl-18:2, but sitosteryl-18:0/18:1 was most abundant and accounted for 37.2-49.6 % and 37.3 % of total steryl/stanyl esters, respectively.

Besides sitosteryl esters, rapeseed oil fatty acid esters additionally consisted of campesteryl and brassicasteryl esters. Brassicasteryl esters were unique compounds of rapeseed oil and represented 8.2-10.3 % of total steryl fatty acid esters. Considering the fatty acid part, linoleic acid esters were dominating in rapeseed oil, followed by C18:3 and C18:0/18:1 esters (~19-25 % and ~16-23 %, respectively); C16 esters made up <1 %.

As mentioned before, sitosteryl esters were the main esters in sunflower oils, but these oils were also characteristic for their high amounts of Δ^7 sitosteryl and 4,4'-dimethylsteryl esters, representing 22.0-37.3 % and 10.7-20.6 % of total esters, respectively. C18:2 and C18:0/18:1 esters were predominant, and C16 esters only minor constituents (<2.3 %).

The compositions of steryl/stanyl fatty acids esters in the olive oils were characterized by a predominance of C18:0/18:1 esters, but also by particularly high percentages of 4,4'-dimethylsteryl esters (27.7-46.2 %). Additionally, the analyzed olive oils exhibited relatively high proportions of C16 esters (15.1-18.1 %), mainly cycloartenyl-16:0/16:1.

Both corn germ oils showed the highest proportions of sitosteryl-18:2 (>50 % of total esters) and were the only type of oil, in which stanyl esters could be detected. Two types of oils, grape seed and sesame seed oil, revealed very similar patterns of steryl/stanyl fatty acid esters with sitosteryl-18:2 and sitosteryl-18:0/18:1 as main esters, together accounting for about 65 %, respectively. The linseed oil was characteristic regarding its high amounts of C18:3 esters (40.5 % of total esters) and 4,4'-dimethylsteryl esters (37.1 % of total esters); the safflower oil, in turn, exhibited a high proportion of Δ^7 steryl esters (22.3 % of total esters), the soybean oil additionally high levels of cycloartenyl esters (13.8 %).

Again, comparison to literature is difficult as most studies did not analyze individual intact steryl/stanyl esters, but reported only the compositions of esterified sterols, which were determined after transesterification or saponification of the isolated steryl ester fractions (cf. 2.2.4). However, the data obtained in the present work regarding the sterol profiles of the

fatty acid esters are predominantly in agreement with previously reported results (Johansson and Appelqvist, 1978; Kalo and Kuuranne, 2001; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a; Cunha *et al.*, 2006; Dulf *et al.*, 2010). Some of those studies additionally detected esterified Δ^5 avenasterol and cholesterol, which could not be identified as intact esters in the oils examined in the present study. In turn, 4,4'-dimethylsterols were not analyzed in most of the cited studies. However, it could be shown that 4,4'-dimethylsteryl fatty acid esters represented a main part of the fatty acid esters in almost all investigated types of oils, but in particular in olive, sunflower, and linseed oils. Azadmard-Damirchi and Dutta (2007) quantified free and esterified 4,4'-dimethylsterols in olive oils and detected mainly cycloartenol and 24-methylene cycloartanol, which is in line with the present data. Intact steryl fatty acid esters in edible plant oils have previously been analyzed either by on-line LC-GC-based methods (Grob *et al.*, 1989; Grob *et al.*, 1990; Artho *et al.*, 1993; Grob *et al.*, 1994) or by isolation of the ester fractions via TLC or preparative HPLC and subsequent GC analysis (Gordon and Griffith, 1992; Gordon and Miller, 1997). For example, sitosteryl esters of C18 fatty acids have been detected in olive, rapeseed, grape seed, soybean, and sunflower oils (Grob *et al.*, 1990; Artho *et al.*, 1993; Grob *et al.*, 1994). Additionally, the presence of brassicasteryl-18 esters was reported for rapeseed oil or that of Δ^7 sitosteryl-18 esters in sunflower oils (Artho *et al.*, 1993; Grob *et al.*, 1994). However, no percentage distributions were given and no separation according to the fatty acid part could be achieved using the applied methods. Other studies characterized steryl esters peaks only on the basis of relative retention times (Gordon and Griffith, 1992; Gordon and Miller, 1997).

The distributions of esterified palmitic/palmitoleic acid and stearic/oleic acid were determined by GC-FID as fatty acid methyl esters after transesterification of the respective steryl/stanyl fatty acid ester fractions isolated via SPE. Two representative samples of the rapeseed, sunflower, and olive oils, the two corn germ oils as well as the single samples of grape seed, linseed, safflower, sesame seed, and soybean oil were analyzed.

A clear predominance of palmitic acid was observed in all oil samples. Palmitoleic acid could not be detected in the sunflower, linseed, safflower, sesame seed, and soybean oils. In the other kinds of oils, the amounts of palmitic acid were 20- to 105-fold higher than those of palmitoleic acid. Furthermore, the proportions of oleic acid within steryl/stanyl fatty acid esters were 2- to 15-fold above those of stearic acid. They were lowest in sunflower, olive, grape seed, linseed, sesame seed, and soybean oil with ratios of oleic/stearic acid in the range of 2:1 and 6:1, and highest in rapeseed, corn germ, and safflower oils with ratios between 8:1 and 15:1 (Appendix Table 37).

The total contents of steryl/stanyl fatty acid esters were by far the highest in the 15 rapeseed oils and 2 corn germ oils, averaging 893.8 ± 104.8 µg/100 mg and 873.1 ± 116.5 µg/100 mg,

respectively. The sunflower, grape seed, linseed, and sesame seed oils showed a range of approximately 200-380 µg/100 mg. Slightly lower levels were determined in the safflower and soybean oil with 180.8 and 152.0 µg/100 mg, respectively. The contents of steryl/stanyl fatty acid esters in the olive oils were <90 µg/100 mg. The amounts quantified in the rapeseed, olive, corn germ, and soybean oils are comparable to some earlier described data (Worthington and Hitchcock, 1984; Grob *et al.*, 1990; Ferrari *et al.*, 1997). Gordon and Miller (1997) reported lower total contents of steryl/stanyl fatty acid esters in rapeseed, sunflower, corn germ, safflower, and soybean oils; the amounts of olive oils are in line. In that study, steryl esters were also analyzed via GC-FID, but individual esters were calculated using an assumed response factor of one, which resulted in an underestimation of the contents.

Steryl/Stanyl Phenolic Acid Esters

Trans-derivatives of steryl/stanyl ferulic acid esters were only detected in the corn germ oils and comprised *trans*-sitostanyl ferulate (~50 %), *trans*-campestanyl ferulate (~35 %), and lower amounts of *trans*-sitosteryl ferulate and *trans*-campesteryl ferulate (each ~7 %). As expected, the total contents in both corn germ oils were lower than in whole corn kernel oils (cf. 4.2.1, Table 18) and amounted to 102.2 ± 5.0 µg/100 mg in oil no. 1 and 78.3 ± 3.0 µg/100 mg in oil no. 2. The levels are slightly higher than earlier reported results (Moreau, 2005; Moreau and Hicks, 2005).

Table 33. Contents and compositions of steryl/stanyl fatty acid esters in rapeseed, sunflower, and olive oils.

	no.	∑ [µg/100 mg oil]	sitosteryl-18:3 [%]	sitosteryl-18:2 [%]	sitosteryl-18:0/18:1 [%]	sitosteryl-16:0/16:1 [%]	campesteryl-18:3 [%]	campesteryl-18:2 [%]	campesteryl-18:0/18:1 [%]
rapeseed oil	1	920.9 ± 25.3	8.1 ± 0.3	30.9 ± 0.3	9.6 ± 0.2	0.4 ± 0.0	8.8 ± 0.5	23.5 ± 0.3	9.4 ± 0.3
	2	1018.6 ± 4.2	8.9 ± 0.1	29.8 ± 0.2	9.3 ± 0.1	0.4 ± 0.0	9.7 ± 0.1	23.1 ± 0.3	8.7 ± 0.2
	3	975.0 ± 1.8	9.6 ± 0.1	30.3 ± 0.1	8.9 ± 0.2	0.4 ± 0.0	9.9 ± 0.1	22.8 ± 0.2	8.2 ± 0.2
	4	1007.0 ± 63.4	9.1 ± 0.2	29.9 ± 0.1	9.9 ± 0.1	0.5 ± 0.0	8.9 ± 0.2	22.4 ± 0.2	9.3 ± 0.1
	5	1009.8 ± 10.5	9.4 ± 0.1	29.9 ± 0.2	8.5 ± 0.0	0.3 ± 0.1	10.0 ± 0.3	23.6 ± 0.1	8.8 ± 0.2
	6	831.1 ± 5.8	8.7 ± 0.3	30.9 ± 0.1	10.1 ± 0.1	0.2 ± 0.0	8.6 ± 0.1	23.4 ± 0.1	8.9 ± 0.1
	7	1050.2 ± 24.4	9.9 ± 0.2	29.8 ± 0.3	7.9 ± 0.2	0.3 ± 0.0	10.3 ± 0.1	23.3 ± 0.0	8.1 ± 0.1
	8	911.8 ± 9.7	10.0 ± 0.1	28.5 ± 0.2	7.9 ± 0.1	0.3 ± 0.0	11.9 ± 0.2	24.4 ± 0.2	8.0 ± 0.1
	9	921.1 ± 26.4	10.6 ± 0.2	30.5 ± 0.2	7.6 ± 0.2	0.3 ± 0.0	10.9 ± 0.2	23.8 ± 0.3	6.8 ± 0.2
	10	849.4 ± 32.5	8.4 ± 0.2	31.4 ± 0.1	9.7 ± 0.1	0.3 ± 0.0	8.9 ± 0.2	23.7 ± 0.2	9.3 ± 0.1
	11	848.3 ± 9.3	10.0 ± 0.1	27.7 ± 0.3	8.7 ± 0.0	0.2 ± 0.0	11.3 ± 0.3	23.7 ± 0.3	8.6 ± 0.1
	12	778.5 ± 15.6	9.7 ± 0.1	29.6 ± 0.1	8.3 ± 0.0	0.2 ± 0.0	10.6 ± 0.3	25.0 ± 0.3	7.8 ± 0.2
	13	700.5 ± 24.8	9.0 ± 0.1	32.3 ± 0.1	12.2 ± 0.1	0.3 ± 0.0	7.7 ± 0.1	19.8 ± 0.1	9.1 ± 0.1
	14	797.0 ± 19.7	9.7 ± 0.2	28.7 ± 0.2	7.9 ± 0.1	0.2 ± 0.0	11.8 ± 0.1	24.2 ± 0.3	8.0 ± 0.1
	15	788.5 ± 16.1	8.6 ± 0.2	32.0 ± 0.2	8.7 ± 0.1	0.3 ± 0.0	8.6 ± 0.2	24.7 ± 0.1	8.0 ± 0.0
sunflower oil	1	335.4 ± 17.8	-[a]	40.9 ± 0.7	12.9 ± 0.6	0.4 ± 0.0	-	3.0 ± 0.1[b]	1.0 ± 0.1
	2	246.2 ± 6.1	-	30.8 ± 0.4	16.8 ± 0.5	0.3 ± 0.0	-	1.9 ± 0.0[b]	0.7 ± 0.1
	3	223.3 ± 5.5	-	24.1 ± 0.6	17.4 ± 0.6	0.4 ± 0.0	-	2.8 ± 0.2[b]	0.9 ± 0.1
	4	295.3 ± 4.9	-	44.9 ± 0.4	13.5 ± 0.4	0.5 ± 0.0	-	3.0 ± 0.1[b]	1.1 ± 0.0
	5	244.7 ± 5.1	-	28.7 ± 0.8	16.3 ± 0.4	0.3 ± 0.0	-	1.9 ± 0.1[b]	0.9 ± 0.0
	6	233.5 ± 2.0	-	24.7 ± 0.4	14.5 ± 0.7	0.2 ± 0.0	-	1.7 ± 0.1[b]	0.8 ± 0.1
	7	227.8 ± 4.1	-	26.3 ± 0.5	16.4 ± 0.5	0.3 ± 0.0	-	2.7 ± 0.1[b]	1.4 ± 0.1
	8	228.8 ± 2.6	-	23.4 ± 0.3	17.0 ± 0.1	0.4 ± 0.0	-	2.8 ± 0.0[b]	1.3 ± 0.0
	9	217.1 ± 7.8	-	28.0 ± 0.5	15.1 ± 0.3	0.4 ± 0.0	-	1.7 ± 0.0[b]	0.7 ± 0.0
	10	235.5 ± 7.8	-	26.7 ± 0.6	17.0 ± 0.4	0.2 ± 0.0	-	1.8 ± 0.1[b]	0.8 ± 0.1
olive oil	1	86.9 ± 6.3	-	20.7 ± 0.4	40.9 ± 0.5	1.9 ± 0.1	-	-	1.2 ± 0.1
	2	63.5 ± 1.4	-	16.9 ± 0.2	42.0 ± 0.7	2.1 ± 0.0	-	-	1.5 ± 0.1
	3	69.0 ± 1.3	-	15.2 ± 0.3	45.7 ± 0.8	2.1 ± 0.1	-	-	1.6 ± 0.1
	4	68.0 ± 3.5	-	18.5 ± 0.2	49.6 ± 0.6	2.7 ± 0.0	-	-	1.7 ± 0.1
	5	56.0 ± 2.7	-	18.3 ± 0.4	42.9 ± 0.6	2.7 ± 0.1	-	-	1.9 ± 0.1
	6	57.4 ± 1.5	-	17.2 ± 0.3	42.1 ± 0.1	2.4 ± 0.1	-	-	1.8 ± 0.0
	7	85.0 ± 3.4	-	13.4 ± 0.3	37.2 ± 0.4	1.7 ± 0.0	-	-	1.5 ± 0.0

Table 33. continued.

	no.	campesteryl-16:0/16:1 [%]	brassicasteryl-18:3 [%]	brassicasteryl-18:2 [%]	brassicasteryl-18:0/18:1 [%]	brassicasteryl-16:0/16:1 [%]	stigmasteryl-18:2 [%]	stigmasteryl-16:0/16:1 [%][d]	Δ^7 sitosteryl-18:2 [%][e]
rapeseed oil	1	0.12 ± 0.01	2.2 ± 0.0	5.0 ± 0.1	2.0 ± 0.0	0.10 ± 0.01	-	-	-
	2	0.21 ± 0.01	2.7 ± 0.1	5.1 ± 0.0	2.0 ± 0.0	0.13 ± 0.01	-	-	-
	3	0.15 ± 0.01	2.7 ± 0.0	5.1 ± 0.0	1.9 ± 0.0	0.13 ± 0.01	-	-	-
	4	0.25 ± 0.03	2.4 ± 0.1	5.0 ± 0.1	2.3 ± 0.1	0.19 ± 0.01	-	-	-
	5	0.10 ± 0.01	2.8 ± 0.1	5.0 ± 0.0	1.5 ± 0.0	0.09 ± 0.01	-	-	-
	6	<LOQ[c]	2.6 ± 0.0	4.7 ± 0.0	1.8 ± 0.0	0.06 ± 0.01			
	7	0.12 ± 0.01	3.3 ± 0.1	5.4 ± 0.0	1.5 ± 0.0	0.07 ± 0.01	-	-	-
	8	0.07 ± 0.00	3.1 ± 0.2	4.5 ± 0.0	1.3 ± 0.0	0.07 ± 0.01	-	-	-
	9	0.05 ± 0.00	3.0 ± 0.1	5.0 ± 0.0	1.3 ± 0.1	0.09 ± 0.00	-	-	-
	10	0.07 ± 0.01	2.0 ± 0.0	4.3 ± 0.0	1.6 ± 0.0	0.26 ± 0.04	-	-	-
	11	0.06 ± 0.01	3.2 ± 0.1	4.7 ± 0.0	1.7 ± 0.0	0.13 ± 0.01	-	-	-
	12	0.10 ± 0.00	2.7 ± 0.1	4.7 ± 0.1	1.3 ± 0.0	0.11 ± 0.00	-	-	-
	13	0.03 ± 0.00	2.5 ± 0.1	4.9 ± 0.0	2.0 ± 0.0	0.09 ± 0.00	-	-	-
	14	0.01 ± 0.00	3.2 ± 0.0	4.8 ± 0.0	1.4 ± 0.0	0.17 ± 0.01	-	-	-
	15	0.03 ± 0.00	2.5 ± 0.1	5.1 ± 0.0	1.4 ± 0.1	0.09 ± 0.01	-	-	-
sunflower oil	1	0.5 ± 0.0	-	-	-	-	2.9 ± 0.2	0.8 ± 0.1	19.3 ± 0.9
	2	0.5 ± 0.0	-	-	-	-	2.3 ± 0.2	0.9 ± 0.1	21.5 ± 0.6
	3	0.5 ± 0.0	-	-	-	-	2.8 ± 0.2	0.9 ± 0.1	24.1 ± 0.6
	4	0.5 ± 0.0	-	-	-	-	3.2 ± 0.0	0.6 ± 0.0	15.9 ± 0.6
	5	0.4 ± 0.0	-	-	-	-	2.7 ± 0.1	0.9 ± 0.0	23.1 ± 0.3
	6	0.3 ± 0.0	-	-	-	-	2.5 ± 0.0	0.6 ± 0.1	27.2 ± 0.4
	7	1.0 ± 0.0	-	-	-	-	2.4 ± 0.1	1.0 ± 0.1	20.6 ± 0.7
	8	0.4 ± 0.0	-	-	-	-	2.6 ± 0.0	0.9 ± 0.0	23.4 ± 0.3
	9	0.6 ± 0.0	-	-	-	-	2.1 ± 0.1	1.0 ± 0.1	21.9 ± 0.9
	10	0.3 ± 0.0	-	-	-	-	2.4 ± 0.1	0.6 ± 0.0	21.2 ± 0.3
olive oil	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-

Table 33. continued.

	no.	Δ^7 sitosteryl-18:0/18:1 [%][f]	cycloartenyl 18:2 [%][d]	cycloartenyl 18:0/18:1 [%][d]	cycloartenyl 16:0/16:1 [%][d]	24-meth.cycl.[g] 18:2 [%][d]	24-meth.cycl.[g] 18:0/18:1 [%][d]	24-meth.cycl.[g] 16:0/16:1 [%][d]	
rapeseed oil	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-
	13	-	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-	-
sunflower oil	1	6.3 ± 0.2	9.8 ± 0.6	2.3 ± 0.1	-	-	-	-	-
	2	7.6 ± 0.2	13.5 ± 0.5	3.2 ± 0.2	-	-	-	-	-
	3	7.1 ± 0.2	15.6 ± 0.5	3.3 ± 0.2	-	-	-	-	-
	4	6.1 ± 0.1	8.6 ± 0.5	2.1 ± 0.1	-	-	-	-	-
	5	7.3 ± 0.2	14.1 ± 0.4	3.4 ± 0.2	-	-	-	-	-
	6	10.1 ± 0.2	13.5 ± 0.2	3.8 ± 0.1	-	-	-	-	-
	7	10.8 ± 0.3	12.8 ± 0.2	4.3 ± 0.2	-	-	-	-	-
	8	7.8 ± 0.2	16.2 ± 0.5	3.8 ± 0.0	-	-	-	-	-
	9	7.9 ± 0.2	16.5 ± 0.9	4.1 ± 0.2	-	-	-	-	-
	10	8.9 ± 0.3	15.5 ± 0.2	4.6 ± 0.0	-	-	-	-	-
olive oil	1	-	3.2 ± 0.3	4.7 ± 0.3	10.0 ± 0.4	6.3 ± 0.2	7.9 ± 0.1	3.2 ± 0.1	-
	2	-	4.0 ± 0.1	6.1 ± 0.3	11.2 ± 0.2	5.8 ± 0.2	7.4 ± 0.2	3.1 ± 0.1	-
	3	-	3.8 ± 0.1	6.2 ± 0.4	10.5 ± 0.2	4.2 ± 0.2	7.7 ± 0.2	3.1 ± 0.1	-
	4	-	3.3 ± 0.2	4.7 ± 0.1	12.1 ± 0.3	2.9 ± 0.1	3.4 ± 0.1	1.3 ± 0.0	-
	5	-	4.8 ± 0.5	6.3 ± 0.1	13.5 ± 0.2	3.5 ± 0.1	4.2 ± 0.0	1.9 ± 0.1	-
	6	-	4.4 ± 0.3	6.2 ± 0.3	12.2 ± 0.3	4.5 ± 0.2	6.5 ± 0.1	2.7 ± 0.2	-
	7	-	3.7 ± 0.2	5.5 ± 0.1	10.4 ± 0.3	8.5 ± 0.1	13.8 ± 0.2	4.3 ± 0.1	-

[a] (-) Content below limit of detection (Table 14 and 0.08 µg/mL i.v. (calculated on the basis of 10 µL i.v)). [b] Coelution with stigmasteryl-18:0/18:1. [c] Content below limit of quantification (Table 14 and 0.17 µg/mL i.v. (calculated on the basis of 10 µL i.v)). [d] Compound calculated with Rf = 1. [e] Compound quantified via calibration of sitosteryl-18:2. [f] Compound quantified via calibration of sitosteryl-18:0/18:1. [g] 24-Methylene cycloartanyl.

Table 34. Contents and compositions of steryl/stanyl fatty acid esters in corn germ, grape seed, linseed, safflower, sesame seed, and soybean oils.

	no.	∑ [µg/100 mg oil]	sitosteryl-18:3 [%]	sitosteryl-18:2 [%]	sitosteryl-18:0/18:1 [%]	sitosteryl-16:0/16:1 [%]	campesteryl-18:3 [%]	campesteryl-18:2 [%]	campesteryl-18:0/18:1 [%]
corn germ oil	1	790.7 ± 18.4	-[a]	51.9 ± 0.4	14.9 ± 0.1	2.7 ± 0.0	-	13.3 ± 0.0[c]	4.6 ± 0.2
	2	955.5 ± 17.3	-	53.9 ± 0.5	13.7 ± 0.4	2.5 ± 0.1	-	13.3 ± 0.1[c]	4.7 ± 0.1
grape seed oil		199.9 ± 3.3	8.6 ± 0.4	46.4 ± 0.4	18.7 ± 0.5	1.7 ± 0.0	5.3 ± 0.2[b]	4.9 ± 0.2[c]	0.9 ± 0.0
linseed oil		350.5 ± 17.3	16.5 ± 0.5	22.7 ± 0.2	9.6 ± 0.5	0.3 ± 0.0	10.0 ± 0.3[b]	3.4 ± 0.1[c]	<LOQ[d]
safflower oil		180.8 ± 7.4	-	25.1 ± 0.1	37.3 ± 0.3	0.5 ± 0.0	-	4.8 ± 0.3[c]	4.9 ± 0.1
sesame seed oil		377.8 ± 5.3	6.5 ± 0.3	43.0 ± 0.3	22.8 ± 0.1	0.9 ± 0.0	5.4 ± 0.1[b]	8.4 ± 0.1[c]	3.2 ± 0.1
soybean oil		152.0 ± 2.9	7.3 ± 0.1	38.8 ± 0.7	12.3 ± 0.3	1.5 ± 0.0	-	4.0 ± 0.1[c]	2.4 ± 0.0

Table 34. continued.

	no.	campesteryl-16:0/16:1 [%]	sitostanyl-18:2 [%]	sitostanyl-16:0/16:1 [%]	stigmasteryl-18:2 [%]	stigmasteryl-16:0/16:1 [%][e]	Δ7 sitosteryl-18:2 [%][f]	Δ7 sitosteryl-18:0/18:1 [%][g]	cycloartenyl 18:3 [%][e]
corn germ oil	1	0.9 ± 0.0	3.8 ± 0.1	0.2 ± 0.0	7.1 ± 0.0	0.6 ± 0.0	-	-	-
	2	0.8 ± 0.0	4.1 ± 0.3	0.2 ± 0.0	6.4 ± 0.1	0.5 ± 0.0	-	-	-
grape seed oil		0.8 ± 0.1	-	-	-	1.0 ± 0.1	-	-	-
linseed oil		0.4 ± 0.1	-	-	-	-	-	-	8.8 - 0.2
safflower oil		-	-	-	5.2 ± 0.0	-	11.4 ± 0.4	10.9 ± 0.2	-
sesame seed oil		1.2 ± 0.0	-	-	-	0.4 ± 0.0	-	-	-
soybean oil		1.2 ± 0.1	-	-	5.8 ± 0.3	1.5 ± 0.3	11.4 ± 0.3	<LOQ	-

Table 34. continued.

	no.	cycloartenyl 18:2 [%][e]	cycloartenyl 18:0/18:1 [%][e]	cycloartenyl 16:0/16:1 [%][e]	24-meth.cycl.[h] 18:3 [%][e]	24-meth.cycl.[h] 18:2 [%][e]	24-meth.cyl.[h] 18:0/18:1 [%][e]
corn germ oil	1	-	-	-	-	-	-
	2	-	-	-	-	-	-
grape seed oil		3.1 ± 0.1	-	3.1 ± 0.3	-	5.3 ± 0.2	-
linseed oil		13.2 ± 0.2	2.0 ± 0.2	-	5.2 ± 0.1	6.6 ± 0.1	1.3 ± 0.1
safflower oil		-	-	-	-	-	-
sesame seed oil		4.5 ± 0.2	1.4 ± 0.1	-	-	1.5 ± 0.1	0.9 ± 0.1
soybean oil		11.4 ± 0.3	<LOQ	2.4 ± 0.1	-	-	-

[a] (-) Content below limit of detection (Table 14 and 0.07 µg/mL i.v. (calculated on the basis of 10 µL i.v)). [b] Coelution with stigmasteryl-18:2. [c] Coelution with stigmasteryl-18:0/18:1. [d] Content below limit of quantification (Table 14 and 0.17 µg/mL i.v. (calculated on the basis of 10 µL i.v)). [e] Compound calculated with Rf = 1. [f] Compound quantified via calibration of sitosteryl-18:2. [g] Compound quantified via calibration of sitosteryl-18:0/18:1. [h] 24-Methylene cycloartanyl.

4.7.3 Distribution Patterns of Free and Esterified Sterols/Stanols

The distribution patterns of those sterols/stanols which occurred free and esterified to fatty acid showed partially obvious differences. However, the observations were not automatically transferable to all kinds of oils. For instance, sitosterol was found to a higher degree within the fraction of steryl/stanyl fatty acid esters in soybean, safflower, corn germ, grape seed, sesame seed, and linseed oils, but not in rapeseed, olive and sunflower oils. Similar percentages of free and esterified sitosterol were detected in rapeseed and olive oils; sunflower oils exhibited a higher proportion of free sitosterol. These observations were predominantly comparable to earlier reported results, which determined the compositions of free and esterified sterols (Johansson, 1979; Johansson and Hoffmann, 1979; Kalo and Kuuranne, 2001; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a; Verleyen *et al.*, 2002b; Cunha *et al.*, 2006; Dulf *et al.*, 2010). Stigmasterol, as another example, was mainly or exclusively found in free form in all analyzed oil samples. Previous studies also described a predominance of free over esterified stigmasterol in several oils samples (Johansson, 1979; Johansson and Hoffmann, 1979; Kalo and Kuuranne, 2001; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a; Verleyen *et al.*, 2002b; Cunha *et al.*, 2006). Brassicasterol, the characteristic sterol of the rapeseed oils, was more abundant in free than in esterified form (~16 versus ~9 %), which also agrees with earlier data (Johansson and Appelqvist, 1978; Kalo and Kuuranne, 2001; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a). Δ^7 Sitosterol was found to a higher degree within the fraction of steryl/stanyl fatty acid esters in sunflower, soybean, and safflower oil, but was only detected in free form in sesame seed oil. A higher proportion of esterified Δ^7 Sitosterol has also previously been described for a soybean and sunflower oil (Johansson, 1979; Johansson and Appelqvist, 1979; Kalo and Kuuranne, 2001). Cycloartenol occurred mainly in esterified form, except for the linseed and safflower oil, where the amounts of free and esterified cycloartenol were almost equal or the free form was predominant, respectively. 24-Methylene cycloartanol was most abundant within steryl/stanyl fatty acid esters in olive, grape seed, sesame seed, and linseed oils. However, in the soybean and safflower oil 24-methylene cycloartanol was only present in free, but not in esterified form.

4.7.4 Distribution Patterns of Total Fatty Acids and of Fatty Acids Esterified to Sterols/Stanols

The total fatty acid profiles of the investigated edible oils are summarized in Appendix Table 42 and Table 43. Oleic acid was the predominant fatty acid in the rapeseed, olive, and safflower oils, representing >62 %. The total fatty acids in the corn germ, sunflower, grape seed, and soybean oils comprised mainly linoleic acid (54-55, 54-67, and 53 %, respectively),

followed by oleic acid (~30, 21-35, and 37 %, respectively). The sesame seed oil exhibited almost equal amounts of both fatty acids. Linseed oil, in turn, showed a distinct profile with linolenic acid as major fatty acid, accounting for 58 % of total fatty acids. Relative high percentages of linolenic acid were also observed in the rapeseed oils and soybean oil (7-10 and 5 %, respectively); the amounts in the other oils were <1 %. Palmitic acid was the fourth major fatty acid type and accounted for 4-12 % of total fatty acids, being the highest in olive, corn germ, soybean, and sesame seed oils (>9 %, respectively). The fatty acid compositions determined in the oils agree very well with official standards or literature data (EU, 1991; CODEX, 1999; Dubois *et al.*, 2007)

As observed for all other samples analyzed in the present study, the distribution patterns of fatty acids esterified to sterols, which were calculated on the basis of intact esters, differed considerably from those of total lipids. However, no clear tendency was observed which was applicable to all types of oil. The steryl fatty acid esters of most oil types contained higher proportions of linoleic acid than the corresponding total lipids, except for grape seed oil, in which the amounts were slightly lower. The percentages of oleic/stearic acid were, in turn, higher in total lipids; only sunflower and grape seed oils revealed almost similar amounts. The proportions of linolenic acid in rapeseed and soybean oil were higher in steryl esters; those of linseed oil higher in total lipids. Moreover, most oils exhibited lower proportions of palmitic/palmitoleic acid within steryl/stanyl esters, except for grape seed oil and olive oils, in which the proportions were either equal or slightly higher, respectively. Previous studies also found higher levels of linoleic acid and lower levels of oleic/stearic acid in the steryl ester fractions of rapeseed, soybean, and corn germ oils (Johansson and Appelqvist, 1978; Worthington and Hitchcock, 1984; Ferrari *et al.*, 1997). However, a higher proportion of linoleic acid was detected in total lipids compared to the fraction of steryl esters for a single sunflower oil sample (Johansson, 1979). The described distribution patterns of C16 fatty acids are inconsistent for the different oil types.

4.7.5 Proportions of Free Sterols/Stanols and Steryl/Stanyl Esters

The sums of the total contents of free sterols/stanols and intact steryl/stanyl esters were most abundant in rapeseed oil (0.99-1.33 mg/100 mg) and corn germ oil (1.23 - 1.34 mg/100 mg), which is due to their high levels of steryl/stanyl fatty acid esters (Figure 37). 1.3-1.7-fold lower amounts were quantified in the linseed oil (0.68 mg/100 mg) and sesame seed oil (0.72 mg/100 mg). The sunflower oils exhibited values in the range of 0.45-0.53 mg/100 mg, which, in turn, were on average approximately 2-times higher than those analyzed in the olive oils (0.18-0.26 mg/100 mg).

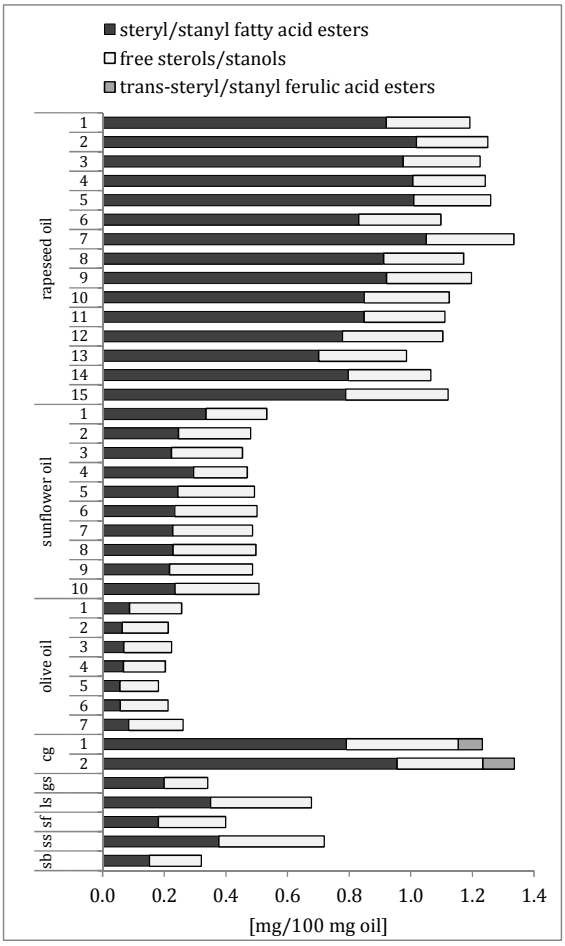

Figure 37. Sums of total contents of steryl/stanyl fatty acid esters and free sterols/stanols in edible plant oils. (The numberings of the samples correspond to those in Table 12.).
cg: corn germ oil; gs: grape seed oil; ls: linseed oil; sf: safflower oil; ss: sesame seed oil; sb: soybean oil.

The different types of oils exhibited not only large differences in their total contents of free sterols/stanols and steryl/stanyl esters, but also in the relative percentage distributions of both substance classes. Steryl/stanyl fatty acid esters made up the majority in rapeseed oils and corn germ oils. The grape seed, linseed, and sesame seed oils as well as three of the ten examined sunflower oils exhibited only slightly higher proportions of steryl/stanyl fatty acid esters than of free sterols/stanols. The levels of free sterols/stanols were, in turn, slightly higher in the soybean oil, safflower oil, and in most of the sunflower oils. The olive oils contained predominantly free sterols/stanols, which accounted for about two-thirds of the sum of the total contents of both sterol classes.

A predominance of free sterols/stanols in olive, grape seed, safflower, and soybean oils have also been described in earlier studies, while corn germ, rapeseed, and sunflower oils contained more esterified sterols/stanols (Worthington and Hitchcock, 1984; Phillips *et al.*, 2002; Verleyen *et al.*, 2002a; Verleyen *et al.*, 2002b; Cunha *et al.*, 2006; Dulf *et al.*, 2010).

4.7.6 Tocopherols, Squalene, and Free Fatty Acids

The tocopherol profiles revealed distinct differences between the various oil types (Table 35 and Table 36). *α*-Tocopherol was predominant in the sunflower, olive, grape seed, and safflower oils, whereas >80 % of the total tocopherols in the corn germ and linseed oils consisted of *γ*-tocopherol. Rapeseed oils mainly contained *γ*-tocopherol and *α*-tocopherol, averaging 54.4 ± 3.5 % and 41.6 ± 3.0 %, respectively. *γ*-Tocopherol was also dominating in soybean oil (67.5 %), but here the levels of *α*- and *δ*-tocopherol were almost equal. Small amounts of *δ*-tocopherol were additionally detected in the rapeseed, corn germ, grape seed, sesame seed, and safflower oils. Three of the seven investigated olive oils exhibited significantly higher percentages of *γ*-tocopherol (14.3-26.7 % versus 4.5-6.9 %). All three samples were produced by the same company in Italy, but an explanation for that observation cannot be given as exact information on origin, cultivar, or state of maturity was not available.

The average total amounts of tocopherols were 66.3 ± 5.7 µg/100 mg in rapeseed oil, 67.9 ± 13.0 µg/100 mg in sunflower oils, and 22.8 ± 3.3 µg/100 mg in olive oils. The other oils exhibited levels in the range of 21.4-102 µg/100 mg. The observed profiles and values are comparable to those described elsewhere (CODEX, 1999; Schwartz *et al.*, 2008).

Particularly high amounts of squalene were quantified in the olive oils, averaging 376.9 ± 68.9 µg/100 mg (Table 35). The levels in the other oils ranged from 1.2-29.8 µg/100 mg, except for the rapeseed oils and soybean oil, in which squalene was not detectable (Table 35 and Table 36). Squalene has already been described as useful marker for the characterization of olive oils as it represents up to 75 % of the unsaponifiable matter (Grob *et al.*, 1992; Villén *et al.*, 1998).

The amounts of free fatty acids are specified for refined and native oils and are usually determined as acid value via titration (EU, 1991; BMELV, 2011). The applied on-line LC-GC-based approach enabled the simultaneous analysis of free fatty acids besides squalene, tocopherols, free sterols, and steryl esters. The preliminary aim of the refining process is the removal of free fatty acids which thus explains the obviously lower amounts of free fatty acid found in refined oils as observed for the rapeseed oils no. 1-9, the sunflower oils no. 1-5, the corn germ oil no. 2 as well as for the grape seed oil, safflower oil, and soybean oil samples (Table 35 and Table 36).

Table 35. Contents and compositions of tocopherols and contents of squalene and free fatty acids in rapeseed, sunflower, and olive oils.

	no.	∑ tocopherols [µg/100 mg oil]	α-tocopherol [%]	γ-tocopherol [%]	δ-tocopherol [%]	squalene [µg/100 mg oil]	∑ free fatty acids [µg/100 mg oil]
rapeseed oil	1	62.9 ± 2.1	45.0 ± 0.3	48.9 ± 0.3	6.1 ± 0.1	-	24.7 ± 1.1
	2	70.0 ± 1.2	41.2 ± 0.1	53.2 ± 0.3	5.6 ± 0.1	-	48.6 ± 0.7
	3	68.9 ± 1.9	41.1 ± 0.3	53.2 ± 0.3	5.6 ± 0.2	-	39.7 ± 1.7
	4	65.7 ± 1.0	40.4 ± 0.3	53.4 ± 0.1	6.2 ± 0.2	-	43.5 ± 3.6
	5	59.8 ± 0.6	43.1 ± 0.1	51.9 ± 0.4	5.0 ± 0.2	-	27.2 ± 1.3
	6	65.4 ± 0.2	39.5 ± 0.2	56.3 ± 0.2	4.2 ± 0.1	-	24.7 ± 3.0
	7	72.0 ± 1.4	44.4 ± 0.2	51.3 ± 0.2	4.2 ± 0.0	-	54.9 ± 10.2
	8	61.2 ± 1.5	41.5 ± 0.2	52.7 ± 0.3	5.8 ± 0.2	-	20.9 ± 5.3
	9	68.1 ± 0.4	39.1 ± 0.1	55.1 ± 0.1	5.7 ± 0.2	-	24.1 ± 0.2
	10	52.2 ± 1.9	32.8 ± 0.1	64.9 ± 0.1	2.3 ± 0.1	-	696.7 ± 29.8
	11	69.8 ± 0.7	43.5 ± 0.3	54.6 ± 0.2	1.9 ± 0.1	-	650.7 ± 19.8
	12	71.1 ± 0.6	42.1 ± 0.1	56.2 ± 0.2	1.8 ± 0.1	-	238.5 ± 3.3
	13	70.1 ± 0.3	42.4 ± 0.4	55.6 ± 0.5	2.0 ± 0.1	-	847.5 ± 38.5
	14	63.3 ± 2.7	42.5 ± 0.6	55.8 ± 0.6	1.7 ± 0.1	-	617.0 ± 29.8
	15	74.4 ± 0.5	45.0 ± 0.2	53.3 ± 0.2	1.7 ± 0.0	-	143.9 ± 2.4
sunflower oil	1	52.0 ± 0.6	92.4 ± 0.7	7.6 ± 0.7	-[a]	2.9 ± 0.3	31.1 ± 1.4
	2	59.1 ± 0.3	94.0 ± 0.2	6.0 ± 0.2	-	5.9 ± 0.1	27.2 ± 2.8
	3	57.1 ± 1.6	91.9 ± 1.0	8.1 ± 1.0	-	8.0 ± 0.3	27.5 ± 3.5
	4	49.6 ± 1.4	92.5 ± 0.3	7.5 ± 0.3	-	1.9 ± 0.1	24.2 ± 1.4
	5	64.5 ± 0.5	93.5 ± 0.5	6.5 ± 0.5	-	7.0 ± 0.1	31.6 ± 0.5
	6	81.9 ± 1.5	94.0 ± 0.3	6.0 ± 0.3	-	1.2 ± 0.4	167.1 ± 0.1
	7	72.0 ± 1.5	93.1 ± 0.3	6.9 ± 0.3	-	10.5 ± 0.1	492.5 ± 5.2
	8	77.5 ± 3.1	92.6 ± 0.4	7.4 ± 0.4	-	14.1 ± 0.5	460.2 ± 2.1
	9	82.8 ± 0.6	93.9 ± 0.3	6.1 ± 0.3	-	12.0 ± 0.3	479.5 ± 15.4
	10	82.0 ± 0.1	94.6 ± 0.3	5.4 ± 0.3	-	17.3 ± 0.2	449.5 ± 12.5
olive oil	1	21.7 ± 0.1	80.7 ± 0.4	19.3 ± 0.4	-	329.0 ± 1.1	233.0 ± 6.3
	2	22.6 ± 0.5	85.7 ± 0.7	14.3 ± 0.7	-	406.0 ± 10.0	209.3 ± 8.1
	3	21.7 ± 0.2	73.3 ± 0.4	26.7 ± 0.4	-	387.4 ± 3.1	297.0 ± 3.1
	4	29.4 ± 1.0	95.5 ± 0.2	4.5 ± 0.2	-	309.0 ± 11.1	232.9 ± 10.8
	5	23.4 ± 0.6	93.1 ± 0.3	6.9 ± 0.3	-	374.5 ± 7.5	199.1 ± 9.3
	6	18.4 ± 0.2	94.6 ± 0.1	5.4 ± 0.1	-	322.9 ± 5.7	245.2 ± 8.5
	7	22.6 ± 0.1	94.6 ± 0.2	5.4 ± 0.2	-	509.8 ± 3.4	123.7 ± 2.9

[a] (-) Content below limit of detection (12 ng/mL i.v.; calculated on the basis of 10 µL i.v).

Table 36. Contents and compositions of tocopherols and contents of squalene and free fatty acids in corn germ, grape seed, linseed, sesame seed, safflower, and soybean oils.

	no.	∑ tocopherols [µg/100 mg oil]	*α*-tocopherol [%]	*γ*-tocopherol [%]	*δ*-tocopherol [%]	squalene [µg/100 mg oil]	∑ free fatty acids [µg/100 mg oil]
corn germ oil	1	77.4 ± 1.0	9.8 ± 0.2	86.1 ± 0.1	4.2 ± 0.1	29.8 ± 0.4	1216.2 ± 39.3
	2	102.0 ± 2.7	15.6 ± 0.4	81.8 ± 0.4	2.6 ± 0.1	16.0 ± 1.5	43.6 ± 2.6
grape seed oil		21.4 ± 0.2	78.8 ± 0.8	18.4 ± 0.5	2.8 ± 0.3	8.3 ± 0.2	42.7 ± 1.4
linseed oil		43.9 ± 1.0	6.4 ± 0.3	93.6 ± 0.3	-[a]	1.4 ± 0.1	162.1 ± 14.6
safflower oil		46.5 ± 2.0	94.0 ± 0.3	4.9 ± 0.2	1.1 ± 0.1	3.0 ± 0.1	20.5 ± 1.2
sesame seed oil		53.6 ± 0.8	1.6 ± 0.5	96.6 ± 0.5	1.8 ± 0.1	1.2 ± 0.0	1071.8 ± 55.2
soybean oil		56.1 ± 0.5	17.4 ± 0.5	67.5 ± 0.4	15.1 ± 0.1	-	19.7 ± 0.4

[a] (-) Content below limit of detection (12 ng/mL i.v.; calculated on the basis of 10 µL i.v).

4.7.7 Comparison of Native and Refined Oils

All investigated oil samples were commercially obtained; 6 of the 15 rapeseed oils as well as 5 of the 10 sunflower oils and 1 of the 2 corn germ oils were declared as cold-pressed native oils. A comparison of the total contents of free sterols/stanols, steryl/stanyl esters, tocopherols, and free fatty acids determined in the refined and native oils is shown in Figure 38.

The free sterol/stanol levels were on average slightly higher in the native oils; differences in the distribution patterns between native and refined oils were not observed. These finding are consistent with previously published results (Phillips *et al.*, 2002; Verleyen *et al.*, 2002b). It has been reported that the contents of free sterols/stanols were decreased during the refining process (Johansson and Hoffmann, 1979; Verleyen *et al.*, 2002b). A reduction in the levels of free sterols/stanols during refining may also be explainable owing to a heat-promoted interesterification of free sterols and fatty acids (Kochhar, 1983; Verleyen *et al.*, 2002b).

The total contents of steryl/stanyl fatty acid esters were higher in the refined than in the native oils. Johansson and Hoffmann (1979) described that steryl esters were much less influenced by refining than free sterols. Verleyen *et al.* (2002b) even observed a considerable increase in the contents of steryl/stanyl esters during the deodorization step of the physical refining process, probably caused by interesterification reactions. However, one study detected a minor decrease (5-10 %) in the contents of steryl esters during the whole refining process (Ferrari *et al.*, 1997).

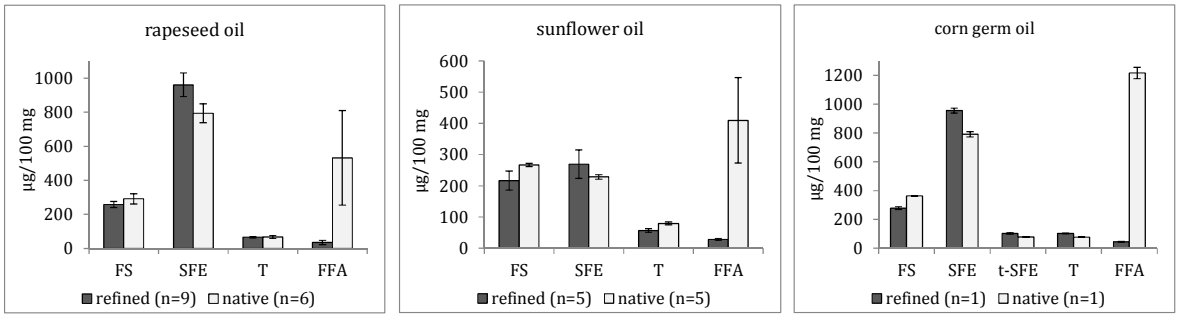

Figure 38. Comparison of total contents of free sterols/stanols (FS), steryl/stanyl fatty acid esters (SFE), steryl/stanyl ferulic acid esters (t-SFE), tocopherols (T), and free fatty acids (FFA) in refined and native oils.

It has been reported that tocopherols can be partially removed during refining depending on the applied conditions (Gordon, 2002). Higher tocopherol contents were observed in cold-pressed rapeseed and olive oil compared to the respective refined oils (Schwartz *et al.*, 2008). Tocopherol levels and compositions determined in the present study were almost comparable for refined and native oils, except for the refined corn germ oil, which exhibited a

higher proportion of α-tocopherol than the native oil (Table 36). This can be explained by the addition of vitamin E to the oil during its production (declaration on the label).
The contents of free fatty acids were clearly lower in the refined oils (<0.05 %), which is not surprising as those were nearly completely removed during neutralization. Although free fatty acids are not considered as essential parameters for authenticity assessments as such, their contents are, however, useful for the differentiation of refined and native oils.

Quantitative data obtained for all individual minor lipids in the rapeseed and sunflower oils were subjected to multivariate PCA (Figure 39). PCA from the combined data set revealed a distinct separation of native and refined oils on the first principal component, representing 49 and 55 % of the total variation in rapeseed oils and sunflower oils, respectively. Particularly, the contents of free fatty acids and steryl/stanyl esters were the major parameters explaining the variation between the two different processed oil types. However, there was also a separation detected within refined sunflower oils. Two samples (no. 1 and 4) exhibited clearly higher amounts and slightly different profiles of total steryl/stanyl fatty acid esters than the other sunflower oils (Table 33). Particularly, the levels of sitosteryl-18:2 were higher, those of Δ^7 sitosteryl-18:2 and cycloartenyl-18:2 lower. An explanation cannot be given as information concerning the conditions of the applied refining process or on cultivar and growing conditions of the used sunflower seeds was not available.

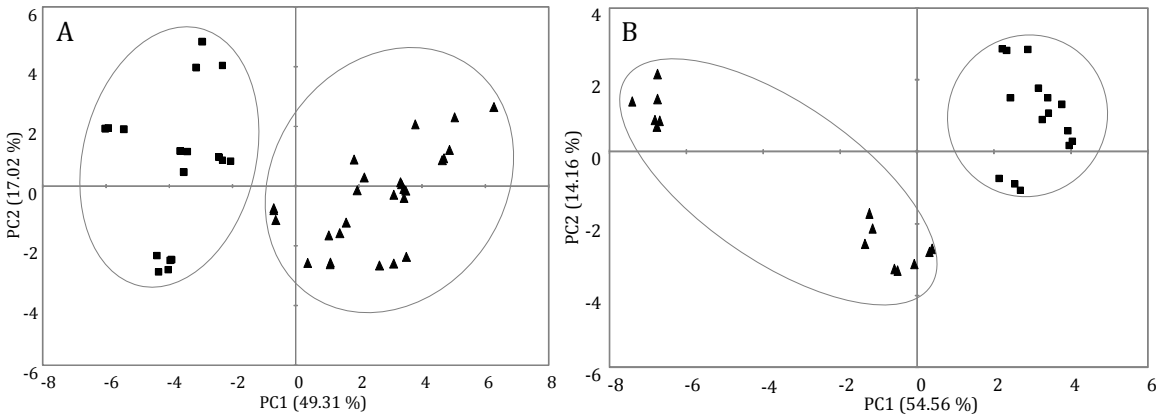

Figure 39. Principal component analyses of individual contents of free sterols/stanols, steryl/stanyl esters, tocopherols, and free fatty acids in (**A**) rapeseed oils and (**B**) sunflower oils: (▲) refined oils and (■) native oils.

4.8 Comparison of Cereals, Nuts, and Edible Plant Oils

As it could be shown that both of the employed analytical approaches (SPE-based and on-line LC-GC-based approach) resulted in comparable amounts of free sterols/stanols and steryl/stanyl esters (cf. 4.1.4), the average total contents of each type of food analyzed in the course of the present study were compared.

Figure 40 shows a comparison of the total amounts calculated on the basis of the raw material. The contents of free sterols/stanols were normally higher in nuts (>470 µg/g fresh weight) than in cereal grains (88-544 µg/g dry matter flour). Only sweet corn exhibited levels that were in the same order of magnitude as those determined in nuts (mean 965 ± 76 µg/g dry matter flour). In contrast, the levels of steryl/stanyl fatty acid esters were in most cereal samples on average approximately 3- to 8-times higher than those analyzed in the nuts, except for pine nuts and pistachios. Steryl/stanyl phenolic acid esters were characteristic compounds in cereals, but could not be detected in proso millet and finger millet. Among the examined cereal grains, total amounts of steryl/stanyl phenolic acid esters were particularly high in the popcorn and teff flours (107-226 µg/g dry matter flour). As expected, steryl/stanyl phenolic acid esters could not be detected in tree nuts and peanuts.

The average total amounts calculated on the basis total lipids are presented in Figure 41. The levels of free sterols/stanols in cereals were in the range of 407-1791 µg/100 mg oil and thus clearly above those quantified in nuts (102-418 µg/100 mg oil) or several other commercially obtained edible plant oils (125-363 µg/100 mg oil). Cereals contained also higher levels of steryl/stanyl fatty acid esters. The average total amounts were approximately 12- to 200-fold above those determined in nuts and were also higher than those quantified in edible plant oils, except for rapeseed and corn germ oil, which exhibited levels comparable to those analyzed in popcorn and sweet corn. Further, the lipid extracts of wheat, rye, teff, and popcorn were particularly rich sources of steryl/stanyl phenolic acid esters (351-734 µg/100 mg oil). Besides nuts, steryl/stanyl phenolic acid esters could also not be detected in seed oils. Only corn germ oil, as an example of a cereal-derived oil, contained small amounts of these esters.

Most of the analyzed samples exhibited distinct distribution patterns of individual free sterols/stanols and steryl/stanyl esters. Sitosterol was the most abundant free sterol/stanol in all samples and sitosteryl esters were predominant within the fraction of steryl/stanyl fatty acids esters, except for sorghum sample no. 1, which contained higher proportions of campesteryl fatty acids esters. The large inter-individual natural variability in the compositions indicates a plant specific synthesis of free sterols/stanols and steryl/stanyl esters, *inter alia* caused by different enzyme specifities and substrate availabilities.

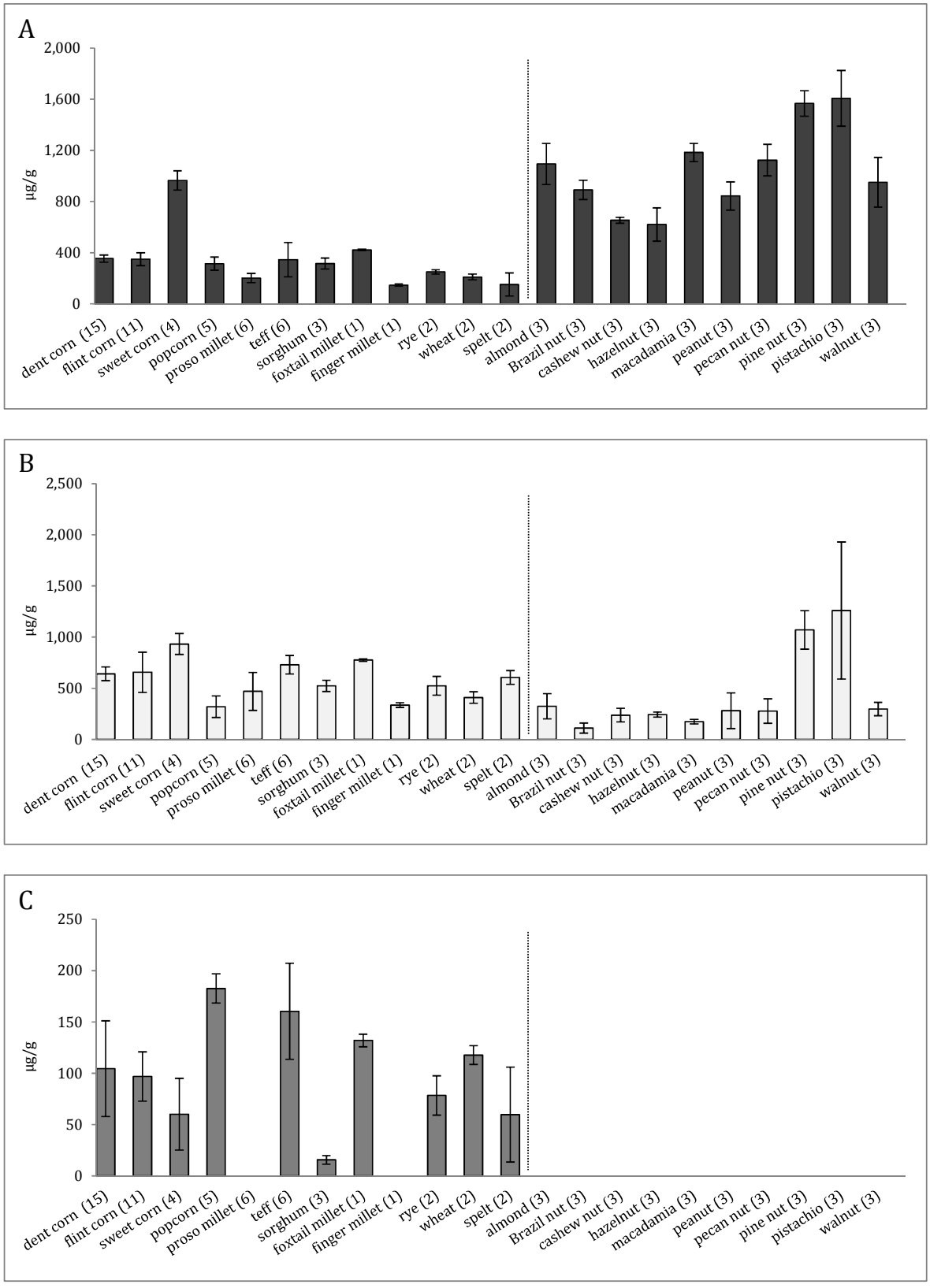

Figure 40. Comparison of total contents of (**A**) free sterols/stanols, (**B**) steryl/stanyl fatty acid esters, and (**C**) steryl/stanyl phenolic acid esters based on µg/g dry matter (cereals) and fresh weight (nuts.)

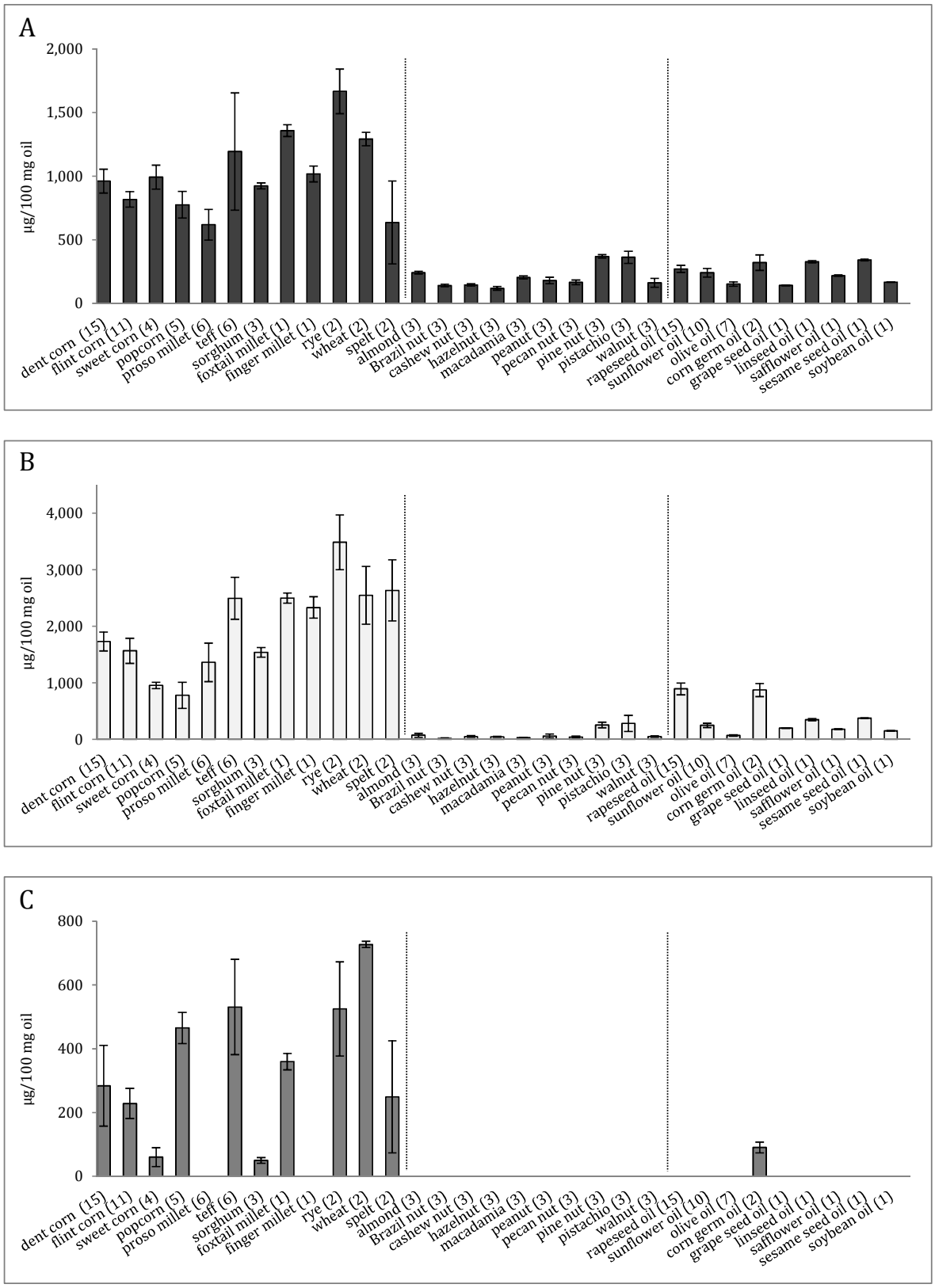

Figure 41. Comparison of total contents of (**A**) free sterols/stanols, (**B**) steryl/stanyl fatty acid esters, and (**C**) steryl/stanyl phenolic acid esters based on µg/100 mg oil.

5 SUMMARY

Phytosterols and their ester derivatives are bioactive secondary plant metabolites and possess several health benefits like cholesterol-lowering properties or anti-oxidative effects. To date, comparative data on the contents and compositions of free sterols/stanols and steryl/stanyl esters are scarce. In particular, individual intact steryl/stanyl fatty acid esters have been less studied, also due to the lack of appropriate analytical methods. Therefore, the aim of the present study was the development of new methodologies for the qualitative and quantitative analysis of free sterols/stanols and steryl/stanyl esters. The methods were validated and applied to different important natural sources of these compounds.

Firstly, an approach based on SPE was developed for the effective separation of the extracted plant lipids into fractions containing free sterols/stanols, steryl/stanyl fatty acid esters, and steryl/stanyl phenolic acid esters. The compositions of the different SPE-fractions were analyzed by GC-FID and GC-MS using an intermediately polar stationary phase. This phase enabled the separation and quantification of the individual free sterols/stanols as well as of the intact steryl/stanyl ester conjugates. The applicability of this methodology was shown for the analysis of the natural variability in compositions and contents of these compounds in different types of grain.

As a second analytical method, an approach based on on-line LC-GC-FID and on-line LC-GC-MS was established in order to provide an automated and more rapid alternative to the SPE-based approach. An on-line LC-GC system using a PTV as interface was employed. The silylated lipid extracts were fractionated via LC on a normal silica gel phase and the fractions containing the different sterol classes were transferred on-line to the GC for the analysis of their individual compositions. This approach enabled the simultaneous analysis of free sterols/stanols and intact steryl/stanyl fatty acid esters in plant lipids. *Trans*-derivatives of steryl/stanyl ferulic acid esters could further be analyzed in a second run. The on-line LC-GC-based method offered also the opportunity for the analysis of other important minor lipids such as tocopherols, squalene, and free fatty acids together with free sterols/stanols and steryl/stanyl fatty acid esters. The suitability of on-line LC-GC for the analysis of free sterols/stanols and steryl/stanyl esters in plant lipids was demonstrated for different cereals, nuts, and edible plant oils.

Whereas the SPE-based approach provided more information on the spectrum of individual steryl/stanyl phenolic acid esters and can easily be established in laboratories, the advantage of the on-line LC-GC method is in particular the far less complex sample preparation. The work up time is strikingly decreased, and less solvent amount is needed compared to the SPE-based approach. Further, due to the automation of the instrumentation and the performance

in a closed system, the risks of sample loss and contamination are reduced, thus enabling a fast and robust analysis. A comparison of both methods revealed similar results regarding certain validation parameters like recovery and repeatability. Further, total amounts of free sterols/stanols and steryl/stanyl esters obtained by the analysis of five different corn samples with both methods were comparable.

The present study provides new and detailed information on the contents and compositions of free sterols/stanols as well as of intact steryl/stanyl fatty acid esters and steryl/stanyl phenolic acid esters in various plant foods. Quantitative differences were detected between the distribution patterns of free sterols/stanols and steryl/stanyl esters within the different investigated types of grains, nuts, and edible plant oils. Steryl/stanyl fatty acid esters were predominant in all cereal crops, except for sweet corn and popcorn, where almost equal amounts of both sterol classes were quantified. In tree nuts and peanuts, free sterols/stanols were more abundant than steryl/stanyl fatty acid esters. The majority of the sterols/stanols in most of the investigated plant oils occurred in form of their fatty acid esters, except for the native sunflower oils, the olive oils, the safflower oil, and the soybean oil, in which the amounts of free sterols/stanols and steryl/stanyl fatty acid esters were either equal or free sterols/stanols were predominant.

The total contents of free sterols/stanols determined on the basis of the raw material were the highest in nuts and sweet corn. The amounts of steryl/stanyl fatty acid esters, in turn, were usually higher in cereals, except for pistachios and pine nuts. Steryl/stanyl phenolic acid esters were unique ingredients of cereals and corn germ oil. Among these samples, the total amounts were particularly high in the flours of popcorn and teff. The average total amounts of free sterols/stanols and steryl/stanyl fatty acid esters calculated on the basis total lipids were significantly higher in the lipid extracts of cereals than in the nut or plant oils. Only the rapeseed and corn germ oils exhibited levels comparable to those determined in popcorn and sweet corn oil.

Regarding the composition, sitosterol was predominant within free sterols/stanols in all investigated samples and sitosteryl esters were the most abundant fatty acids esters, except for one sorghum sample. The distribution patterns of free versus esterified sterols/stanols as well as the profiles of fatty acids in total lipids compared to those esterified with sterols/stanols varied considerable. However, no general rules could be assigned and applied to all investigated types of food.

In conclusion, both of the presented methodologies provide very detailed information on the natural variability of free sterols/stanols and of individual intact steryl/stanyl esters and can be used as efficient tools for the analytical characterization of these compounds in various plant foods.

6 ZUSAMMENFASSUNG

Phytosterole und deren Ester sind bioaktive sekundäre Pflanzenstoffe, denen zahlreiche gesundheitsfördernde Eigenschaften, wie zum Beispiel eine cholesterolsenkende und antioxidative Wirkung zugesprochen werden. Bisher gibt es aber nur wenig vergleichbare Daten bezüglich der Gehalte und Zusammensetzungen freier Sterole/Stanole und Steryl-/Stanylester. Vor allem individuelle intakte Steryl-/Stanylfettsäureester wurden nur selten analysiert, was unter anderem auch am Mangel geeigneter Analysemethoden liegt. Daher war das Ziel der vorliegenden Arbeit, neue Methoden zur qualitativen und quantitativen Analytik von freien Sterolen/Stanolen und Steryl-/Stanylestern zu entwickeln. Diese Methoden wurden validiert und zur Analytik dieser Verbindungen in wichtigen natürlichen Quellen eingesetzt.

Zuerst wurde eine Methode basierend auf Festphasenextraktion entwickelt. Diese diente dazu, die extrahierten Pflanzenlipide in Fraktionen mit freien Sterolen/Stanolen, Steryl-/Stanylfettsäureestern und Steryl-/Stanylphenolsäureestern aufzutrennen. Die Analytik der einzelnen SPE-Fraktionen erfolgte mittels GC-FID und GC-MS an einer mittelpolaren stationären Phase. Diese Phase erwies sich als geeignet zur Untersuchung individueller freier Sterole/Stanole und intakt vorliegender Steryl-/Stanylester. Die Tauglichkeit dieses Ansatzes wurde anhand der Analytik der natürlichen Variabilität bezüglich der Gehalte und Zusammensetzungen individueller Steryl-/Stanylderivate in unterschiedlichen Getreidearten gezeigt.

Als zweite Methode wurde ein Ansatz basierend auf on-line LC-GC-FID und on-line LC-GC-MS entwickelt mit dem Ziel, eine automatisierte und schnellere Alternative zum SPE-basierenden Ansatz zur Verfügung zu stellen. Dabei wurde ein on-line LC-GC System mit PTV Interface eingesetzt. Die silylierten Lipide wurden mittels LC anhand einer Normalphase bestehend aus Silicagel vorgetrennt. Die Fraktionen, welche die unterschiedlichen Sterolklassen enthielten, wurden dann on-line zur GC transferiert, wo wiederum die Analytik der individuellen Zusammensetzung der transferierten Fraktionen erfolgte. Dieser Ansatz ermöglichte die simultane Analytik freier Sterole/Stanole und intakter Steryl-/Stanylfettsäureester. *Trans*-derivate der Steryl-/Stanylferulasäureester wurden in einem zweiten Analysenlauf analysiert. Darüber hinaus bot der on-line LC-GC basierte Ansatz auch die Möglichkeit andere wichtige Minorlipide wie Tocopherole, Squalen oder freie Fettsäuren neben freien Sterolen/Stanolen und Steryl-/Stanylfettsäureestern zu analysieren. Die Eignung der on-line LC-GC zur Analytik freier Sterole/Stanole und Steryl-/Stanylester in pflanzlichen Lipiden wurde an verschiedenen Getreiden, Nüssen und pflanzlichen Ölen demonstriert.

Während mit dem SPE-basierenden Ansatz mehr Informationen bezüglich des Spektrums individueller Steryl-/Stanylphenolsäureester erhalten wurden und dieser recht einfach in Laboratorien etabliert werden kann, liegen die Vorteile der on-line LC-GC Methode vor allem in der wesentlich vereinfachten Probenvorbereitung. Die Aufarbeitungszeit wurde deutlich verringert und im Vergleich zum SPE basierten Ansatz wird wesentlich weniger Lösemittel verbraucht. Des Weiteren wird aufgrund der Automatisierung und Geschlossenheit des Systems das Risiko von Probenverlusten und Kontaminationen reduziert, wodurch eine schnelle und robuste Analytik ermöglicht wird. Beide Methoden zeigten vergleichbare Ergebnisse bezüglich verschiedener Validierungsparameter wie Wiederfindungsraten oder Wiederholbarkeit. Auch die Gesamtgehalte freier Sterole/Stanole und Steryl-/Stanylester, die durch die Aufarbeitung fünf unterschiedlicher Maisproben mit beiden Methoden erhalten wurden, waren vergleichbar.

Die vorliegende Arbeit liefert neue und detaillierte Informationen zu den Gehalten und Zusammensetzungen freier Sterole/Stanole sowie intakter Steryl-/Stanylfettsäureester und Steryl-/Stanylphenolsäureester in verschiedenen pflanzlichen Lebensmitteln. Dabei wurden quantitative Unterschiede in den Verteilungsprofilen der freien Sterole/Stanole und Steryl-/Stanylester innerhalb der verschiedenen untersuchten Getreide-, Nuss- und Ölarten detektiert. Steryl-/Stanylfettsäureester dominierten in allen Getreidearten, ausgenommen von Zuckermais und Popcorn, in denen annähernd gleiche Gehalte beider Sterolklassen quantifiziert wurden. In Nüssen überwogen hingegen freie Sterole/Stanole. In den meisten untersuchten pflanzlichen Ölen lag die Mehrheit der Sterole/Stanole in Form ihrer Fettsäureester vor, mit Ausnahme der nativen Sonnenblumenöle, der Olivenöle sowie des Distel- und Sojaöls, in denen entweder annähend gleiche Gehalte an freien Sterolen/Stanolen und Steryl-/Stanylfettsäureestern vorlagen oder freie Sterole/Stanole dominierten.
Die höchsten Gesamtgehalte freier Sterole/Stanole bezogen auf das Rohmaterial wurden in Nüssen und Zuckermais bestimmt. Die Gehalte an Steryl-/Stanylfettsäureestern waren wiederum in der Regel am höchsten in Getreiden, mit Ausnahme der Pistazien- und Pinienkernproben. Steryl-/Stanylphenolsäureester waren einzigartig für Getreide und Maiskeimöl. Unter diesen Proben waren die Gehalte besonders hoch in den Popcorn- und Teffmehlen. Die mittleren Gesamtgehalte an freien Sterolen/Stanolen und Steryl-/Stanylfettsäureestern in den jeweiligen Lipidextrakten waren signifikant höher in den Getreidelipiden als in den Nuss- oder Pflanzenölen. Nur in den Raps- und Maiskeimölen waren die Gehalte vergleichbar zu denen, die den in Popcorn- und Zuckermaisölen bestimmt wurden.
Sitosterol war in allen untersuchten Proben das dominierende freie Sterol/Stanol und Sitosterylester machten die Mehrheit innerhalb der Fettsäureester aus, mit Ausnahme von einer Sorghumprobe. Die Verteilungsprofile freier versus veresterter Sterole/Stanole sowie

die Fettsäureprofile der Gesamtlipide im Vergleich zu denen, die verestert mit Sterolen/Stanolen vorlagen, zeigen teils deutliche Unterschiede. Allerdings konnten keine allgemeinen Regeln abgeleitet werden, die für alle untersuchten Lebensmittelproben gültig gewesen wären.

Abschließend lässt sich sagen, dass beide der hier vorgestellten Methoden sehr detaillierte Informationen zur natürlichen Variabilität freier Sterole/Stanole und individueller intakter Steryl-/Stanylester liefern. Sie können somit als effiziente Analysenmethoden zur Charakterisierung dieser Verbindungen in verschiedenen pflanzlichen Lebensmitteln eingesetzt werden.

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8 APPENDIX

The ratios of C16:0/16:1 and C18:0/18:1 determined as fatty acid methyl esters in the steryl/stanyl fatty acid ester fractions of selected small millet, sorghum, nut, and edible plant oil samples are shown in Table 37.

Table 37. Ratios of C16:0/16:1 and C18:0/18:1 in the steryl/stanyl fatty acid ester fractions of selected small millet, sorghum, nut, and edible plant oil samples.

	no.	ratio C16:0/16:1	ratio C18:0/18:1
small millets and sorghum			
proso millet	1	-[a]	1:12
	4	50:1	1:6
teff	2	-	1:8
	3	-	1:5
sorghum	1	18:1	1:9
	2	16:1	1:10
foxtail millet		-	1:3
finger millet		-	1:19
tree nuts and peanuts			
almond	1	-	1:13
Brazil nut	1	118:1	1:2
cashew nut	2	-	1:5
hazelnut	2	73:1	1:17
macadamia	1	1:1	1:7
	2	1:1	1:6
	3	1:1	1:9
peanut	1	-	1:13
pecan nut	2	94:1	1:10
pine nut	3	-	1:6
pistachio	2	-	1:19
walnut	1	-	1:4
edible plant oils			
rapeseed oil	1	20:1	1:15
	10	33:1	1:14
sunflower oil	1	-	1:3
	7	-	1:5
olive oil	1	43:1	1:4
	7	35:1	1:6
corn germ oil	1	105:1	1:8
	2	68:1	1:10
grapeseed oil		28:1	1:3
linseed oil		-	1:2
safflower oil		-	1:10
sesame seed oil		-	1:4
soybean oil		-	1:2

[a] (-) No palmitoleic acid detected.

The fatty acid methyl ester compositions of the lipids extracted from cereals and nuts as well as those of the edible plant oils are listed in Table 38-Table 43.

Table 38. Fatty acid methyl ester compositions of rye, wheat, and spelt lipids

	no.	C14:0 [%]	C16:0 [%]	C16:1 [%]	C17:0 [%]	C18:0 [%]	C18:1 [%]
rye	1	0.07 ± 0.00	13.73 ± 0.04	0.12 ± 0.01	0.08 ± 0.00	0.67 ± 0.04	17.74 ± 0.04
	2	0.06 ± 0.00	13.38 ± 0.00	0.17 ± 0.01	0.07 ± 0.01	0.81 ± 0.01	18.11 ± 0.02
wheat	1	0.08 ± 0.00	15.42 ± 0.03	0.11 ± 0.00	0.10 ± 0.01	0.68 ± 0.01	13.88 ± 0.04
	2	0.07 ± 0.01	14.94 ± 1.51	0.16 ± 0.01	0.09 ± 0.01	0.88 ± 0.02	14.95 ± 0.26
spelt	1	0.07 ± 0.01	13.83 ± 0.20	0.13 ± 0.00	0.09 ± 0.00	0.93 ± 0.04	20.29 ± 0.12
	2	0.05 ± 0.00	13.65 ± 0.33	0.14 ± 0.00	0.08 ± 0.02	0.85 ± 0.01	21.52 ± 1.76

Table 38. continued.

	no.	C18:2 [%]	C18:3 [%]	C20:0 [%]	C20:1 [%]	C22:0 [%]	C24:0 [%]
rye	1	57.67 ± 0.11	7.62 ± 0.06	0.38 ± 0.00	1.61 ± 0.08	0.15 ± 0.01	0.15 ± 0.01
	2	59.21 ± 0.18	6.15 ± 0.18	0.23 ± 0.01	1.43 ± 0.00	0.19 ± 0.00	0.18 ± 0.01
wheat	1	63.83 ± 0.09	4.73 ± 0.02	0.70 ± 0.05	0.13 ± 0.01	0.12 ± 0.00	0.13 ± 0.00
	2	63.09 ± 1.12	4.56 ± 0.12	0.14 ± 0.00	0.84 ± 0.02	0.13 ± 0.01	0.15 ± 0.02
spelt	1	60.30 ± 0.22	2.90 ± 0.09	0.18 ± 0.03	0.62 ± 0.00	0.35 ± 0.04	0.32 ± 0.02
	2	58.73 ± 1.27	3.48 ± 0.10	0.16 ± 0.02	1.08 ± 0.02	0.13 ± 0.00	0.13 ± 0.00

Table 39. Fatty acid methyl ester compositions of dent corn, flint corn, sweet corn and popcorn lipids.

	no.	C14:0 [%]	C16:0 [%]	C16:1 [%]	C17:0 [%]	C18:0 [%]	C18:1 [%]	C18:2 [%]	C18:3 [%]	C20:0 [%]	C20:1 [%]	C22:0 [%]	C24:0 [%]
dent corn	1	0.02 ± 0.00[a]	9.37 ± 0.03	0.08 ± 0.00	0.06 ± 0.00	1.74 ± 0.01	30.56 ± 0.00	56.15 ± 0.05	0.90 ± 0.06	0.51 ± 0.01	0.32 ± 0.00	0.16 ± 0.01	0.14 ± 0.01
	2	0.02 ± 0.00	10.89 ± 0.02	0.09 ± 0.00	0.07 ± 0.01	1.74 ± 0.01	28.52 ± 0.04	56.53 ± 0.04	1.13 ± 0.01	0.43 ± 0.00	0.29 ± 0.02	0.13 ± 0.00	0.16 ± 0.01
	3	0.01 ± 0.00	11.07 ± 0.05	0.09 ± 0.00	0.06 ± 0.00	1.86 ± 0.12	29.01 ± 0.06	55.92 ± 0.05	1.13 ± 0.03	0.36 ± 0.02	0.27 ± 0.03	0.08 ± 0.00	0.12 ± 0.02
	4	0.02 ± 0.00	9.59 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	2.20 ± 0.01	24.50 ± 0.01	61.31 ± 0.04	1.25 ± 0.04	0.46 ± 0.01	0.22 ± 0.01	0.13 ± 0.00	0.18 ± 0.01
	5	0.02 ± 0.00	12.06 ± 0.02	0.09 ± 0.00	0.07 ± 0.00	1.58 ± 0.02	24.01 ± 0.03	59.92 ± 0.02	1.34 ± 0.02	0.40 ± 0.00	0.26 ± 0.01	0.11 ± 0.00	0.15 ± 0.01
	6	0.02 ± 0.00	9.51 ± 0.02	0.10 ± 0.00	0.07 ± 0.00	1.82 ± 0.01	25.64 ± 0.28	60.78 ± 0.22	1.13 ± 0.06	0.40 ± 0.00	0.27 ± 0.00	0.11 ± 0.00	0.14 ± 0.02
	7	0.02 ± 0.00	10.74 ± 0.05	0.07 ± 0.00	0.06 ± 0.00	1.54 ± 0.00	23.47 ± 0.03	61.99 ± 0.01	1.18 ± 0.03	0.39 ± 0.01	0.26 ± 0.01	0.12 ± 0.01	0.14 ± 0.02
	8	0.02 ± 0.00	10.36 ± 1.53	0.09 ± 0.02	0.07 ± 0.00	1.78 ± 0.07	26.33 ± 0.85	59.32 ± 2.38	1.12 ± 0.08	0.40 ± 0.02	0.27 ± 0.01	0.11 ± 0.00	0.14 ± 0.01
	9	0.02 ± 0.00	11.96 ± 0.01	0.09 ± 0.01	0.06 ± 0.00	1.62 ± 0.01	26.25 ± 0.02	57.79 ± 0.04	1.19 ± 0.06	0.40 ± 0.01	0.31 ± 0.00	0.13 ± 0.01	0.17 ± 0.00
	10	0.02 ± 0.00	12.59 ± 0.02	0.08 ± 0.00	0.06 ± 0.00	1.75 ± 0.02	27.03 ± 0.01	55.87 ± 0.05	1.42 ± 0.02	0.47 ± 0.01	0.35 ± 0.00	0.15 ± 0.00	0.21 ± 0.00
	11	0.02 ± 0.00	10.50 ± 0.04	0.12 ± 0.00	0.06 ± 0.00	1.44 ± 0.01	26.51 ± 0.03	59.49 ± 0.03	0.98 ± 0.02	0.38 ± 0.01	0.29 ± 0.01	0.10 ± 0.01	0.12 ± 0.01
	12	0.02 ± 0.00	10.22 ± 0.03	0.10 ± 0.00	0.06 ± 0.00	1.64 ± 0.01	26.00 ± 0.06	59.91 ± 0.12	1.07 ± 0.10	0.41 ± 0.01	0.26 ± 0.02	0.13 ± 0.01	0.18 ± 0.01
	13	0.01 ± 0.00	8.97 ± 0.07	0.08 ± 0.00	0.06 ± 0.00	1.98 ± 0.01	23.20 ± 0.06	63.71 ± 0.07	1.06 ± 0.03	0.45 ± 0.01	0.25 ± 0.01	0.13 ± 0.01	0.10 ± 0.02
	14	0.01 ± 0.00	10.12 ± 0.02	0.09 ± 0.00	0.06 ± 0.00	2.17 ± 0.01	25.19 ± 0.07	60.62 ± 0.12	0.88 ± 0.03	0.40 ± 0.01	0.22 ± 0.01	0.10 ± 0.01	0.14 ± 0.01
	15	0.02 ± 0.00	10.46 ± 0.02	0.09 ± 0.01	0.07 ± 0.00	1.76 ± 0.01	25.26 ± 0.09	59.86 ± 0.12	1.37 ± 0.05	0.44 ± 0.02	0.31 ± 0.01	0.14 ± 0.01	0.21 ± 0.00
flint corn	1	-[a]	9.48 ± 0.02	0.09 ± 0.00	0.07 ± 0.01	1.83 ± 0.01	25.81 ± 0.01	60.67 ± 0.04	1.10 ± 0.04	0.41 ± 0.00	0.27 ± 0.00	0.12 ± 0.00	0.16 ± 0.01
	2	0.02 ± 0.00	9.72 ± 0.09	0.11 ± 0.01	0.07 ± 0.00	2.40 ± 0.02	30.14 ± 0.65	55.58 ± 0.54	0.93 ± 0.03	0.52 ± 0.02	0.26 ± 0.01	0.12 ± 0.01	0.14 ± 0.02
	3	0.02 ± 0.00	9.15 ± 0.04	0.10 ± 0.00	0.07 ± 0.00	2.44 ± 0.05	35.80 ± 0.39	50.58 ± 0.36	0.93 ± 0.03	0.48 ± 0.02	0.24 ± 0.01	0.10 ± 0.01	0.10 ± 0.02
	4	-	10.03 ± 0.01	0.08 ± 0.00	0.06 ± 0.00	1.70 ± 0.01	29.08 ± 0.03	56.38 ± 0.10	1.57 ± 0.10	0.46 ± 0.01	0.33 ± 0.01	0.13 ± 0.00	0.16 ± 0.01
	5	0.02 ± 0.00	9.91 ± 0.12	0.08 ± 0.00	0.06 ± 0.00	2.50 ± 0.03	28.49 ± 0.71	57.06 ± 0.60	0.89 ± 0.03	0.53 ± 0.11	0.26 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
	6	0.02 ± 0.00	10.27 ± 0.01	0.09 ± 0.00	0.07 ± 0.00	2.36 ± 0.01	28.37 ± 0.01	56.94 ± 0.04	0.85 ± 0.03	0.51 ± 0.00	0.26 ± 0.01	0.11 ± 0.00	0.14 ± 0.01
	7	0.02 ± 0.00	10.27 ± 0.05	0.09 ± 0.00	0.07 ± 0.00	2.36 ± 0.03	28.37 ± 0.03	56.94 ± 0.04	0.85 ± 0.04	0.51 ± 0.01	0.26 ± 0.01	0.11 ± 0.01	0.14 ± 0.01
	8	0.02 ± 0.00	9.69 ± 0.05	0.11 ± 0.00	0.07 ± 0.00	2.36 ± 0.03	29.68 ± 0.65	55.88 ± 0.58	1.15 ± 0.05	0.51 ± 0.03	0.26 ± 0.01	0.12 ± 0.01	0.15 ± 0.01
	9	0.01 ± 0.00	9.91 ± 0.04	0.09 ± 0.01	0.07 ± 0.00	2.22 ± 0.02	26.94 ± 0.01	58.84 ± 0.07	1.06 ± 0.06	0.41 ± 0.02	0.21 ± 0.01	0.11 ± 0.01	0.12 ± 0.02
	10	0.02 ± 0.00	12.00 ± 0.05	0.08 ± 0.00	0.06 ± 0.00	1.56 ± 0.08	28.31 ± 0.04	55.99 ± 0.08	1.13 ± 0.03	0.35 ± 0.01	0.28 ± 0.00	0.10 ± 0.01	0.11 ± 0.02
	11	0.02 ± 0.00	12.26 ± 0.04	0.10 ± 0.00	0.06 ± 0.02	2.13 ± 0.02	29.57 ± 0.01	54.18 ± 0.01	0.83 ± 0.01	0.43 ± 0.02	0.24 ± 0.01	0.08 ± 0.01	0.10 ± 0.01
sweet corn	1	0.03 ± 0.00	15.30 ± 0.02	0.15 ± 0.01	0.08 ± 0.00	3.07 ± 0.00	36.94 ± 0.01	41.77 ± 0.02	1.34 ± 0.01	0.64 ± 0.01	0.34 ± 0.00	0.17 ± 0.00	0.16 ± 0.00
	2	0.02 ± 0.00	12.50 ± 0.02	0.15 ± 0.00	0.07 ± 0.00	2.95 ± 0.00	40.19 ± 0.03	42.06 ± 0.05	0.92 ± 0.01	0.59 ± 0.00	0.27 ± 0.00	0.14 ± 0.00	0.13 ± 0.00
	3	0.03 ± 0.00	14.61 ± 0.09	0.16 ± 0.00	0.09 ± 0.00	3.00 ± 0.02	38.51 ± 0.22	41.66 ± 0.23	0.75 ± 0.56	0.62 ± 0.00	0.29 ± 0.00	0.15 ± 0.00	0.14 ± 0.00
	4	0.02 ± 0.00	14.02 ± 0.01	0.15 ± 0.00	0.07 ± 0.00	2.42 ± 0.02	35.49 ± 0.02	45.50 ± 0.03	1.18 ± 0.03	0.56 ± 0.01	0.30 ± 0.02	0.14 ± 0.00	0.14 ± 0.00
popcorn	1	0.02 ± 0.00	10.31 ± 0.02	0.12 ± 0.00	0.06 ± 0.00	1.87 ± 0.00	26.01 ± 0.01	59.72 ± 0.01	0.81 ± 0.00	0.50 ± 0.00	0.27 ± 0.00	0.15 ± 0.00	0.17 ± 0.00
	2	0.02 ± 0.00	10.94 ± 0.01	0.11 ± 0.00	0.07 ± 0.00	1.94 ± 0.00	22.40 ± 0.00	62.57 ± 0.03	0.95 ± 0.03	0.45 ± 0.00	0.24 ± 0.00	0.13 ± 0.00	0.18 ± 0.00
	3	0.02 ± 0.00	11.35 ± 0.02	0.15 ± 0.00	0.07 ± 0.00	1.98 ± 0.00	28.15 ± 0.01	56.38 ± 0.04	0.81 ± 0.04	0.46 ± 0.00	0.31 ± 0.00	0.14 ± 0.00	0.19 ± 0.00
	4	0.02 ± 0.00	11.49 ± 0.04	0.12 ± 0.00	0.07 ± 0.00	2.04 ± 0.00	28.59 ± 0.01	55.73 ± 0.06	0.85 ± 0.03	0.46 ± 0.00	0.30 ± 0.00	0.14 ± 0.00	0.19 ± 0.00
	5	0.02 ± 0.00	11.48 ± 0.02	0.12 ± 0.00	0.08 ± 0.00	1.86 ± 0.00	24.50 ± 0.05	59.81 ± 0.13	0.95 ± 0.10	0.51 ± 0.00	0.30 ± 0.00	0.15 ± 0.00	0.21 ± 0.00

[a] (-) Content below limit of detection.

Table 40. Fatty acid methyl ester compositions of small millet and sorghum lipids.

	no.	C14:0 [%]	C16:0 [%]	C16:1 [%]	C17:0 [%]	C18:0 [%]	C18:1 [%]	C18:2 [%]	C18:3 [%]	C20:0 [%]	C20:1 [%]	C22:0 [%]	C24:0 [%]
proso m.	1	0.02 ± 0.00	7.18 ± 0.13	0.16 ± 0.00	0.04 ± 0.00	2.02 ± 0.20	22.85 ± 0.60	64.49 ± 0.70	0.81 ± 0.07	1.22 ± 0.34	0.45 ± 0.00	0.45 ± 0.02	0.31 ± 0.00
	2	0.03 ± 0.00	10.15 ± 0.03	0.23 ± 0.01	0.05 ± 0.00	1.59 ± 0.03	21.79 ± 0.18	62.83 ± 0.10	1.19 ± 0.14	0.89 ± 0.02	0.48 ± 0.01	0.46 ± 0.00	0.32 ± 0.00
	3	0.02 ± 0.00	7.23 ± 0.01	0.15 ± 0.00	0.04 ± 0.00	1.31 ± 0.05	22.93 ± 0.04	65.68 ± 0.05	0.93 ± 0.02	0.70 ± 0.00	0.47 ± 0.01	0.32 ± 0.00	0.23 ± 0.00
	4	0.02 ± 0.00	7.13 ± 0.05	0.16 ± 0.01	0.03 ± 0.00	1.40 ± 0.05	23.18 ± 0.21	65.53 ± 0.10	0.86 ± 0.10	0.61 ± 0.07	0.46 ± 0.01	0.36 ± 0.03	0.26 ± 0.02
	5	0.01 ± 0.00	7.52 ± 0.03	0.16 ± 0.00	0.05 ± 0.00	1.65 ± 0.01	23.70 ± 0.09	64.11 ± 0.14	0.91 ± 0.05	0.76 ± 0.03	0.42 ± 0.01	0.39 ± 0.00	0.30 ± 0.00
	6	0.03 ± 0.00	12.18 ± 0.08	0.15 ± 0.01	0.05 ± 0.00	2.37 ± 0.11	21.09 ± 0.08	58.95 ± 0.10	1.49 ± 0.07	2.32 ± 0.14	0.40 ± 0.03	0.58 ± 0.02	0.38 ± 0.01
teff	1	0.03 ± 0.00	16.90 ± 0.80	0.16 ± 0.01	0.11 ± 0.01	5.15 ± 0.54	28.15 ± 0.21	40.55 ± 2.84	6.97 ± 1.52	1.09 ± 0.09	0.37 ± 0.04	0.33 ± 0.03	0.19 ± 0.01
	2	0.02 ± 0.00	15.54 ± 0.03	0.20 ± 0.00	0.10 ± 0.00	4.40 ± 0.02	28.00 ± 0.01	44.72 ± 0.02	5.28 ± 0.01	0.91 ± 0.01	0.35 ± 0.01	0.29 ± 0.00	0.17 ± 0.00
	3	0.02 ± 0.00	15.25 ± 0.05	0.23 ± 0.00	0.09 ± 0.00	5.27 ± 0.01	31.31 ± 0.04	41.45 ± 0.05	4.57 ± 0.09	1.01 ± 0.01	0.33 ± 0.00	0.29 ± 0.00	0.17 ± 0.00
	4	0.03 ± 0.00	15.64 ± 0.04	0.19 ± 0.00	0.10 ± 0.00	4.37 ± 0.00	28.21 ± 0.01	44.77 ± 0.03	4.98 ± 0.01	0.94 ± 0.00	0.35 ± 0.00	0.27 ± 0.00	0.15 ± 0.00
	5	0.02 ± 0.00	11.29 ± 0.03	0.11 ± 0.00	0.11 ± 0.00	4.56 ± 0.02	27.05 ± 0.02	48.17 ± 0.02	7.00 ± 0.06	0.90 ± 0.07	0.40 ± 0.01	0.25 ± 0.00	0.15 ± 0.00
	6	0.03 ± 0.00	15.07 ± 0.04	0.18 ± 0.00	0.13 ± 0.00	4.65 ± 0.01	27.43 ± 0.01	44.57 ± 0.17	6.08 ± 0.17	1.05 ± 0.03	0.36 ± 0.02	0.30 ± 0.00	0.16 ± 0.00
sorghum	1	0.02 ± 0.00	12.06 ± 0.01	0.70 ± 0.00	0.06 ± 0.00	1.47 ± 0.00	34.76 ± 0.00	48.51 ± 0.03	1.79 ± 0.02	0.19 ± 0.00	0.27 ± 0.02	0.08 ± 0.00	0.10 ± 0.00
	2	0.02 ± 0.00	12.22 ± 0.02	0.53 ± 0.00	0.07 ± 0.00	1.26 ± 0.00	36.50 ± 0.03	47.21 ± 0.08	1.35 ± 0.03	0.29 ± 0.04	0.33 ± 0.07	0.11 ± 0.00	0.13 ± 0.00
	3	0.02 ± 0.00	10.76 ± 0.05	0.42 ± 0.00	0.06 ± 0.00	1.07 ± 0.01	32.18 ± 0.08	52.45 ± 0.04	2.42 ± 0.02	0.16 ± 0.01	0.28 ± 0.01	0.08 ± 0.00	0.10 ± 0.00
foxtail m.		0.03 ± 0.00	6.08 ± 0.01	0.08 ± 0.00	0.07 ± 0.00	0.93 ± 0.00	16.00 ± 0.01	72.04 ± 0.08	3.26 ± 0.07	0.40 ± 0.02	0.56 ± 0.01	0.37 ± 0.00	0.18 ± 0.00
finger m.		0.04 ± 0.00	23.08 ± 0.03	0.32 ± 0.01	0.15 ± 0.00	1.94 ± 0.00	44.45 ± 0.03	25.49 ± 0.01	3.10 ± 0.00	0.53 ± 0.01	0.52 ± 0.02	0.22 ± 0.00	0.16 ± 0.00

Table 41. Fatty acid methyl ester compositions of tree nuts and peanut lipids.

	no.	C12:0 [%]	C14:0 [%]	C16:0 [%]	C16:1 [%]	C17:0 [%]	C18:0 [%]	C18:1 [%]
almond	1	-	0.02 ± 0.00	5.60 ± 0.03	0.53 ± 0.00	0.05 ± 0.00	2.17 ± 0.01	73.97 ± 0.02
	2	-	0.04 ± 0.00	6.27 ± 0.01	0.51 ± 0.00	0.05 ± 0.00	1.23 ± 0.01	70.91 ± 0.07
	3	-	0.03 ± 0.00	6.41 ± 0.00	0.44 ± 0.00	0.05 ± 0.00	1.47 ± 0.00	62.48 ± 0.01
Brazil nut	1	-	0.04 ± 0.00	14.40 ± 0.01	0.35 ± 0.00	0.08 ± 0.00	10.87 ± 0.00	31.88 ± 0.00
	2	-	0.04 ± 0.00	13.61 ± 0.08	0.34 ± 0.01	0.08 ± 0.00	10.33 ± 0.02	34.64 ± 0.02
	3	-	0.03 ± 0.00	13.81 ± 0.06	0.36 ± 0.00	0.08 ± 0.00	10.45 ± 0.05	33.08 ± 0.05
cashew nut	1	-	0.02 ± 0.00	9.76 ± 0.01	0.33 ± 0.00	0.13 ± 0.00	9.29 ± 0.00	59.76 ± 0.01
	2	-	0.02 ± 0.00	10.56 ± 0.07	0.35 ± 0.00	0.13 ± 0.01	9.93 ± 0.05	58.12 ± 0.03
	3	-	0.01 ± 0.00	8.52 ± 0.01	0.34 ± 0.00	0.11 ± 0.00	7.91 ± 0.01	63.49 ± 0.00
hazelnut	1	-	0.02 ± 0.00	4.88 ± 0.08	0.16 ± 0.00	0.04 ± 0.00	2.12 ± 0.09	79.34 ± 0.85
	2	-	0.02 ± 0.00	4.26 ± 0.26	0.13 ± 0.01	0.04 ± 0.00	1.99 ± 0.13	77.23 ± 0.14
	3	-	0.02 ± 0.00	5.95 ± 0.01	0.23 ± 0.00	0.05 ± 0.00	2.54 ± 0.00	83.98 ± 0.01
macadamia	1	0.07 ± 0.01	0.76 ± 0.01	7.73 ± 0.03	21.49 ± 0.05	0.02 ± 0.00	3.32 ± 0.47	56.50 ± 0.40
	2	0.04 ± 0.00	0.58 ± 0.01	8.43 ± 0.03	21.41 ± 0.12	0.03 ± 0.00	4.11 ± 0.01	56.43 ± 0.09
	3	0.04 ± 0.00	0.52 ± 0.03	7.51 ± 0.06	18.60 ± 0.12	0.03 ± 0.01	2.88 ± 0.01	61.26 ± 0.20
peanut	1	-	0.03 ± 0.00	9.64 ± 0.01	0.04 ± 0.00	0.14 ± 0.00	2.86 ± 0.01	49.37 ± 0.10
	2	-	0.03 ± 0.00	9.70 ± 0.01	0.04 ± 0.00	0.14 ± 0.00	2.88 ± 0.00	49.65 ± 0.00
	3	-	0.02 ± 0.00	7.25 ± 0.05	0.07 ± 0.00	0.10 ± 0.00	2.83 ± 0.02	69.36 ± 0.22
pecan nut	1	-	0.03 ± 0.00	5.50 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	2.96 ± 0.00	64.05 ± 0.01
	2	-	0.05 ± 0.00	6.34 ± 0.01	0.06 ± 0.00	0.06 ± 0.00	2.46 ± 0.00	54.39 ± 0.01
	3	-	0.03 ± 0.00	5.79 ± 0.02	0.06 ± 0.00	0.06 ± 0.00	1.95 ± 0.03	63.91 ± 0.01
pine nut	1	-[a]	0.03 ± 0.00	4.86 ± 0.01	0.06 ± 0.00	0.05 ± 0.00	2.14 ± 0.00	40.85 ± 0.01
	2	-	0.04 ± 0.00	6.31 ± 0.01	0.13 ± 0.00	0.07 ± 0.00	3.53 ± 0.01	38.54 ± 0.01
	3	-	0.04 ± 0.00	6.22 ± 0.05	0.08 ± 0.00	0.06 ± 0.00	3.48 ± 0.10	39.43 ± 0.69
pistachio	1	-	0.07 ± 0.00	11.34 ± 0.05	1.08 ± 0.01	0.04 ± 0.00	1.19 ± 0.00	52.63 ± 0.03
	2	-	0.08 ± 0.00	9.53 ± 0.01	0.81 ± 0.00	0.04 ± 0.00	1.01 ± 0.01	56.18 ± 0.01
	3	-	0.08 ± 0.00	8.55 ± 0.19	0.69 ± 0.02	0.03 ± 0.00	0.96 ± 0.03	56.71 ± 0.72
walnut	1	-	0.02 ± 0.00	6.94 ± 0.06	0.12 ± 0.00	0.04 ± 0.00	2.47 ± 0.02	19.07 ± 0.06
	2	-	0.02 ± 0.00	7.00 ± 0.04	0.13 ± 0.00	0.04 ± 0.00	2.32 ± 0.00	15.94 ± 0.02
	3	-	0.01 ± 0.00	6.43 ± 0.01	0.08 ± 0.00	0.04 ± 0.00	2.44 ± 0.00	17.51 ± 0.01

Table 41. continued.

	no.	C18:2 [%]	C18:3 [%]	C20:0 [%]	C20:1 [%]	C22:0 [%]	C22:1 [%]	C24:0 [%]
almond	1	17.43 ± 0.01	0.05 ± 0.00	0.08 ± 0.00	0.07 ± 0.01	0.02 ± 0.00	-	0.01 ± 0.00
	2	20.67 ± 0.08	0.05 ± 0.00	0.07 ± 0.00	0.09 ± 0.00	0.02 ± 0.00	-	0.01 ± 0.00
	3	28.89 ± 0.00	0.03 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.02 ± 0.00	-	0.01 ± 0.00
Brazil nut	1	41.88 ± 0.01	0.07 ± 0.01	0.28 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	-	0.03 ± 0.00
	2	40.43 ± 0.04	0.08 ± 0.00	0.30 ± 0.01	0.07 ± 0.01	0.06 ± 0.00	-	0.03 ± 0.01
	3	41.69 ± 0.05	0.08 ± 0.01	0.27 ± 0.00	0.07 ± 0.00	0.05 ± 0.00	-	0.02 ± 0.00
cashew nut	1	19.36 ± 0.00	0.16 ± 0.00	0.70 ± 0.00	0.18 ± 0.00	0.16 ± 0.00	-	0.16 ± 0.00
	2	19.62 ± 0.02	0.14 ± 0.01	0.72 ± 0.02	0.15 ± 0.01	0.14 ± 0.01	-	0.13 ± 0.01
	3	18.40 ± 0.00	0.14 ± 0.00	0.59 ± 0.00	0.21 ± 0.00	0.13 ± 0.00	-	0.15 ± 0.00
hazelnut	1	12.36 ± 0.13	0.12 ± 0.01	0.13 ± 0.00	0.17 ± 0.01	0.03 ± 0.00	-	0.01 ± 0.00
	2	15.34 ± 0.60	0.15 ± 0.01	0.11 ± 0.01	0.15 ± 0.01	0.02 ± 0.00	-	-
	3	6.84 ± 0.00	0.07 ± 0.00	0.14 ± 0.00	0.16 ± 0.00	0.03 ± 0.00	-	
macadamia	1	2.14 ± 0.01	0.17 ± 0.00	2.93 ± 0.13	3.10 ± 0.08	0.96 ± 0.01	0.38 ± 0.01	0.44 ± 0.02
	2	1.90 ± 0.01	0.17 ± 0.01	3.00 ± 0.02	2.47 ± 0.02	0.82 ± 0.02	0.25 ± 0.00	0.36 ± 0.01
	3	2.33 ± 0.03	0.19 ± 0.01	2.54 ± 0.01	3.00 ± 0.01	0.52 ± 0.45	0.30 ± 0.02	0.28 ± 0.01
peanut	1	30.85 ± 0.07	0.05 ± 0.00	1.37 ± 0.01	1.01 ± 0.00	3.13 ± 0.20	0.05 ± 0.00	1.47 ± 0.01
	2	31.02 ± 0.00	0.05 ± 0.00	1.38 ± 0.00	1.01 ± 0.00	2.52 ± 0.01	0.06 ± 0.00	1.47 ± 0.00
	3	13.08 ± 0.02	-	1.40 ± 0.01	1.65 ± 0.01	2.74 ± 0.13	0.13 ± 0.01	1.38 ± 0.15
pecan nut	1	25.74 ± 0.01	1.11 ± 0.01	0.14 ± 0.00	0.30 ± 0.01	0.03 ± 0.00	-	0.01 ± 0.00
	2	34.78 ± 0.04	1.37 ± 0.04	0.13 ± 0.00	0.31 ± 0.01	0.03 ± 0.00	-	0.01 ± 0.00
	3	26.59 ± 0.02	1.17 ± 0.02	0.11 ± 0.00	0.28 ± 0.01	0.03 ± 0.00	-	0.01 ± 0.00
pine nut	1	50.08 ± 0.01	0.30 ± 0.02	0.57 ± 0.00	0.84 ± 0.02	0.17 ± 0.00	-	0.06 ± 0.00
	2	48.73 ± 0.02	0.48 ± 0.02	0.61 ± 0.00	0.85 ± 0.01	0.15 ± 0.00	-	0.04 ± 0.00
	3	48.70 ± 0.47	0.56 ± 0.03	0.57 ± 0.07	0.72 ± 0.04	0.12 ± 0.01	-	0.03 ± 0.00
pistachio	1	32.53 ± 0.02	0.47 ± 0.01	0.14 ± 0.00	0.39 ± 0.00	0.09 ± 0.00	-	0.04 ± 0.00
	2	31.33 ± 0.00	0.34 ± 0.01	0.14 ± 0.00	0.40 ± 0.01	0.09 ± 0.00	-	0.04 ± 0.00
	3	32.02 ± 0.47	0.34 ± 0.01	0.12 ± 0.01	0.40 ± 0.04	0.07 ± 0.01	-	0.02 ± 0.00
walnut	1	58.15 ± 0.22	12.86 ± 0.08	0.09 ± 0.00	0.19 ± 0.00	0.03 ± 0.00	-	0.01 ± 0.00
	2	60.41 ± 0.09	13.84 ± 0.10	0.12 ± 0.07	0.14 ± 0.09	0.03 ± 0.00	-	0.01 ± 0.00
	3	59.37 ± 0.03	13.76 ± 0.03	0.09 ± 0.00	0.21 ± 0.00	0.02 ± 0.00	-	0.03 ± 0.00

[a] (-) Content below limit of detection.

Table 42. Fatty acid methyl ester compositions of rapeseed oils, sunflower oils, and olive oils.

	no.	C14:0 [%]	C16:0 [%]	C16:1 [%]	C17:0 [%]	C18:0 [%]	C18:1 [%]	C18:2 [%]
rapeseed oil	1	0.04 ± 0.00	4.26 ± 0.01	0.20 ± 0.00	0.05 ± 0.00	1.67 ± 0.00	63.07 ± 0.01	19.85 ± 0.02
	2	0.04 ± 0.00	4.22 ± 0.00	0.20 ± 0.00	0.05 ± 0.00	1.68 ± 0.00	63.42 ± 0.00	19.49 ± 0.03
	3	0.04 ± 0.00	4.19 ± 0.00	0.20 ± 0.00	0.05 ± 0.00	1.64 ± 0.00	63.32 ± 0.00	19.53 ± 0.02
	4	0.03 ± 0.01	4.26 ± 0.00	0.21 ± 0.00	0.05 ± 0.00	1.66 ± 0.01	63.31 ± 0.02	19.84 ± 0.01
	5	0.04 ± 0.00	4.27 ± 0.09	0.21 ± 0.01	0.05 ± 0.00	1.73 ± 0.03	63.01 ± 0.79	19.66 ± 0.43
	6	0.04 ± 0.00	4.19 ± 0.05	0.19 ± 0.00	0.05 ± 0.00	1.87 ± 0.01	65.05 ± 0.06	18.56 ± 0.01
	7	0.04 ± 0.00	4.21 ± 0.01	0.21 ± 0.00	0.05 ± 0.00	1.69 ± 0.01	62.15 ± 0.01	19.96 ± 0.01
	8	0.03 ± 0.00	4.16 ± 0.00	0.18 ± 0.00	0.05 ± 0.00	1.64 ± 0.00	62.75 ± 0.00	19.71 ± 0.01
	9	0.04 ± 0.00	4.21 ± 0.01	0.19 ± 0.00	0.05 ± 0.00	1.73 ± 0.00	62.41 ± 0.00	19.20 ± 0.02
	10	0.04 ± 0.00	4.30 ± 0.02	0.19 ± 0.00	0.04 ± 0.00	1.85 ± 0.00	64.74 ± 0.01	18.62 ± 0.07
	11	0.04 ± 0.00	3.88 ± 0.01	0.17 ± 0.00	0.04 ± 0.00	1.66 ± 0.00	63.00 ± 0.00	19.19 ± 0.02
	12	0.04 ± 0.00	4.25 ± 0.02	0.18 ± 0.00	0.05 ± 0.00	1.70 ± 0.00	61.96 ± 0.02	20.71 ± 0.12
	13	0.04 ± 0.00	4.23 ± 0.01	0.19 ± 0.00	0.05 ± 0.00	1.79 ± 0.02	67.46 ± 0.01	16.99 ± 0.06
	14	0.03 ± 0.00	3.90 ± 0.00	0.17 ± 0.00	0.05 ± 0.01	1.65 ± 0.01	62.93 ± 0.01	19.17 ± 0.01
	15	0.04 ± 0.00	4.27 ± 0.01	0.20 ± 0.00	0.05 ± 0.00	1.88 ± 0.00	65.70 ± 0.01	18.27 ± 0.07
sunflower oil	1	0.05 ± 0.00	5.82 ± 0.01	0.06 ± 0.00	0.04 ± 0.00	3.94 ± 0.01	22.51 ± 0.02	65.94 ± 0.02
	2	0.05 ± 0.00	6.23 ± 0.02	0.08 ± 0.00	0.04 ± 0.00	3.56 ± 0.01	27.75 ± 0.17	60.73 ± 0.21
	3	0.05 ± 0.00	6.09 ± 0.04	0.07 ± 0.00	0.04 ± 0.00	3.83 ± 0.02	23.63 ± 0.44	64.55 ± 0.36
	4	0.05 ± 0.00	5.80 ± 0.03	0.06 ± 0.00	0.04 ± 0.00	3.97 ± 0.00	21.28 ± 0.01	67.24 ± 0.02
	5	0.05 ± 0.00	6.12 ± 0.01	0.08 ± 0.00	0.04 ± 0.00	3.52 ± 0.00	25.52 ± 0.00	63.14 ± 0.01
	6	0.06 ± 0.00	6.48 ± 0.10	0.11 ± 0.01	0.03 ± 0.00	3.15 ± 0.14	27.08 ± 0.21	61.55 ± 0.24
	7	0.05 ± 0.00	5.65 ± 0.01	0.09 ± 0.00	0.03 ± 0.00	3.26 ± 0.00	34.98 ± 0.01	54.08 ± 0.01
	8	0.05 ± 0.00	5.94 ± 0.01	0.10 ± 0.00	0.04 ± 0.00	3.71 ± 0.00	27.22 ± 0.00	61.25 ± 0.02
	9	0.05 ± 0.00	5.70 ± 0.01	0.08 ± 0.00	0.04 ± 0.00	3.40 ± 0.00	27.50 ± 0.01	61.75 ± 0.02
	10	0.06 ± 0.00	5.98 ± 0.12	0.09 ± 0.00	0.04 ± 0.00	2.93 ± 1.93	30.31 ± 0.61	59.08 ± 1.17
olive oil	1	0.01 ± 0.00	11.50 ± 0.01	1.03 ± 0.00	0.07 ± 0.00	2.89 ± 0.00	73.42 ± 0.00	9.50 ± 0.00
	2	0.01 ± 0.00	11.05 ± 0.01	0.74 ± 0.00	0.06 ± 0.00	2.79 ± 0.00	77.40 ± 0.06	6.21 ± 0.00
	3	0.01 ± 0.00	10.52 ± 0.09	0.67 ± 0.00	0.05 ± 0.00	3.17 ± 0.02	78.69 ± 0.10	5.24 ± 0.01
	4	0.01 ± 0.00	11.77 ± 0.17	0.76 ± 0.00	0.05 ± 0.00	3.02 ± 0.01	75.88 ± 0.12	6.39 ± 0.03
	5	0.01 ± 0.00	11.30 ± 0.33	0.83 ± 0.01	0.05 ± 0.00	2.53 ± 0.01	77.35 ± 0.27	6.29 ± 0.05
	6	0.01 ± 0.00	10.57 ± 0.05	0.64 ± 0.01	0.05 ± 0.00	2.47 ± 0.00	77.37 ± 0.04	7.20 ± 0.01
	7	0.01 ± 0.00	10.52 ± 0.01	0.76 ± 0.00	0.05 ± 0.00	3.28 ± 0.00	78.51 ± 0.01	5.35 ± 0.01

Table 42. continued.

	no.	C18:3 [%]	C20:0 [%]	C20:1 [%]	C22:0 [%]	C22:1 [%]	C24:0 [%]
rapeseed oil	1	7.96 ± 0.01	0.60 ± 0.01	1.46 ± 0.00	0.36 ± 0.00	0.34 ± 0.00	0.14 ± 0.00
	2	8.41 ± 0.03	0.58 ± 0.00	1.31 ± 0.00	0.32 ± 0.00	0.16 ± 0.00	0.13 ± 0.01
	3	8.43 ± 0.02	0.58 ± 0.00	1.37 ± 0.00	0.33 ± 0.00	0.18 ± 0.00	0.13 ± 0.00
	4	7.76 ± 0.01	0.59 ± 0.00	1.42 ± 0.00	0.35 ± 0.00	0.38 ± 0.00	0.15 ± 0.02
	5	8.44 ± 0.19	0.59 ± 0.01	1.36 ± 0.03	0.33 ± 0.01	0.19 ± 0.01	0.13 ± 0.00
	6	7.22 ± 0.02	0.61 ± 0.00	1.42 ± 0.00	0.32 ± 0.00	0.34 ± 0.00	0.14 ± 0.00
	7	8.93 ± 0.01	0.60 ± 0.00	1.41 ± 0.00	0.35 ± 0.00	0.29 ± 0.00	0.13 ± 0.00
	8	8.65 ± 0.01	0.57 ± 0.00	1.42 ± 0.00	0.35 ± 0.03	0.37 ± 0.03	0.12 ± 0.00
	9	8.90 ± 0.02	0.60 ± 0.00	1.53 ± 0.00	0.34 ± 0.00	0.67 ± 0.00	0.12 ± 0.00
	10	7.60 ± 0.09	0.67 ± 0.01	1.32 ± 0.01	0.37 ± 0.00	0.09 ± 0.00	0.17 ± 0.00
	11	9.62 ± 0.02	0.56 ± 0.00	1.33 ± 0.00	0.30 ± 0.00	0.10 ± 0.00	0.12 ± 0.00
	12	8.66 ± 0.15	0.59 ± 0.00	1.31 ± 0.00	0.34 ± 0.00	0.10 ± 0.00	0.13 ± 0.00
	13	6.65 ± 0.09	0.63 ± 0.00	1.29 ± 0.01	0.39 ± 0.01	0.12 ± 0.01	0.17 ± 0.01
	14	9.64 ± 0.01	0.56 ± 0.00	1.35 ± 0.00	0.30 ± 0.00	0.13 ± 0.00	0.12 ± 0.00
	15	7.27 ± 0.07	0.62 ± 0.00	1.20 ± 0.00	0.32 ± 0.00	0.04 ± 0.00	0.15 ± 0.00
sunflower oil	1	0.21 ± 0.01	0.26 ± 0.00	0.19 ± 0.00	0.74 ± 0.00	0.01 ± 0.00	0.22 ± 0.00
	2	0.07 ± 0.02	0.25 ± 0.00	0.18 ± 0.00	0.79 ± 0.01	-	0.25 ± 0.00
	3	0.14 ± 0.01	0.26 ± 0.00	0.21 ± 0.00	0.77 ± 0.01	0.14 ± 0.00	0.23 ± 0.00
	4	0.13 ± 0.01	0.26 ± 0.00	0.18 ± 0.00	0.76 ± 0.02	0.01 ± 0.00	0.23 ± 0.00
	5	0.06 ± 0.00	0.25 ± 0.00	0.17 ± 0.00	0.79 ± 0.00	- [a]	0.25 ± 0.00
	6	0.08 ± 0.01	0.25 ± 0.00	0.18 ± 0.00	0.75 ± 0.02	-	0.27 ± 0.01
	7	0.33 ± 0.00	0.26 ± 0.00	0.23 ± 0.00	0.77 ± 0.00	0.01 ± 0.00	0.25 ± 0.00
	8	0.23 ± 0.03	0.28 ± 0.00	0.20 ± 0.00	0.73 ± 0.00	0.01 ± 0.00	0.24 ± 0.00
	9	0.05 ± 0.00	0.24 ± 0.00	0.17 ± 0.00	0.76 ± 0.00	-	0.25 ± 0.00
	10	0.06 ± 0.00	0.28 ± 0.01	0.16 ± 0.00	0.75 ± 0.01	-	0.25 ± 0.00
olive oil	1	0.67 ± 0.00	0.43 ± 0.00	0.30 ± 0.00	0.13 ± 0.00	-	0.06 ± 0.00
	2	0.67 ± 0.00	0.48 ± 0.00	0.35 ± 0.00	0.15 ± 0.00	0.01 ± 0.00	0.07 ± 0.00
	3	0.64 ± 0.00	0.47 ± 0.00	0.33 ± 0.00	0.14 ± 0.00	0.01 ± 0.00	0.06 ± 0.00
	4	0.92 ± 0.01	0.57 ± 0.00	0.36 ± 0.00	0.19 ± 0.00	-	0.08 ± 0.00
	5	0.64 ± 0.00	0.46 ± 0.00	0.32 ± 0.01	0.15 ± 0.00	-	0.07 ± 0.00
	6	0.66 ± 0.02	0.44 ± 0.00	0.39 ± 0.00	0.13 ± 0.00	-	0.06 ± 0.00
	7	0.67 ± 0.01	0.41 ± 0.00	0.27 ± 0.00	0.12 ± 0.00	-	0.05 ± 0.00

[a] (-) Content below limit of detection.

Table 43. Fatty acid methyl ester compositions of corn germ, grape seed, linseed, safflower, sesame seed, and soybean oil.

	no.	C14:0 [%]	C16:0 [%]	C16:1 [%]	C17:0 [%]	C18:0 [%]	C18:1 [%]	C18:2 [%]
corn germ oil	1	0.03 ± 0.00	11.56 ± 0.03	0.10 ± 0.00	0.06 ± 0.00	1.89 ± 0.00	29.95 ± 0.03	54.46 ± 0.05
	2	0.02 ± 0.00	10.55 ± 0.01	0.09 ± 0.00	0.06 ± 0.00	1.88 ± 0.00	30.21 ± 0.00	55.39 ± 0.01
grape seed oil		0.03 ± 0.00	6.52 ± 0.02	0.10 ± 0.00	0.06 ± 0.00	3.91 ± 0.02	18.59 ± 0.57	70.10 ± 0.53
linseed oil		0.03 ± 0.00	4.85 ± 0.01	0.05 ± 0.00	0.06 ± 0.00	4.59 ± 0.00	16.36 ± 0.02	15.47 ± 0.13
safflower oil		0.05 ± 0.00	4.59 ± 0.04	0.08 ± 0.00	0.03 ± 0.00	2.26 ± 0.00	76.21 ± 0.01	15.14 ± 0.01
sesame seed oil		0.01 ± 0.00	9.18 ± 0.09	0.11 ± 0.00	0.06 ± 0.00	5.83 ± 0.04	41.75 ± 0.46	41.74 ± 0.32
soybean oil		0.06 ± 0.00	10.17 ± 0.02	0.10 ± 0.00	0.09 ± 0.00	3.36 ± 0.00	26.62 ± 0.02	53.08 ± 0.05

Table 43. continued.

	no.	C18:3 [%]	C20:0 [%]	C20:1 [%]	C22:0 [%]	C22:1 [%]	C24:0 [%]
corn germ oil	1	0.74 ± 0.01	0.48 ± 0.00	0.29 ± 0.00	0.16 ± 0.00	0.01 ± 0.00	0.20 ± 0.00
	2	0.68 ± 0.01	0.44 ± 0.00	0.29 ± 0.00	0.14 ± 0.00	0.01 ± 0.00	0.18 ± 0.00
grape seed oil		0.22 ± 0.01	0.17 ± 0.00	0.21 ± 0.00	0.07 ± 0.00	-[a]	0.03 ± 0.00
linseed oil		58.08 ± 0.12	0.15 ± 0.00	0.13 ± 0.00	0.14 ± 0.00	0.01 ± 0.00	0.09 ± 0.00
safflower oil		0.11 ± 0.00	0.50 ± 0.00	0.35 ± 0.00	0.39 ± 0.01	0.08 ± 0.00	0.21 ± 0.00
sesame seed oil		0.25 ± 0.01	0.64 ± 0.00	0.21 ± 0.00	0.13 ± 0.00	-	0.10 ± 0.00
soybean oil		5.35 ± 0.08	0.35 ± 0.00	0.24 ± 0.01	0.42 ± 0.07	0.01 ± 0.00	0.15 ± 0.00

[a] (-) Content below limit of detection.

9 PUBLICATIONS AND PRESENTATIONS

PUPLICATIONS (peer-reviewed)

Esche, R.; Scholz, B.; Engel, K.-H., GC and on-line LC-GC: Useful tools for the qualitative and quantitative analysis of phytosterols and their esters. In *Instrumental Methods for the Analysis of Bioactive Molecules*, Patil, B. S., Jayaprakasha, G. K. & Pellati, F. (Eds.); American Chemical Society: Washington, DC; **2013**, *submitted*.

Esche, R.; Müller, L.; Engel, K.-H., On-line LC-GC-based analysis of minor lipids in various tree nuts and peanuts *J. Agric. Food Chem.* **2013**, *61*, 11636-11644.

Esche, R.; Scholz, B.; Engel, K.-H., On-line LC-GC analysis of free sterols/stanols and intact steryl/stanyl esters in cereals *J. Agric. Food Chem.* **2013**, *61*, 10932-10939.

Esche, R.; Scholz, B.; Engel, K.-H., Analysis of free phytosterols/stanols and their intact fatty acid and phenolic acid esters in various corn cultivars *J. Cereal Sci.* **2013**, *58*, 333-340

Esche, R.; Barnsteiner, A.; Scholz, B.; Engel, K.-H., Simultaneous analysis of free phytosterols/phytostanols and intact phytosteryl/phytostanyl fatty acid and phenolic acid esters in cereals. *J. Agric. Food Chem.* **2012**, *60*, 5330-5339.

ORAL PRESENTATIONS

Esche, R.; Scholz, B.; Engel, K.-H., GC and on-line LC-GC: Useful tools for the qualitative and quantitative analysis of phytosterols and their esters. *246th American Chemical Society National Meeting & Exposition, Symposium: Instrumental Methods for the Analysis of Bioactive Molecules* **2013**, Indianapolis, IN, USA.

Esche, R.; Scholz, B.; Engel, K.-H., LC-GC-MSD Kopplung: Praxisanwendung, Leistung und Grenzen. *Agilent GC/GC-MSD Anwendertreffen* **2013**, Garching, Germany.

Esche, R.; Scholz, B.; Engel, K.-H., Phytosterole und Phytosterylester - ernährungsphysiologisch interessante Inhaltsstoffe von Getreiden. *5. Wissenschaftliches Symposium des Verbandes Deutscher Mühlen e.V.* **2012**, Würzburg, Germany.

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POSTER PRESENTATIONS

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Esche, R.; Scholz, B.; Engel, K.-H., Methoden zur simultanen Bestimmung der Profile freier Phytosterole/-stanole sowie intakter Phytosteryl/-stanylester in Getreide. *49. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung e.V.* **2012**, Freising, Germany.

OTHERS

Esche, R.; Günther, J.; Engel, K.-H., Simultane Analytik freier Phytosterole, intakter Phytosterylester und anderer Minorlipide in pflanzlichen Ölen. *Lebensmittelchemie* **2013**, *67*, 68-69.

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10 CURRICULUM VITAE

PERSONAL INFORMATION

Name:	Rebecca Esche
Date of birth:	10.01.1986
Place of birth:	Frankenberg, Germany
Current address:	Frühlingstr. 2 85354 Freising, Germany

WORK EXPERIENCE

03/2014 – to date	Study Director Analytics BSL BIOSERVICE Scientific Laboratories GmbH, Planegg, Germany
05/2010 – 10/2013	Research assistant (PhD Student) Technische Universität München, Center of Life and Food Sciences Weihenstephan, Chair of General Food Technology (Univ.-Prof. Dr. Karl-Heinz Engel), Freising, Germany PhD Thesis: "New Methodologies for the Analysis of Phytosterols and Their Intact Esters: Applications to Cereals, Nuts, and Edible Plant Oils.
10/2008 – 02/2009	Internship in Research and Development Chocoladenfabriken LINDT & SPRÜNGLI GmbH, Aachen, Germany

ACADEMIC STUDIES AND EDUCATION

02/2010	Degree in Food Chemistry (Diplom-Lebensmittelchemikerin) Technische Universität Dresden, Faculty of Science, Department of Chemistry and Food Chemistry, Chair of Special Food Chemistry and Food Production (Prof. Dr. Karl Speer), Dresden, Germany Diplom Thesis: „Methodenentwicklung zur Analytik organischer Säuren in Honig"
08/2008	Degree in Food Chemistry (1. Staatsexamen) Technische Universität Dresden, Faculty of Science, Department of Chemistry and Food Chemistry, Dresden, Germany
10/2004 – 03/2010	Academic Studies of Food Chemistry Technische Universität Dresden, Faculty of Science, Department of Chemistry and Food Chemistry, Dresden, Germany
09/1998 – 07/2004	Allgemeine Hochschulreife Städtisches Gymnasium, Mittweida, Germany