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Extraction and gastro-intestinal digestion of phytosteryl ferulates and phytosteryl glycosides

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ABSTRACT

Phytosterols are known to decrease the atherogenic LDL-cholesterol. In earlier studies, free phytosterols and their fatty acid esters have been extensively studied for their cholesterol-lowering properties. However, the metabolic fate of phytosterols still need investigations. This dissertation focused on phytosteryl ferulates and phytosteryl glycosides. The aim was to study the digestion of phytosteryl ferulates and phytosteryl glycosides under stomach and small intestinal conditions.

Two methods were tested for phytosteryl ferulates and phytosteryl glycosides extraction. Secondly, stigmasterol, phytosteryl ferulates and phytosteryl glycosides were digested under stomach and small intestinal conditions.

Soxhlet extraction, followed by fractionation by NP-SPE was found to be suitable for phytosteryl ferulates and phytosteryl glycosides extraction. The pH was found to have an effect on phytosteryl ferulates. Phytosteryl ferulates and phytosteryl glycosides fractions and stigmasterol were tested for their stability under stomach and small intestinal conditions. The amounts of stigmasterol recovered and the RP-HPLC chromatograms showed that stigmasterol was stable under stomach and small intestinal conditions. No new peaks were detected when phytosteryl ferulates and phytosteryl glycosides samples were digested under stomach conditions. The disappearance of peaks and formation of new peaks, as showed by the RP-HPLC chromatograms, gave an indication that phytosteryl ferulates and phytosteryl glycosides samples contained compounds which were unstable under small intestinal conditions.

LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
ACAT	Acyl-CoA Cholesterol Acyl Transferase
ANOVA	Analysis of Variance
APPI	Atmospheric pressure photoionization
ASG	Acylated steryl glycoside
ATP	Adenosine Tri-Phosphate
BEH	Ethylene Bridged Hybrid
CaCO	Colon Adenocarcinoma Cell Line
EB	Enzyme blank
EU	European Union
FDA	Food and Drug Administration
FS	Free Sterol
GC	Gas Chromatography
LDL	Low Density Lipoprotein
LOD	Limit of Detection
LOQ	Limit of Quantitation
LXR	Liver X Receptor
m/z	Mass/charge
MeOH	Methanol
MS	Mass Spectrometry
n.d	Not Detected
NPC1L1	Niemann-Pick C1-Like Protein
NP-SPE	Normal Phase Solid Phase Extraction
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RSD	Relative Standard Deviation
RXR	Retinoid X Receptor
SB	Substrate blank
SCF	Scientific Committee on Food
SD	Standard Deviation
SE	Steryl Ester
SG	Steryl Glycoside
SPE	Solid Phase Extraction
UPLC	Ultra High Performance Chromatography
USA	United States of America
v/v	Volume/Volume
w/w	Weight/Weight

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Chapter 1 INTRODUCTION

Phytosterols, or plant sterols, are bioactive compounds, which are naturally present in plants. They are only synthesized in plants and not in the human body (Garti & Romer, 2006), which means that phytosterols found in human body come from the diet. More than 200 different types of phytosterols have been reported to be present in different plants (Farre *et al*., 2006; Piironen *et al*., 2000a). Phytosterols are heterogeneous compounds, but the three most abundant forms of phytosterols in foods are β-sitosterol, stigmasterol and campesterol (Amiot *et al*., 2011; Moreau *et al*., 2002). In some literature, the term "phytosterols" is collectively used to denote both phytosterols and their saturated forms, phytostanols. As an example, sitostanol is the saturated form of sitosterol, whereas saturation of campesterol gives rise to campestanol.

Phytosterols can occur as free sterols (FS) (phytosterol with a free hydroxyl group), and as steryl esters (SE) of fatty acids or phenolic acids, mostly ferulic acid. They can also form glycosidic linkages with sugars giving rise to phytosteryl glycosides (SG), which in turn may be esterified with a fatty acid to form acylated steryl glycosides (ASG) (Habib-Jiwan *et al*., 2003; Nystrom *et al*., 2008; Nystrom *et al*., 2007b). SE, SG and ASG are also called phytosterol conjugates.

Phytosterols and cholesterol are structurally related. The only differences are the modifications at C-24 position of the hydrophobic carbon chain of phytosterols. However, in spite of the structural similarities, the absorption efficiency of phytosterols and that of cholesterol in humans is different. The percent absorption of phytosterols ranges from 2-5% (Ostlund *et al*., 2002a) while that of cholesterol is nearly 60% (Bosner *et al*., 1999).

Phytosterols have received attention because of their health benefits. Several studies have demonstrated their ability to lower serum total and low-density lipoprotein (LDL) cholesterol levels in the human body (Demonty *et al*., 2009; Scholle *et al*., 2009; Weststrate & Meijer, 1998). However, the exact mechanisms by which phytosterols reduce the absorption of cholesterol are not fully understood (Hearty *et al*., 2009). Pelletier *et al*. (1995) and Jones *et al*. (1997) reported that up to 10% and 15% reduction of total and low-density lipoprotein (LDL) cholesterol, respectively, could be achieved by consuming phytosterol-enriched diet. Phytosterols reduce the LDL-cholesterol levels in a dose-dependent manner (Richelle *et al.*, 2004). However, there is no additional effect from an intake above 3g/day (Ostlund, 2002).

The form of phytosterols is of vital importance for their bioactivity as well as for their technological applications (Piironen *et al.*, 2002). Phytosterols are thought to be effective in

inhibiting cholesterol absorption in their free form (Christiansen *et al.*, 2001b). However, free phytosterols are insoluble in water and marginally soluble in fats (Salo & Wester, 2005). Attempts to improve their solubility included emulsification of free phytosterols in lecithin (Ostlund *et al*., 1999), use of microcrystalline form (Christiansen *et al*., 2001b) and microencapsulated form of free phytosterols (Quilez *et al*., 2003a).

Esterification of free phytosterols and phytostanols with fatty acids was used in order to increase their solubility in lipids and to be able to incorporate them into lipid-based food products such as margarines, spreads and salad dressings (Jandacek *et al*., 1977; Quilez *et al*., 2003a). The esterification of free phytosterols and phytostanols increased their solubility by a ten-fold and provided a means of administering reasonable amount of phytosterols necessary to reduce cholesterol absorption (Salo & Wester, 2005).

In addition to free phytosterols and their fatty acid esters, phytosteryl ferulates and phytosteryl glycosides are other bioactive forms of phytosterols. Because of their polar groups, phytosteryl ferulates and phytosteryl glycosides may be solubilized in aqueous food products, and as such, they could be another alternative to deliver phytosterols. Besides the cholesterol-lowering effect of phytosteryl ferulates (Orthoefer, 2004), the latter also possess anti-oxidation properties (Kim *et al*., 2001; Nystrom *et al*., 2007a; Nystrom *et al*., 2005). Their anti-inflammation properties were also reported by Akihisa *et al*. (2000) but not extensively studied. On the other hand, the bioactivity of phytosteryl glycosides in humans was studied by Lin *et al*. (2009). The results of their study indicated that phytosteryl glycosides reduce cholesterol absorption by 37.6%. This effect was also reported in other studies that used free phytosterols or phytosterol esters (Law, 2000; Vanstone *et al*., 2002).

In some foods, especially in cereals (Nystrom *et al*., 2007b), considerable amounts of the total phytosterols occur as phytosteryl glycosides and phytosteryl ferulates. In whole wheat flour and cornmeal, they make up to 25% and 55% of the total sterol content, respectively (Nystrom *et al*., 2007b). Taken altogether, phytosteryl ferulates and phytosteryl glycosides are worth attention.

Objectives of the research

Overall objective

The overall aim of this research is to study the behavior of phytosteryl ferulates and phytosteryl glycosides in the upper gastro-intestinal tract.

Specific objectives

In order to achieve the aim, phytosteryl ferulates and phytosteryl glycosides had to be extracted from wheat germs before they could be digested. Then, their behavior under stomach and small intestinal conditions was studied.

Chapter 2 REVIEW OF THE LITERATURE

2.1 Phytosterols – Plant components

Phytosterols are widely found in plant kingdom where they play different roles. It has been found that phytosterols are precursors of plant hormones (e.g. brassinosteroids) that play a role in the embryonic growth and development of plants (Schrick *et al*., 2002; Yokota, 1997). Phytosterols are important structural elements of plant cells where they act as cell membrane reinforcers (Ribeiro *et al*., 2007), and like cholesterol in vertebrates, they stabilize phospholipid bilayers in the plant cell membrane by regulating its fluidity and permeability (Hartmann, 1998).

2.2 Structure and classification of phytosterols

2.2.1 General structure

The general structure of phytosterols consists of a five-membered ring skeleton with an hydroxyl group at the C-3 position of the A-ring, and an alkyl side chain attached to the C-17 of the D-ring (Figure 1). Phytosterols are divided into three groups according to the number of methyl groups present on the C-4 position. Those lacking a methyl group are referred to as 4-desmethylsterols, whereas 4-monomethylsterols and 4-dimethylsterols have one and two methyl groups, respectively (Moreau *et al*., 2002a). Based on the presence or absence of a double bond in the ring skeleton, phytosterols are divided into two classes: (1) phytosterols, with a double bond in their ring skeleton (figure 2), and (2) phytostanols, which have a α-reduced double bond (figure 3). Phytostanols are obtained by hydrogenation of phytosterols either by plant enzymes or during industrial hydrogenation (Bradford & Awad, 2007).

Phytosterols may also be classified into Δ5-sterols and Δ7-sterols based on the position of the double bond in their ring skeleton. The former class of phytosterols is predominant in foods as compared to the latter (Breinholder *et al*., 2002). Figure 2 illustrates the structures of the phytosterols most commonly found in foods, and of cholesterol.

2.2.2 Similarities between phytosterols and cholesterol

Phytosterols are exclusively synthesized in plants (Garti & Romer, 2006) but they are closely related to cholesterol. Cholesterol is an essential sterol which is produced in animals, including humans. Chemically, the structure of phytosterol resembles that of cholesterol except that phytosterols contain an additional aliphatic side chain at C-24 position (Amiot *et al*., 2011) and, in some cases, an unsaturated bond at the C-22 position. For example, both

stigmasterol and sitosterol have an ethyl group at C-24, whereas campesterol has a methyl group at the same position (figure 2).

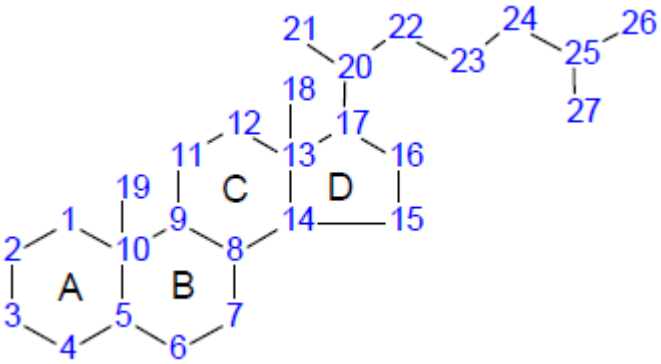

Figure 1 Basic skeleton of (phyto) sterols

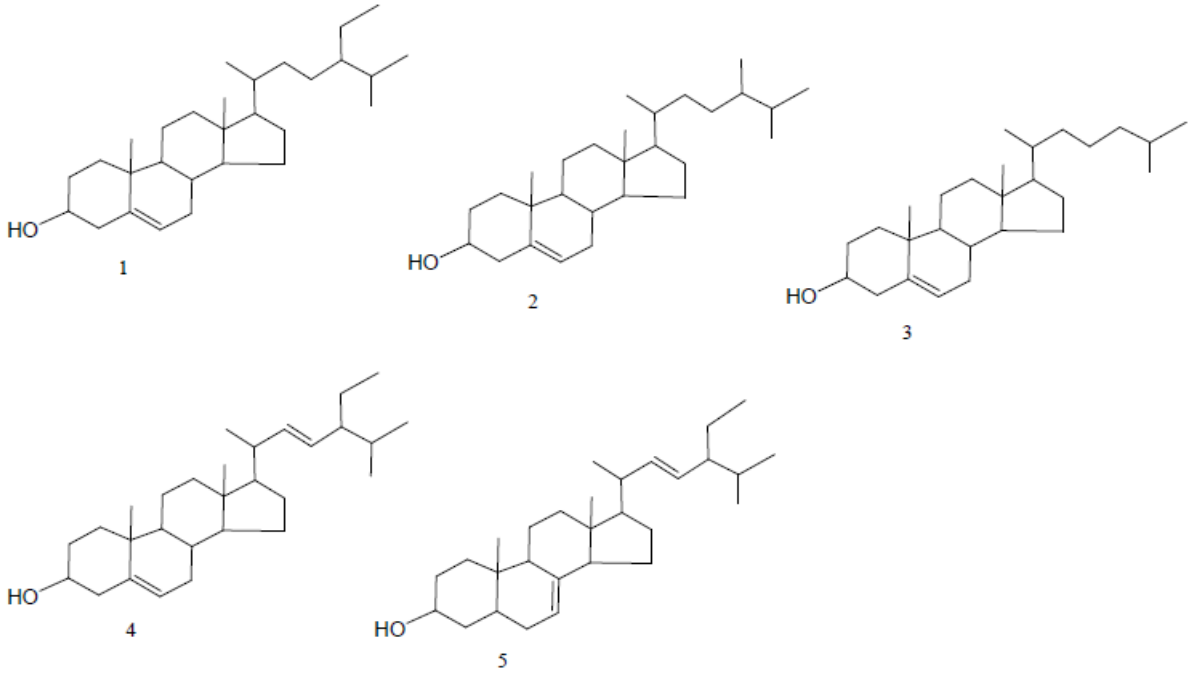

Figure 2 Chemical structures of the common phytosterols and cholesterol (Δ5-sterols and Δ7-sterol); 1=sitosterol, 2=campesterol, 3=cholesterol, 4= stigmasterol, 5=Δ7-stigmasterol. Source: Moreau *et al.* (2002)

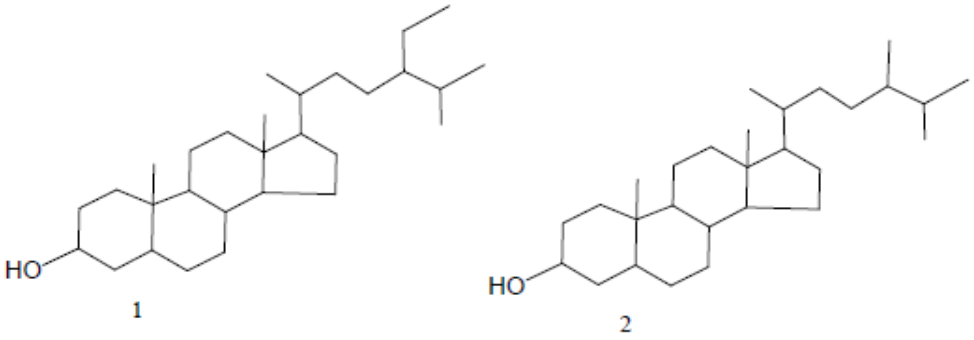

Figure 3 Examples of chemical structures of phytostanols. 1=Sitostanol, 2=campestanol; Source: Breinholder *et al*. (2002)

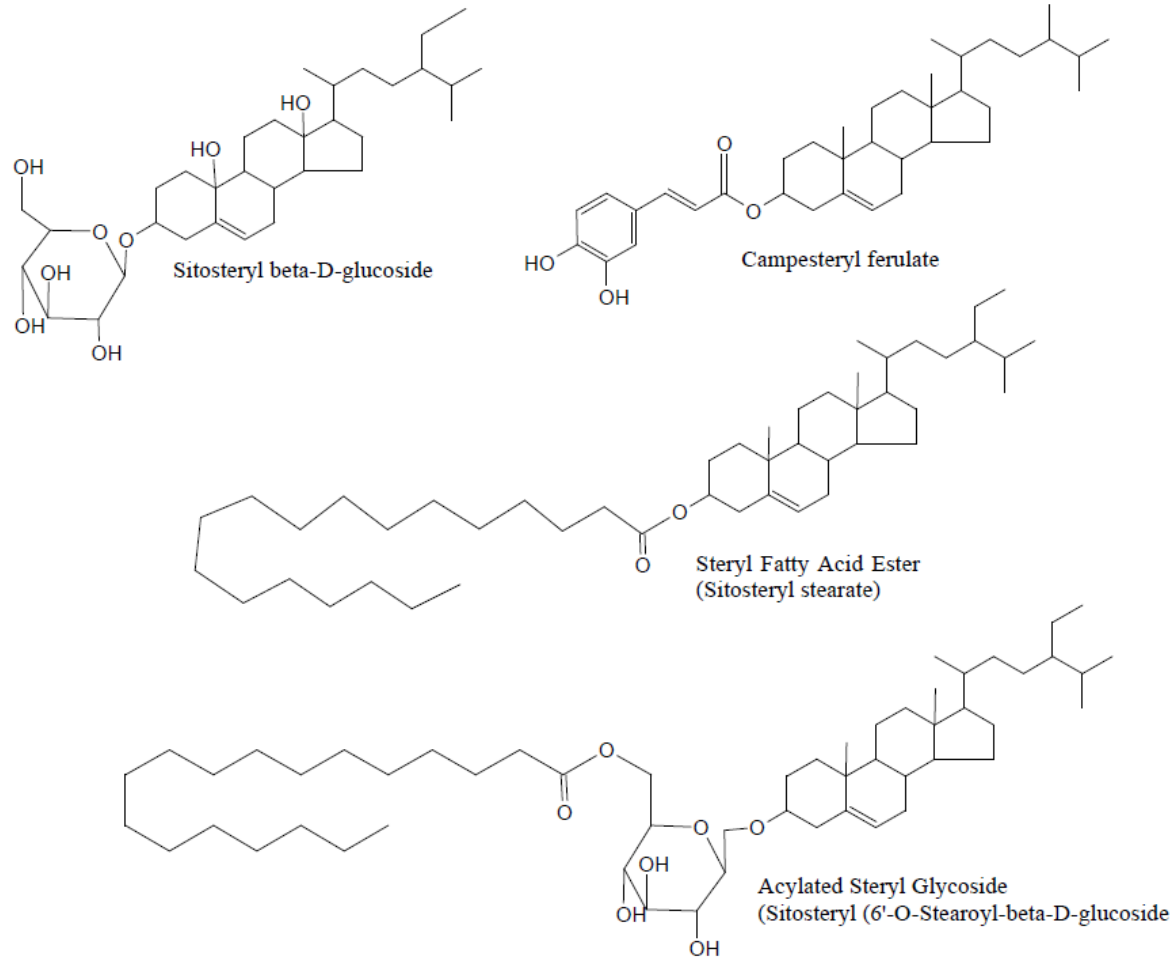

Figure 4 Examples of chemical structures of phytosterol conjugates.

Source: Moreau *et al*. (2002)

2.2.3 Phytosterol conjugates

Phytosterol conjugates refer to the forms of phytosterols in which the hydroxyl group at C-3 position of free phytosterols is coverently bound with another compound. The first type of phytosterol conjugates are formed when the hydroxyl group is linked with an fatty acid to form fatty acid esters. The second type of phytosterol conjugates known as phytosteryl glycosides, are formed through a β-glycosidic bond between the hydroxyl group of free phytosterols and a sugar (mostly glucose). The hydroxyl group at C-6 position of glucose moiety may also be esterified with a fatty acid to form acylated phytosteryl glucoside. The fourth type of phytosterol conjugates are esters of free phytosterols and hydroxycinnamic acids (mostly ferulic and *p*-coumaric acid). Some examples of phytosterol conjugates are shown in the figure 4.

2.3 Occurrence of phytosterols in foods

2.3.1 Total phytosterols content in different foods

Phytosterols are naturally found in the regular human diet particularly in plant food items in which they occur as part of the lipid fraction (Farre *et al*., 2006). Phytosterols occur as free sterols or as phytosterol derivatives (Breinholder *et al*., 2002). β-sitosterol, stigmasterol and campesterol are the most abundant phytosterols in foods and make 95% of the total phytosterols (Maguire *et al*., 2004). Out of these, β-sitosterol is predominant (Farre *et al*., 2006; Normen *et al*., 2002). Phytosterols gained research attention due to their physiological effects. As a result thereof, their levels in different foods, whether natural or added, have been determined and reviewed (Farre *et al*., 2006; Phillips *et al*., 2005; Piironen *et al*., 2000b).

The sources of dietary of phytosterols include vegetable oils (especially unrefined oils), nuts, legumes, cereals (wheat, rye, corn, rice), fruits and vegetables (Farre *et al*., 2006). Vegetable oils are the richest natural sources of phytosterols, followed by cereals and cereal products (Piironen *et al*., 2000a). Among vegetable oils analysed by Piironen *et al*. (2000b), rapeseed and maize oils had total phytosterol content higher than 700 mg/100 g. Gupta *et al*. (2011) reported that the levels of phytosterols found in wheat germ, corn, rapeseed oils were 919 mg/100 g, 909 mg/100 g and 666 mg/100 g, respectively.

Compared to vegetable oils, the total sterol content in cereals is low. Piironen *et al*. (2002) compared the phytosterol (free and bound sterols) content of the main cereals i.e. rye, wheat, barley, oats, grown in the same year in Finland. The results of their study indicated that phytosterol content of rye was the highest with 95.5 mg/100 g on average. The phytosterol content of wheat, barley, and oats was found to be 69.0 mg/100 g, 76.1 mg/100 g and 44.7

mg/100 g, respectively. Sitosterol was reported to be predominant accounting for 90% of the total phytosterol content in all cereals (Piironen *et al*., 2002). Similar results on the phytosterol content of rye were also reported in a study by Nystrom *et al*. (2007b). The total phytosterol content of wheat, on the other hand, was slightly higher (78.3 mg/100 g) than was reported by Piironen *et al*. (2002). However, due to the high consumption of cereals and cereal products (Cordain, 1999), the latter may supply about 40% of daily intake of phytosterols (Valsta *et al*., 2004). In a study by Normen *et al*. (2002), the total phytosterol content in cereal products ranged between 4.1-200 mg/100 g. Bread and other cereal products have been found to be the major contributors to phytosterol intake in Belgium (Sioen *et al*., 2011).

Although vegetable oils and cereals are known to be the richest sources of phytosterols, the data show that the consumption of fruits and vegetables with relatively low amounts of phytosterols also contribute considerably to the total phytosterol intake (Han *et al*., 2008; Normen *et al*., 1999). A study by Han *et al*. (2008) indicated that per 100 g edible portion, the total phytosterol contents in fruits and vegetables ranged from 1.6–32.6 mg and from 1.1–53.7 mg, respectively. Among the fruits and vegetables studied, mango, navel orange, tangerine, pea, cauliflower and broccoli were found to contain high amount of phytosterols (Han *et al*., 2008). Piironen *et al*. (2000b) also reported that the phytosterol content of vegetables ranged from 5–37 mg/100 g and from 250–410 mg/100 g on fresh weight and dry weight basis, respectively. Phytosterol content in fruits and berries ranged from 6–75 mg/100 g on fresh weight basis and from 37–293 mg/100 g on dry weight basis (Piironen *et al*., 2000b).

In addition to the natural sources, phytosterol-enriched food products are currently available on the market and constitute another important source of phytosterols (Amiot *et al*., 2011). The first phytosterol-enriched product was a spread but salad dressing, milk, yoghurt, soy and fruit drinks among others to which phytosterols have been added are also available at the EU market (Weingartner *et al*., 2009).

2.3.2 Phytosteryl ferulates and phytosteryl glycosides in foods

Phytosteryl ferulates and phytosteryl glycosides are less studied. It is speculated that this might be due the their absence or the fact that they are barely present in vegetable oils which is the most studied sources of phytosterols (Nystrom *et al*., 2007b). Popov *et al*. (1975) reported that phytosteryl glycosides could not be detected after refining crude sunflower and corn oils initially containing 30-50 mg/ 100 g of these compounds. Another study by Yoon and Kim (1994) also reported that more than 50% of γ-oryzanol, a mixture of phytosteryl

ferulates isolated from rice bran oils is removed by degumming, alkali refining, bleaching and deodorization (Islam *et al*., 2008).

However, the proportions of phytosteryl ferulates and phytosteryl glycosides are considerable in foods. According to Nystrom *et al*. (2007b), each form may represent about 10–15% of the total sterol content in cereals. In their study, the same authors found that the amount of phytosteryl glycosides in wheat and rye grains was 7.5 mg/100 g and 8.3 mg/100 g on dry weight basis, respectively (Nystrom *et al*., 2007b). The authors further reported that the concentration of phytosteryl ferulates was 5.2 mg/100 g and 4.4 mg/100 g in wheat and rye, respectively (Nystrom *et al*., 2007b). Phillips *et al*. (2005) analyzed the steryl glucosides in different foods and dietary supplements and found that sitosteryl, campesteryl and stigmasteryl glucosides ranged from 9-37% of the total sterol content. Recently, β-sitosterol glucoside was found to be 24.3 mg/100 g in fleshly harvested broccoli (Gajewski *et al*., 2011).

2.4 Estimated phytosterol intake by different populations

2.4.1 Phytosterol intake from natural sources

The dietary phytosterols intake among different populations ranges from 160-400 mg/day (Ahrens & Boucher, 1978; Han *et al*., 2007; Jones *et al*., 1997). However, intake may vary among people. In a nutritional survey by Cerqueira *et al*. (1979), a daily intake of more than 400 mg of phytosterols was recorded in the Tarahumara Indians of Mexico whose diet contains high amounts of beans and corn. Given the differences in culture and dietary patterns, it is not surprising that the intake of phytosterols differs between countries and within individuals or population groups depending on the type and the amount of food consumed. Table 1 shows the estimated average daily intake of naturally occurring phytosterols in some countries. In this table, phytosterol intake is expressed as the sum of individual free phytosterols.

Phytosterol intake depends on the type of food consumed. Vegetarians may consume more phytosterols (Jenkins *et al*., 2003; Piironen *et al*., 2000a). According to Slavin (2004), the vegetarians' diet may supply up to 500 mg of phytosterols daily. Nair *et al*. (1984) compared the phytosterol intake of Seventh-day Adventists (SDA) pure vegetarians, SDA lacto-ovo vegetarians, SDA non-vegetarians and non-vegetarians from the general population in the United States. The ingested (β-sitosterol + stigmasterol)/cholesterol ratio was found to be the highest (16.0) in SDA pure vegetarians. In SDA lacto-ovo vegetarians, the ratio was 3.3, compared to 1.1 in SDA non-vegetarians. In non-vegetarians from the general population, the

ingested (β-sitosterol + stigmasterol)/cholesterol ratio was found to be 0.5. This shows that vegetarians' phytosterol intake is higher compared to non-vegetarians.

Table 1 Average intake of naturally occurring phytosterols in different populations

	Intake of naturally occurring phytosterols (mg/day)			
Country(year)	**Population**	**Men**	**Women**	**Reference**
Belgium (2004)	–	301(131)	229(98)	Sioen *et al*. (2011)
China (2002)	322.41(-)	–	–	Han *et al*. (2007)
Finland (1997)	–	305(-)	237(-)	Valsta *et al*. (2004)
UK (2004)	–	310(108)	303(100)	Andersson *et al*. (2004)
Japan (1957–1982)	373(-)	–	–	Hirai *et al*. (1986)
The Netherlands (1986)	–	307.3(103.9)	262.9(83.7)	Normen *et al*. (2001)
Mexico (1973–1974)	–	473(67)	402(34)	Cerqueira *et al*. (1979)
Spain (2000)	276(-)	–	–	Jimenez-Escrig *et al*. (2006)

SD= standard deviation

2.4.2 Phytosterol-enriched products

In addition to the natural sources, phytosterol-enriched food products are currently available in many countries worldwide (Moreau *et al*., 2002) and constitute another important source of phytosterols in the human diet (Amiot *et al*., 2011). In this category of products, Benecol® and Take Control® margarines were the first to be marketed for lowering of cholesterol. Benecol® (Raisio Plc., Raisio, Finland) was launched in Finland in 1995 and contains 1650 mg phytostanol esters per serving (Bradford & Awad, 2007). The recommended daily intake of Benecol and Benecol products with added phytosterols (fresh cheese, salad dressing, yoghurt and snack bars) is based on 2 g phytostanols (SCF, 2002). Take Control® was first introduced in the USA in 1999 by Unilever. It contains 850 mg phytosterol esters per serving (Bradford & Awad, 2007). In the same year and later in 2000, Take Control® margarine was marketed (under the brand Flora/Becel Pro.Activ) in Australia, New Zealand, Brazil and Switzerland and in the EU. The recommended intake was 28 g/ day, providing 2.2 g phytosterol esters (SCF, 2002). Examples of products with added phytosterol/ phytostanols that are available at the EU market include yellow fat spreads, milk type products, cheese type products, cream cheese, rye bread, etc.(EFSA, 2008).

2.5 Safety aspects concerning phytosterols

At present time, the trend of enrichment of food products with phytosterols is becoming popular (Hearty *et al*., 2009). Like any other substance present in food that may have effect on the consumer health, phytosterols also pose a public health concern. The areas of concern about the consumption of phytosterols are their effects on the serum levels of phytosterols, on fat-soluble vitamins and on the sex hormones (Tikkanen, 2005).

The safety concern regarding the effect of phytosterols on the serum phytosterols and phytostanols concentrations arises from the insight that elevated serum phytosterols is associated with accelerated atherosclerosis and premature coronary heart disease (Bhattacharyya & Connor, 1974; Lee *et al*., 2001a) in a rare autosomally inherited lipid metabolic disorder called phytosterolemia or sitosterolemia. Sitosterolemic patients are marked with high blood levels of phytosterols due to hyperabsorption and decreased excretion of phytosterols into the bile (Lee *et al*., 2001a).

2.5.1 Effect of phytosterols on the absorption of fat-soluble (pro)/ vitamins

Fat-soluble vitamins and other fat-soluble nutrients are absorbed along with lipids in the gastro-intestinal tract. The interference in the intestinal absorption and reduction in carotenoids levels in blood has been reported as being some of the undesired effect of phytosterols (Quilez *et al*., 2003a).

From the study by Judd *et al*. (2002), it was concluded that the consumption of salad dressing containing phytosterols results in a significant reduction in α- and β-carotene and β-cryptoxanthin in the blood. In a randomized parallel-controlled study by Maki *et al*. (2001), the concentration of lycopene (0.10 µmol/L) was found to be out of the US National Health and Nutrition Survey reference range (0.13–0.83 µmol/L) after phytosterol intervention. The authors also reported significant reductions in blood α- and β-carotenoid concentrations in blood even though the values remained within the reference range (Maki *et al*., 2001). Similarly, Weststrate and Meijer (1998a) also found that the consumption of 3 g/day of phytosterol esters resulted in reductions of plasma lycopene (from 85 µg/L to 63 µg/L) and in α- and β-carotenoids levels (from 220 µg/L to 168 µg/L) in normocholesterolemic and mildly hypercholesterolemic individuals. Hendriks *et al*. (1999) also reported that the consumption of spreads providing 0.83, 1.61, 3.24 g of phytosterols reduced α- and β-carotene by 12%, 11% and 19%, respectively. The consumption of 1.61 and 3.24 g of phytosterols decreased α –tocopherol by 6 and 8%, respectively (Hendriks *et al*., 1999).

The data on the effect of phytosterols on fat-soluble nutrients are equivocal. As previously discussed, some studies show that the consumption of phytosterol or phytostanol esters decreases the concentration of fat-soluble (pro) vitamins in blood. However, other studies suggest that these nutrients remain unaffected (Raeini-Sarjaz *et al*., 2002). Hallikainen *et al*. (1999) investigated the effect of low-fat margarines enriched with phytostanol esters on the concentrations of serum carotenoids. From their study, no change in serum retinol concentration was reported after daily consumption of 2.3 g of phytostanol esters for 8 weeks. The authors further concluded that low-fat stanol ester do not significantly lower neither the concentration of lycopene nor that of α- and β-carotene. In another study by Gylling and Miettinen (1999), it was also found that when phytostanol esters were consumed, the serum retinol and vitamin D concentrations as well as α-tocopherol/cholesterol ratio remained unchanged. According to Hendriks *et al*. (1999), the consumption of spreads enriched with phytosterols does not decrease the plasma concentration of vitamins K1 and D.

In any case, the possible negative effect of phytosterol consumption on the fat-soluble vitamins should not be ruled out. Therefore, it is suggested that human studies that investigate the cholesterol-lowering effect of phytosterols should be optimized in such a way to effectively lower the blood cholesterol levels while minimizing the effect on other fat-soluble nutrients (Hendriks *et al*., 1999). In addition, the possible decrease in plasma carotenoids levels can be compensated for by taking more fruits and green leafy vegetables and other good sources of carotenoids while taking phytosterol-enriched products (Noakes *et al.*, 2002; Quilez *et al.*, 2003c).

For the sake of consumer protection, the EU Commission Regulation (EC) No 608/2004 concerning phytosterol-enriched products made it obligatory for food industry to label those products in order to allow users to control their intake. In this regulation, particular attention is paid to children under the age of five years, pregnant and lactating women. Moreover, it is stated that a daily intake of more than 3 g phytosterols/phytostanols should be avoided. (http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32004R0608:EN:HTML).

2.5.2 Effect of phytosterols on hormonal metabolism

Some animal studies suggest that phytosterols may have estrogenic effects (Maclatchy & Vanderkraak, 1995; Mellanen *et al*., 1996) probably due to the resemblance of their ring structure with that of estrogens (Tikkanen, 2005). Mellanen *et al*. (1996) have reported development of infertility when fish were exposed to high levels of wood pulp containing sitosterol. However, an extensive safety evaluation by Baker *et al*. (1999) using *in vitro* and *in*

vivo assays indicates that phytosterols do not bind to estrogen receptors. The authors further reported that phytosterols do not stimulate the transcriptional activity of the human estrogen receptors in a recombinant yeast strain.

A number of clinical studies have concluded that phytosterols are clinically safe when consumed within the recommended intake of 1.5-3.0 g/ day (SCF, 2002). In a study by Hendriks *et al*. (2003), both efficacy and safety of long-term consumption of phytosterol esters-enriched spread were evaluated in 185 individuals. Changes in sex hormone levels in blood served as safety markers. The results of this study reported no hormonal change in males and females after a daily consumption of 20 g of spread enriched with 1.6 g phytosterol for a period of one year. The authors reported that the consumption of spreads enriched with phytosterols is safe and can be used for an extended period of time. In another study, the consumption of 8.6 g phytosterols per day for a period of 21 and 28 consecutive days in males and females, respectively, did not have any effect on serum reproductive hormone levels in females (Weststrate *et al*., 1999b).

2.5.3 Views of regulatory bodies on the safety of phytosterols

As far as safety is concerned, short-term studies (Hendriks *et al.*, 1999; Volpe *et al.*, 2001) and a one-year follow up study (Hendriks *et al*., 2003) did not report any adverse effect of phytosterols. In the USA, phytosterols and phytostanols possess the GRAS (generally recognized as safe) status issued by the Food and Drug Administration (FDA) which approved spreads that contain 20% of phytosterols and their esters (FDA, 2000; Quilez *et al*., 2003a). After a thorough review of the safety of phytosterols, the European Union Scientific Committee on Food (SCF) also confirmed that phytosterols are safe for human consumption and authorized marketing of products with added phytosterols and phytostanols and their esters (SCF, 2000). Nevertheless, as a precaution, the consumption of high doses is not advised due to some negative effects as discussed in the previous section. De Jong *et al*. (2004) suggested that surveillance is necessary and safety studies should be done on consumption of doses above 8.6 g given that consumers are becoming exposed to large variety of phytosterol-enriched products.

2.6 Cholesterol-lowering properties of phytosterols

High blood cholesterol concentration is regarded as one of the risk factors for coronary heart diseases (CHD) (Ebrahim *et al*., 1998; Tanasescu *et al*., 2004). In the last decade, phytosterols/phytostanols and their esters have attracted the interest of researchers due to their

effectiveness in the reduction of intestinal absorption of cholesterol (Ostlund *et al*., 2002b; Plat *et al*., 2000). These natural compounds are used as plant-based, alternative to drug therapy (Garti & Romer, 2006) and one of the dietary options to reduce blood cholesterol (Mussner *et al*., 2002; Pollak, 1953).

The cholesterol-lowering effect of phytosterols has been known since the 1950s (Pollak, 1953). Since then, several studies have been conducted to investigate this effect. Different types of phytosterols, i.e. free phytosterols/phytostanols and their esters have been administered in different doses using different food formats. Table 1 shows the effect of phytosterols/ phytostanols consumption on the serum total and LDL-cholesterol concentration and cholesterol absorption. Evidence from numerous clinical trials indicates that a daily consumption of at least 0.8 g of phytosterols/phytostanols is effective for the total and LDL-cholesterol reduction (Hendriks *et al*., 1999; Miettinen & Vanhanen, 1994). Additionally, several studies that have compared their efficacy, concluded that phytosterols are as effective as phytostanols in reducing the serum total and LDL-cholesterol (Hallikainen *et al*., 2000; Vanstone *et al*., 2002; Volpe *et al*., 2001; Weststrate & Meijer, 1998).

As it can be seen in table 2, different studies achieved different results in regard to the reduction of blood cholesterol. As reviewed by Quilez *et al*. (2003a), 15% is the maximum LDL-cholesterol reduction that can be achieved at a daily intake of 1.5-3 g phytosterols. According to Berger *et al*. (2004), the difference in the percent reduction is partly due to the difference in the solubility of phytosterols in the food vehicles used for phytosterol administration. In a study by Clifton et al. (2004), phytosterol esters were more effective when solubilized in low-fat milk than in breads and cereals.

2.7 Proposed mechanisms by which phytosterols lower LDL-cholesterol

Despite the scientific evidence of cholesterol-lowering properties of phytosterols, the principles of the underlying mechanism are still poorly understood. However, several theories have been suggested (Trautwein *et al*., 2003) based on the different stages of cholesterol absorption.

2.7.1 Physico-chemical effects

2.7.1.1 Competitive solubilization between phytosterols and cholesterol in dietary mixed **micelles**

For the dietary cholesterol to be efficiently absorbed, cholesterol needs to be in its unconjugated form. Therefore, its esters must first be hydrolysed by pancreatic cholesterol

Table 2 Effect of free sterols, sterol and stanol esters on total cholesterol, LDL cholesterol and cholesterol absorption

Phytosterols	**Vehicle**	**# Study population**	**Dose (g/day)**	**Duration (weeks)**	**Effect**	**Reference**
Plant sterols	Margarine	95	3.2	3.5	↓ TC 8%, ↓ LDL 13%	Weststrate and Meijer (1998)
Free sterols	Spread	100	0.8	3.5	↓ TC 4.9%, ↓ LDL 6.7%	
			1.6		↓ TC 5.9%, ↓ LDL 8.5%	
			3.2		↓ TC 6.8%, ↓ LDL 9.9%	Hendriks *et al*. (1999)
Free sterols Plant stanols	Spread	15	1.8	3	↓ LDL 13%, ↓ C-abs 36% ↓ LDL 6%, ↓ C-abs 26%	Jones *et al*. (2000)
Plant sterols	Margarine	60	2.1	3	↓ TC 5%, ↓ LDL 9%	Vissers *et al*. (2000)
Sterol esters	Spread	224	1.1	5	↓ TC 5.2%, ↓ LDL 7.6%	
			2.2		↓ TC 6.6%, ↓ LDL 8.1%	Maki *et al*. (2001)
Plant sterols	Ground beef	34	2.7	4	↓ TC 9.3%, ↓ LDL 14.6%	Matvienko *et al*. (2002)
Plant sterols	Bakery products (croissant, muffin)	57	3.2	8	↓ TC 5.5%, ↓ LDL 10.4%	Quilez et al. (2003b)
Plant stanols	Limonade	21	1.9	10	↓ C-abs 32%	
	Egg white				↓ C-abs 38%	Spilburg *et al*. (2003)
Sterol esters	Milk	58	1.6	3	↓ TC 8.7%, ↓ LDL 15.9%	
	Yoghurt				↓ TC 5.6 %, ↓ LDL 8.6%	
	Bread				↓ LDL 6.5%	
	Cereal				↓ LDL 5.4%	Clifton *et al*. (2004)
Plant sterols	Yoghurt	184		4	↓ LDL 9.4%	Doornbos *et al*. (2006)
Plant sterols	Fermented milk	116	1.6	3	↓ LDL 7.91%	
				6	↓ LDL 9.44%	Mannarino *et al*. (2009)

↓= reduction; TC= total cholesterol; LDL= low density lipoprotein; C-abs= cholesterol absorption

esterase into free cholesterol which is subsequently solubilized in the bile to form mixed micelles (Brown *et al*., 2010). Likewise, solubilization of free phytosterols in the mixed micelles is the first requirement (Armstrong & Carey, 1987) in order to get to the brush-border membrane and to be absorbed into the circulation (Haikal *et al*., 2008). A mixed micelle is an amphiphilic mixture of bile salts, fatty acids, phospholipids, and free cholesterol. Micelle play an important role in the absorption of lipophilic compounds, by incorporating them into the apolar core and transporting them toward the intestinal brush-border membrane (Trautwein *et al*., 2003). It is thought that phytosterols exhibit their cholesterol-lowering properties through competitive incorporation into mixed micelles. This belief was examined *in vivo* (Ikeda & Sugano, 1983; Ikeda *et al*., 1989) and *in vitro* (Mel'nikov *et al*., 2004) by using radioactive phytosterols in order to study the competitive solubilization between phytosterols and cholesterol in the mixed micelles. From these experiments, the authors found that phytosterols exhibited a greater affinity for micellar solubilization than cholesterol, and displaced cholesterol from the mixed micelles. Similarly, a study by Jesch and Carr (2006) also indicated that phytosterols limit the incorporation of cholesterol into the mixed micelles, hence decreasing its access to the brush border membrane for absorption

2.7.1.2 Co-crystallization of cholesterol and phytosterols

Another molecular mechanism by which phytosterols could lower cholesterol absorption is thought to be the co-crystallization of phytosterols and cholesterol during lipid digestion which leads to the formation of poorly absorbable mixed crystals. The co-crystallization phenomenon was demonstrated by Christiansen *et al*. (2001a). In their study, mixed crystals were formed upon solvent (ethanol) evaporation from a mixed solution of cholesterol:β-sitosterol (β-sitosterol < 80% by weight). Hence, it is suggested that the formation of non-absorbable complex or mixed crystals between phytosterols and cholesterol might inhibit the intestinal absorption of cholesterol. However, there is no information available regarding co-crystallisation under *in vivo* conditions. Moreover, the findings of Christiansen *et al*. (2001a) further demonstrated that sitostanol does not co-crystallize to the same degree as sitosterol when mixed with cholesterol. Due to the fact that β-sitosterol and sitostanol behave differently with respect to the formation of mixed crystals with cholesterol, it is assumed that, even if co-crystallization between phytosterols and cholesterol may occur in *in vivo* conditions, it would have little importance in the overall phytosterol-induced reduction of intestinal cholesterol absorption (Mel'nikov *et al*., 2004; Trautwein *et al*., 2003).

2.7.2 Effect on hydrolysis by cholesterol esterase

Of the total cholesterol consumed daily, 20% occur as cholesterol ester (Garti & Romer, 2006). As already mentioned, dietary cholesterol ester must be hydrolysed by pancreatic cholesterol esterase prior to its absorption in enterocytes. According to Ikeda *et al*. (2002), pancreatic cholesterol esterase accelerates cholesterol absorption by hydrolyzing and releasing free cholesterol from other lipids such as phospholipids (e.g. phosphatidylcholine). Phytosterol fatty esters may serve as substrates for the marginal specific cholesterol esterase due to the structural similarities with the cholesterol esters (Ikeda *et al*., 2002; Trautwein *et al*., 2003). This is also supported by Moreau and Hicks (2004) who demonstrated that the same enzyme is responsible for the hydrolysis of phytosterol esters. Phytosterol esters can competitively interact with the enzyme, thereby limiting its activity towards cholesterol esters. As a result, the amount of absorbable cholesterol from the diet is reduced. It is also suggested that if the enzyme does not hydrolyse the lipophilic phytosterol esters, they will remain in the intestinal lumen, thereby serving as a solvent for other lipophilic compounds, cholesterol and its esters inclusive. By doing so, part of dietary and hepatic cholesterol can be transported to the distal intestine where absorption is limited or even impossible (Trautwein *et al*., 2003).

2.7.3 Intra-cellular trafficking

Intra-cellular trafficking of cholesterol describes the process by which cholesterol is transported from the intestinal lumen to lymph. This process can be divided into two steps (Garti & Romer, 2006):

1) Passage through the epithelial layer of the intestinal mucosa and
2) Re-esterification in the intestinal mucosal cell

2.7.3.1 Competition between cholesterol and phytosterols for cholesterol transporters

There is evidence that cholesterol uptake from the mixed micelle to the enterocytes is a protein mediated process. The Niemann-Pick C 1-Like 1 (NPC1L1) protein localized in the small intestine brush border membrane was found to be the primary cholesterol transporter (Altmann *et al*., 2004). Based on their structural resemblance, phytosterols instead of cholesterol could be transported into the enterocytes, hence decreasing absorption of cholesterol (Trautwein *et al*., 2003).

Another transporter protein, ATP-binding cassette (ABC) transporter A 1 (ABCA1), which is expressed in small intestine (Vasiliou *et al*., 2009), has also been shown to be involved in cholesterol transport across the cell membrane. It has been demonstrated by Plat and Mensink

(2002) that the incorporation of sitostanol into *in vitro* prepared micelles induced ABCA1 gene expression and led to transport of free cholesterol back to the intestinal lumen. This concept was also supported by Yu *et al*. (2002), who indicated that over-expression of ATP-binding cassette G5 (ABCG5) and ATP-binding cassette G8 (ABCG8) in transgenic mice resulted in 50% reduction in cholesterol absorption. ABCG5 and ABCG8 are two-half transporters of the ABC transporter family (Trautwein *et al*., 2003). Phytosterols have been shown to activate liver X receptor (LXR) and retinoid X receptor (RXR) which regulate ABCA1 gene expression (Janowski *et al*., 1996; Plat *et al*., 2005).

2.7.3.2 Inhibition of acyl coenzyme A cholesterol acyltransferase (ACAT) activity

Intestinal acyl coenzyme A cholesterol acyltransferase (ACAT) has been found to play an important role in cholesterol and, to a lesser extent, sitosterol esterification in the enterocytes (Buhman *et al*., 2000; Lee *et al*., 2000b). The resulting esters are packaged into the chylomicrons and thereafter secreted into the lymph (Garti & Romer, 2006; Hernandez *et al*., 2000). An *in vitro* study by Field and Mathur (1983) demonstrated that ACAT-induced esterification of cholesterol is 60 times higher than that of phytosterols. Because they are poorly esterified, free phytosterols accumulate in the enterocytes, and impair the ACAT activity leading to the reduction in cholesterol esterification (Garti & Romer, 2006). As a result, the amount of cholesterol esters to be incorporated in chylomicron will be reduced. This suggestion is supported by the evidence that ACAT inhibition resulted in reduced cholesterol absorption in rats (Krause *et al*., 1993) and in the intestinal cell line CaCo-2 (Field *et al*., 2001).

2.8 Absorption and metabolism of phytosterols

The absorption of phytosterols appears to occur under the same conditions as that of cholesterol because the hydrolysis of cholesterol and phytosterol esters requires the same enzymes (Brown *et al*., 2010; Garti & Romer, 2006). However, in contrast to cholesterol, the absorption of phytosterols in low. While between 30–60% of dietary cholesterol is absorbed, the absorption of campesterol ranges between 9.4–14.8%. It is 3 times higher than that of sitosterol (3.1–4.5%) and stigmasterol between (4%) (Bosner *et al*., 1999). The differences in the absorption efficiency of phytosterols are attributed to their molecular structure. It increases with the presence of the double bond between C5-C6 and decreases as the number of carbon atoms in the side chain increases (Heinemann *et al*., 1993; Ostlund *et al*., 2002a).

There is limited information regarding the distribution of phytosterols in different body tissues. In a study by Sanders *et al*. (2000), labeled phytosterols were found in adrenal glands, ovaries, testis and intestinal epithelia of animals. It has been reported that phytosterols are converted into cholesterol, then steroid hormones or bile acids (Douglass *et al*., 1981; Svoboda *et al*., 1980) in prawns and insects. Although Salen *et al*. (1970) reported the conversion of intravenously administered [22, 23-^{3}H]sitosterol into cholic and chenodeoxycholic acid in humans, later animal and human studies could not prove the conversion of phytosterols neither into cholesterol nor normal C_{24}-bile acids (Boberg *et al*., 1990; Lund *et al*., 1991). Rather phytosterols were excreted as polar compounds, namely di- and trihydroxylated C_{21}-derivatives (Boberg *et al*., 1990). Phytosterols and cholesterol are excreted via the biliary route (Igel *et al*., 2003; Sanders *et al*., 2000). This process is more rapid for phytosterols than cholesterol (Ling & Jones, 1995). Poor absorption and faster excretion of phytosterols via bile explain their low concentration in serum of healthy individuals, as opposed to sitosterolemic individuals (Piironen *et al*., 2000a).

In the small intestine, small amount of free phytosterols is absorbed (Bosner *et al*., 1999), and a large proportion ends up in the feces (Miettinen *et al*., 2000; Weststrate *et al*., 1999). Before they are excreted in the feces, it has been reported that unabsorbed free phytosterols might undergo microbial conversion in the colon. According to Weststrate *et al*. (1999), free phytosterols undergo oxidation at the C-3 position and saturation at the C_5-C_6 position in the colon. Song *et al*. (2000) also identified some metabolites, namely androstadienedione, androstedienedione and androstanedione, in rat colon.

Chapter 3 MATERIALS AND METHODS

3.1 Materials, reagents and enzymes

Wheat germs were obtained from Markal Company (St-Marcel-lès-Valence, France). Acetone (99.98%, analytical grade), methanol (99.9%, LC-grade), ethyl acetate (99.8%, LC-grade) and isopropanol (99.8%, LC-grade), sodium bicarbonate ($NaHCO_3$) (>95.5%), hexane (analytical grade), diethyl ether (99.5%, analytical grade), potassium hydroxide (KOH) and hydrochloric acid (HCl) were obtained from Chem-Lab (Zedelgem, Belgium). Pepsin from porcine stomach mucosa and pancreatin from porcine pancreas, stigmasterol (>95%), sitosterol from soybeans (~60%) and bile salts (~50% sodium cholate, ~50% sodium deoxycholate) were obtained from Sigma (St-Louis, MO, USA).

3.2 Extraction of phytosteryl glycosides and phytosteryl ferulates

3.2.1 Extraction of lipids

Phytosteryl ferulates and phytosteryl glycosides were extracted from wheat germs. Wheat germs were first milled in batches (20 g per batch) using a coffee bean miller for 3-5 min per batch. Two methods were tested to extract lipids. In the first method, 2 g of wheat germs were placed in kjeldahl tube and 80 ml acetone was added. The sample-solvent mixture was then heated for 15 min in the 2020 Digestor heating block at 150^{o}C. Extra acetone was added to complete 15 min heating step. After 15 min heating, the kjeldhal tube was cooled down, and the mixture was then transferred to the extraction thimble and refluxed in the Soxhlet apparatus for 30 min. The lipid extracts were collected in round bottom bulb, and the solvent was evaporated using a rotary evaporator. After evaporation, the lipid extracts were redissolved in hexane-diethyl ether (9:1, v/v) and stored at -20^{o}C. In the second method, the lipids were extracted from wheat germs with acetone using Soxhlet extraction apparatus. A 20 g sample of wheat germs was extracted by Soxhlet extraction for 2 hours. The lipids were collected in a round bottom bulb, evaporated and redissolved in hexane-diethyl ether (9:1, v/v). The lipid extracts were kept at -20^{o}C until further analysis.

3.2.2 Fractionation of extracted lipids into phytosteryl glycosides and phytosteryl ferulates

The lipid extracts were fractionated by normal phase solid phase extraction (NP-SPE) under vacuum using SPE cartridges (Maxi-clean SPE 900mg silica) from Alltech (Lokeren, Belgium). The silica cartridge was conditioned with 3 column volumes hexane (i.e. 3 ml). For

each run, a 0.5 ml of lipid extracts was loaded and the fractions were allowed to elute in a drop wise flow. The first eluting fraction named as load fraction was collected. Then, two fractions of phytosteryl ferulates were eluted. The first fraction of phytosteryl ferulates was eluted with 2 column volumes hexane–diethyl ether (9:1, v/v), while the second fraction was eluted with 2 column volumes hexane–diethyl-ether (1:1, v/v). The fraction containing phytosteryl glycosides was eluted with 2 column volumes of acetone. The load fraction and the two fractions containing phytosteryl ferulates were combined, dried to dryness under a nitrogen flow and stored at -20^{o}C.

3.2.3 Acid-base washing of phytosteryl ferulates

3.2.3.1 Removal of residual fatty acids and triglycerides

After fractionation by using NP-SPE, dried under a nitrogen flow and redissolved in methanol. The pH was increased to 10 by adding 0.6% KOH and then liquid-liquid extraction was performed twice. To 3 ml phytosteryl ferulates in methanol, 3 ml hexane was added. It was mixed by vortexing for 1 min and centrifuged for 5 min at 3000 g at room temperature. The hexane layer containing the fatty acids and triglycerides was removed.

3.2.3.2 Removal of salts

The pH of the remaining methanol layer was decreased to 1 using 6 M HCl followed by 3 times liquid-liquid extraction. To 3 ml of methanol layer, 2 ml hexane was added, mixed on a vortex mixer, centrifuged for 5 min and the hexane layer containing the phytosteryl ferulates was collected in a new tube. All the hexane layers from the first, second and the third step were combined, evaporated under nitrogen flow at room temperature and stored at -20^{o}C until analyis.

3.2.4 Determination of the influence of the pH on the stability and extractability of phytosteryl ferulates

NP-SPE samples of 3 ml were taken and their pH were set at 8, 10, 12 and 14. For each pH, six samples were taken in test tubes and divided into two series. For the first series of three samples of phytosteryl ferulates suspended in methanol, liquid-liquid extraction with hexane was performed immediately. The second series of three samples was incubated for 24 hours at room temperature while shaking on an orbital shaker prior to liquid-liquid extraction. Liquid-liquid extraction was performed as described in 3.2.3.1 and 3.2.3.2. The sample: solvent ratio was 2:3.

3.3 Digestion of stigmasterol, phytosteryl glycosides and phytosteryl ferulates under stomach conditions

For stomach digestion, stigmasterol (>95% purity) standard solution (2.5 mg/ml) was prepared by dissolving appropriate amount of stigmasterol in methanol: isopropanol (MeOH: IPA) mixture (1:1, v/v). For phytosteryl glycosides and phytosteryl ferulates digestion, extracts were used to prepare 5 mg/ml of their respective stock solutions. The resulting stigmasterol stock solution was sonicated for 5 min and then diluted 20 times with demineralized water to obtain 5% (v/v) organic solvent. Substrate blank was prepared by dissolving MeOH: IPA (1:1, v/v) in demineralized water. The concentration of solvent was 5% (v/v). The pH of both stigmasterol stock solution and substrate blank was set at 2 using 1 M HCl and 1 M $NaHCO_3$. Stigmasterol stock solution was distributed into four samples with 10ml each. Three samples served as replicates and one as enzyme blank. Pepsin (1 mg) was then added to stigmasterol samples and stigmasterol blank. All samples were incubated for two hours in a water bath of 37^{o}C while mixing. Samples of 3 ml each were taken at the beginning (0 hour), 1 hour and 2 hours of incubation. After sampling, the enzyme activity was stopped by heating for 10 min at 95^{o}C, and then increasing the pH to 8.5. Similar steps were used for the digestion of phytosteryl glycosides and phytosteryl ferulates.

3.4 Digestion of stigmasterol, phytosteryl glycosides and phytosteryl ferulates under small intestinal conditions

Stigmasterol (>95% purity) standard solution (2.5 mg/ml) was prepared by dissolving stigmasterol in MeOH: IPA (1:1 v/v). The resulting stock solution was diluted 20 times with demineralized water to obtain a stigmasterol solution containing 5% organic solvent. The pH was then set at 7.5 by adding 1 M $NaHCO_3$. Pancreatic juice was prepared by dissolving 62.5 g/l $NaHCO_3$, 30 g/l bile salts and 4.5 g/l pancreatin in demineralized water. 1 ml of freshly prepared pancreatic juice was then added to 9 ml stigmasterol solution in triplicate. Substrate blank consisted of 9 ml 5% MeOH: IPA (1:1 v/v) to which 1 ml pancreatic juice was added. Enzyme blank consisted of diluted stigmasterol solution without pancreatic juice. After addition of pancreatic juice, the pH was re-adjusted to 7.5. The samples were incubated for 3 hours in a water bath at 37^{o}C while mixing. Samples were taken at the beginning (0 hour), after 1.5 hour and after 3 hours of incubation. The enzyme activity was stopped by heating for 10 min at 95^{o}C and then increasing the pH to 9.5. The described procedure for stigmasterol digestion under small intestinal conditions was the same for phytosteryl glycosides and

phytosteryl ferulates, with the exception that for the two latter substrates, a concentration of 5 mg/ml stock solution was used.

3.5 Extraction of stigmasterol, phytosteryl glycosides and phytosteryl ferulates from digested samples

After digestion under stomach and small intestine conditions, stigmasterol, phytosteryl ferulates and phytosteryl glycosides were extracted from their respective digested samples in by 3 times liquid-liquid extraction using ethyl acetate (HPLC grade). Ethyl acetate was added in 2:3 (sample: solvent) ratio. The samples were mixed with a vortex mixer for 1 min and centrifuged at 3000 g for 5 min. The solvent layer was separated from aqueous phase. The solvent layers from the first, second and third liquid-liquid extraction steps were combined, evaporated to dryness under a flow of nitrogen and stored at -20^{o}C until analysis.

3.6 Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) analysis

After acid-base washing step described in 3.2.3, dried hexane and methanol fractions were redissolved in 1 ml methanol (HPLC grade) and phytosteryl ferulates were analysed by RP-HPLC. After digestion experiments, RP-HPLC was also used to analyse stigmasterol, phytosteryl ferulates and phytosteryl glycosides samples. The dried samples digested under stomach and small intestinal conditions were reconstituted in 0.5 ml MeOH: IPA (1:1, v/v), filtered through 0.45µm filters, and subsequently analysed with RP-HPLC.

Table 3 RP-HPLC method gradients

0-5 min	Isocratic at 100% A
5-15 min	Linear gradient from 100% A to 100% B
15-20 min	Isocratic at 100% B
20-40 min	Linear gradient from 100% C to 100% C
40-50 min	Isocratic at 100% C
50-60 min	Linear gradient from 100% D to 100% B
60-70 min	Linear gradient from 100% C to 100% A
70-80 min	Isocratic at 100% A

The samples were analyzed on a Thermo HPLC consisting of an automatic sampler, high pressure pump, degasser, column compartment and a Thermo Surveyor PDA detector at 206, 234 and 280 nm. The HPLC was equipped with Supercosil LC-18 column (Supelcosil, 5µm, 250 x 4.6 mm, Supelco, Bellafonte, PA, USA). A flow rate of 0.7 ml/min was used. A

gradient as described in table 3 was used, with solvent A= 40% MeOH, B= 100%MeOH and C= isopropanol.

3.7 Ultra High Performance Liquid Chromatography (UPLC) analysis

Phytosteryl ferulate and phytosteryl glycoside extracts were analysed using LC-MS. The analysis was done by an Acquity Ultra High Performance Liquid Chromatography (UPLC) (Waters, Milford, MA, USA) equipped with a BEH C18 column (1.7 µm, 150 x 2.1 mm, Waters, Milford, MA, USA). The UPLC was coupled to a Synapt High Definition Mass Spectrometer (Waters). The mobile phase used was 50% MeOH (A) and 100% MeOH (B) (ULC-grade Biosolve, Valkenswaard, The Netherlands). The flow was set at 0.3 ml/min and the column temperature at 20^{o}C. The gradient used was as follows: From 0 to 1 min, isocratic at 99% A ; from 1 to 5 min, linear gradient from 99% to 1% A; from 5 to 22 min, isocratic at 1% A; from 22 to 25 min, linear gradient from 1% to 99% A; and from 25 to 30 min, isocratic at 99% A. The chromatographic peaks were detected by a PDA detector at the wavelength between 190 to 320 nm. After separation, the flow from the UPLC was introduced in the mass spectrometer. The compounds were ionized by atmospheric pressure photoionization (APPI) with toluene as dopant (0.03 ml/min) and in a positive mode. The results were processed using the MassLynx software. The chromatographic peaks were assigned by their mass/charge (*m/z*) ratio with *z* equals to 1.

3.8 Data analysis

To obtain standard curves, peak areas were plotted against their corresponding concentrations. Standard errors of the intercept and the slope of the standard curves were obtained using LINEST function of MS excel (2007). The limit of detection (LOD) was defined as three times standard errors of the intercept/the slope and the limit of quantitation (LOQ) was expressed as ten times the standard error of the intercept/the slope. The reproducibility was checked by percent relative standard deviation (%RSD) of stigmasterol peak area obtained at different days. Analysis of variance (one-way repeated measures ANOVA) was used to compare recoveries and peak areas at different time points of digestion. It was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego Calfornia, USA). Turkey's test was used to determine differences among the data. The values of significant difference were taken at $p< 0.05$.

Chapter 4 RESULTS AND DISCUSSION

4.1 Choice of extraction method

Phytosterols constitute the largest proportion of unsaponifiable fraction of lipids (Careri *et al*., 2001). They are, therefore, extracted along with lipids. The comparison of the two methods was made based the amounts of lipids extracted. The lipid levels were determined after rotary evaporation. Lipid yield was calculated by dividing the amount of lipid extract by the initial mass of wheat sample. Extraction was done on triplicate.

$$\text{Lipid yield}(\%) = \frac{\text{mass}_{\text{extract}}(\text{g})}{\text{mass}_{\text{sample}}(\text{g})} \text{x} 100$$

The first method, with some modifications, was described by Nystrom *et al*. (2007b). It consisted of the second method with an extra 15 min heating step. The second method was the Soxhlet extraction method which is also known as the standard method of lipids extraction (de Castro & Garcia-Ayuso, 1998). It was expected that during the heating step, more phytosteryl ferulates and phytosteryl glycosides would be released. Surprisingly, the results did not meet our expectations. The results from the two extraction methods are presented in table 4. From these results, Soxhlet extraction method (method 2) gave lipid yield of about 16% (w/w) whereas the lipid yield of method 1 was 12% (w/w).

The results from RP-HPLC analysis of phytosteryl ferulates served as a second basis for comparison of the two methods. Figure 5 shows the chromatograms obtained from phytosteryl ferulates analysis. The chromatograms from the two methods show similar pattern between 20 and 26 min, but with more intense peaks in the chromatograms from the second method than the first method. Between 26 and 37 min, almost all the peaks detected in the chromatograms from the second method were not found in the chromatograms from the first method. This also gave an indication that the amount of phytosteryl ferulates extracted could be higher when the second extraction method was used than with the first method. However, it is important to note that the identity of the peaks detected by RP-HPLC analysis were not determined.

Furthermore, the choice was partly based on practical reasons. During the first step –heating at 150^{o}C for 15 min– of the first method, almost all the solvent (acetone) evaporated before 15 min. Based on the lipid yield, the second method was found to give the highest yield. The first method was regarded as less suitable for phytosterol extraction and above all, it was found to be more laborious than the second method.

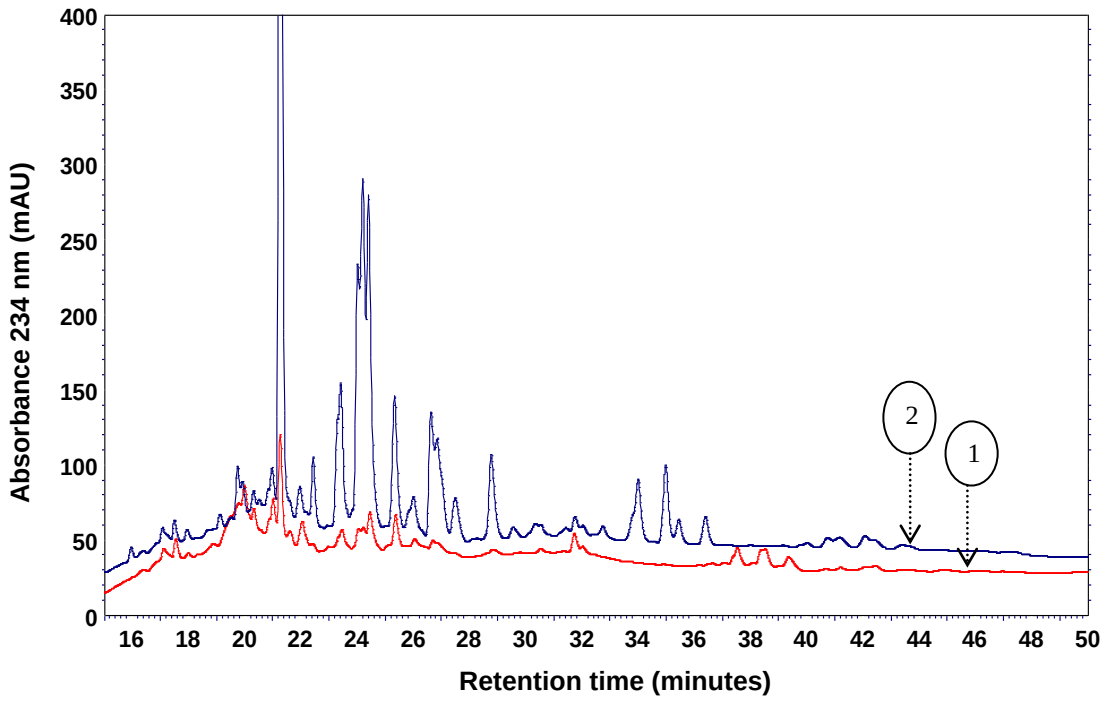

Figure 5 RP-HPLC chromatograms of phytosteryl ferulates (fraction 2) obtained using different methods. 1 denotes method one, 2 denotes method two.

Table 4 Comparison of lipid yields obtained using different extraction methods

	Method 1	Method 2
Sample weight (g)	2.01 ± 0.002	20.01 ± 0.004
Extracted lipids (g)	0.25 ± 0.01	3.11 ± 0.1
% (w/w)	12.2 ± 0.01	15.5 ± 0.5

The values are means (±SD) of three replications.

Previous studies have compared the lipid yield from wheat germs using Soxhlet extraction and other extraction methods. Dunford and Zhang (2003) extracted wheat germ oil using an accelerated solvent extraction as a means of reducing the time used in Soxhlet extraction method. The authors reported that accelerated solvent extraction and Soxhlet extraction methods yielded 11.0% (w/w) and 10.8% (w/w), respectively. Ostlund *et al*. (2003) reported 10.3% (w/w) lipids content in wheat germ. The data presented by Barnes (1982) indicate that a lipid content of 13.4-14.8% (w/w) is obtained from wheat germs by using acetone as extraction solvent. These results are comparable or even lower than those obtained in this thesis.

4.2 Effect of pH on the extractability of phytosteryl ferulates

The effect of pH on the extractability of phytosteryl ferulates was examined after extraction, acid-base wash and RP-HPLC analysis of phytosteryl ferulates. The chromatograms of

samples that were immediately washed and those washed after 24 hours incubation were compared. The percent mean differences between peak areas before and after 24 hours incubation are presented in table 5. The tables do not show results from samples treated at pH 14 because when the alkaline solution was added to set the pH to 14, the samples turned into a thickened state, and could therefore not be analysed.

The peaks detected in the chromatograms of non-incubated samples were also detected the in the incubated samples. However, the peaks had different intensities. Some peaks increased and some decreased after incubation at different pH values (8, 10 and 12). At pH 8, the peak areas of 6 peaks increased after incubation and 9 decreased. Of the 9 peaks which decreased, 2 peaks could not be detected any more. Similarly, but not for the same peaks, 6 peaks increased and 9 decreased after incubation at pH 10. Again, 2 peaks could not be detected after incubation at pH 10. At both pH 8 and pH 10, the peak area of three peaks with retention times 19.3±0.1 min, 20.4±0.2 min, and 24.5±0.4 min increased in the incubated samples. Also, at the same pH values, three peaks with retention times 22.1±0.1 min, 31.8±0.1 min, and 42.7±0.2 min decreased after incubation. When samples were incubated at pH 12, the peak areas of 6 peaks increased and 3 decreased, of which 2 could not be detected after incubation. At the same pH value, 2 peaks were only detected in after incubation while 4 peaks were detected neither before nor after incubation. The peak with retention time 39.6 ±0.1 min increased at both pH 10 and pH 12.

Based on the increases/decreases in peak areas observed at pH 8, pH 10 and pH 12, the extractability of phytosteryl ferulates was influenced by the pH. However, there was no particular trend in the changes observed in samples with or without incubation. In the literature, it was reported that ester linkages of phytosteryl ferulates are cleaved during acid-base wash step of phytosterol analysis (Moreau *et al*., 2002) to liberate free phytosterols. It was expected that, if phytosteryl ferulates are hydrolysed at pH 8, more hydrolysis would take place at higher pH, which was apparently not the case. In addition, no peak was detected at the retention time of stigmasterol (29.9 min). At the retention time of sitosterol (30.1 min), a peak was detected in non-incubated samples at pH 8, but could not be detected after incubation at the same pH. At pH 10, a peak was detected at 30.1 min in non-incubated samples but it decreased when samples were incubated. Also, at the retention time of sitosterol, a peak was detected in the incubated samples but not in non-incubated samples at pH 12. Based on these changes, there was no indication that stigmasterol and sitosterol have been liberated as a result of pH effect.

Table 5 Change in peak areas after incubation of phytosteryl ferulates at different pHs

Rt	pH	Peak area before incubation	Peak area after incubation	% difference
19.3±0.1	8	211.5±110.4	2927.8±1292.0	1284.5
	10	2885.3±3327.5	3395.1±1095.8	17.7
	12	4928.5±4097.1	32648.9±24760.4	562.4
20.4±0.2	8	1037.0±714.1	17435.7±7890.9	1581.4
	10	5630.1±1168.5	26788.2±6283.3	375.8
	12	9043.8±16072.8	n.d	-100
21.3±0.1	8	813.8±23.1	830.7±80.6	2.1
	10	848.1±184.1	695.6±45.5	-18.0
	12	759.4±136.2	55741.9±47891.3	7240.7
22.1±0.1	8	242.3±234.2	137.4±12.6	-43.3
	10	2315.4±3091.6	547.8±453.7	-76.3
	12	990.1±1146.7	2443.4±1984.3	146.8
24.5±0.4	8	189.0±82.4	468.4±460.5	147.8
	10	1221.7±483.7	1372.6±379.5	12.3
	12	3431.6±2863.4	2834.7±1550.3	-17.4
25.5±0.4	8	412.1±168.2	280387±231.0	-32.0
	10	89.5±47.3	n.d	-100
	12	200.5±179.4	4103.1±3062.4	1946.3
26.4±0.4	8	294.1±222.1	122.4±14.5	-58.3
	10	3067.9±2359.3	2473.8±1424.2	19.3
	12	152.7±57.7	n.d	-100
30.1±0.1	8	40.3±31.7	n.d	-100
	10	304.4±213.6	262.9±198.4	-13.6
	12	n.d	523.1±381.0	-100
31.8±0.1	8	112.5±63.6	100.7±7.3	-10.5
	10	1358.9±530.0	960.1±462.1	-29.3
	12	88.4±34.8	185.9±244.5	110.1
32.6±0.1	8	80.9±71.5	n.d	-100
	10	759.9±191.1	445.4±149.0	-41.3
	12	n.d	n.d	–
35.6±0.1	8	125.5±2.5	693.3±920.9	452.4
	10	172.1±73.4	n.d	-100
	12	145.5±10.5	165.2±23.0	13.5
37.7±0.1	8	993.9±713.8	308.6±87.4	-68.9
	10	1997.4±1867.3	4776.9±1370.6	139.1
	12	n.d	n.d	–
38.6±0.1	8	1206.6±746.0	367.8±113.1	-69.4
	10	2268.7±2155.8	5498.4±2145.7	142.3
	12	n.d	n.d	n.d
39.6±0.1	8	402.1±294.7	122.0±37.2	-69.6
	10	1045.5±751.8	1720.3±625.2	64.5
	12	n.d	1350.8±534.8	100
42.7±0.2	8	188.0±82.1	52.3±18.6	-72.2
	10	847.3±420.2	373.7±383.4	-55.8
	12	n.d	n.d	–

The values are means ± SD (n=3); Rt= average retention time in minutes; n.d= not detected.

4.3 Identification of phytosteryl ferulates and phytosteryl glycosides

UPLC coupled with MS was used to ensure the presence of phytosteryl ferulates and phytosteryl glycosides in their respective extracts. Stigmasteryl, sitosteryl and campesteryl ferulates/glycosides were selected because they are the most abundant forms of phytosterol conjugates. Tables 6 and 7 present the mass-to-charge ratios (*m/z*) and the retention times of the compounds that are likely to be formed when phytosteryl ferulates/glycosides are ionized. MS chromatograms of those compounds are presented in appendices A and B.

The retention times of the ionized forms of stigmasteryl and sitosteryl ferulates showed that these compounds eluted before stigmasterol. However, the retention times of the ionized compounds from campesteryl ferulate were different. But it is not possible for one compound to have two different retention times. The compound with retention time of 6.92 min is most likely to be campestryl ferulate because its retention time was shorter than that of stigmasterol. The results also showed that the *m/z* ratios of the compounds from campesteryl ferulates were identical to those from sitosteryl glycosides. Therefore, no clear conclusion could be made with regard to the presence of campesteryl ferulate or/and with regard to the presence of sitosteryl glucoside in the extract.

The results from table 7 indicate that the retention times of phytosteryl glucosides in the form of $[M\text{-}H_20+H]^+$ were different from the retention times phytosteryl glucosides in their $[M+H]^+$ forms. As mentioned earlier, this is not possible because one compound cannot elute at two different retention times. Based on the intensities of the $[M+H]^+$ forms, which were much higher than that of $[M\text{-}H_20+H]^+$ forms, it was suggested that phytosteryl glucosides were most likely to appear in the form of $[M+H]^+$, with the assumption that the ion with the highest intensity is the "real" ion.

Table 6 The *m/z* ratios and retention times of the selected ions present in the phytosteryl ferulates extract

		Phytosterol		
	Stigmasterol	Stigmasteryl ferulate	Sitosteryl ferulate	Campesteryl ferulate
$[M+H]^+$	n.d	589.42	591.44	577.41
RT	–	7.95	8.21	13.36
$[M\text{-}H_20+H]^+$	396.37	571.41	573.43	559.41
RT	11.2	7.99	8.21	6.92

RT= retention time; $[M+H]^+$ and $[M\text{-}H_20+H]^+$ are *m/z* ratios

Table 7 The *m/z* ratios and retention times of the selected ions present in the phytosteryl glycosides extract

	Phytosterol			
	Stigmasterol	Stigmasteryl glycoside	Sitosteryl glcoside	Campesteryl glycoside
$[M+H]^+$	n.d	575.4	577.4	563.4
RT	–	11.67	13.4	10.95
$[M-H_20+H]^+$	396.37	557.4	559.4	545.4
RT	11.2	7.58	7.73	9.68

RT= retention time; $[M+H]^+$ and $[M-H_20+H]^+$ are *m/z* ratios

4.4 Limit of detection, limit of quantitation and reproducibility of the RP-HPLC method used to determine stigmasterol

In order to ensure the suitability of the RP-HPLC method to consistently quantify stigmasterol, three parameters, i.e. the limit of detection, limit of quantitation and reproducibility, were determined on five different days. The average limit of detection, limit of quantitation and reproducibility (expressed by the percent relative standard deviation) are presented in tables 8 and 9.

4.4.1 Limit of detection (LOD) and limit of quantitation (LOQ)

The average LOD and LOQ were 0.10 mg/ml and 0.35 mg/ml, respectively. The LOD obtained was below the lowest concentration, suggesting that the RP-HPLC method used to determine stigmasterol could reliably quantify stigmasterol at the concentration of 0.16 mg/ml. The regression coefficients obtained on five different days were above 98%.

Table 8 The limit of detection (LOD) and limit of quantitation (LOQ) obtained on different days

	Mean (n= 5)	SD
Limit of detection (mg/ml)	0.10	0.09
Limit of quantitation (mg/ml)	0.35	0.31
r^2	0.995	0.00

SD= standard deviation; r^2= regression coefficient.

However, the LOD and LOQ obtained in this thesis are lower than those reported in the literature. The LOD and LOQ obtained by Careri *et al.*, (2001) were 0.42 µg/ml and 0.54 µg/ml, respectively. Rodriguez-Parmelo *et al.*, (1994) obtained 1.12 ng/g and 3.18 ng/g for

LOD and LOQ, respectively. In comparing these findings, it is important to note that the methods used by those authors were different from our method. Rodriguez-Parmelo *et al.,* (1994) used gas chromatography (GC) whereas Careri *et al.,* (2001) used a narrow bore RP-HPLC. However, their results show that our method is not as sensitive as their methods.

4.4.2 Reproducibility

The reproducibility of the RP-HPLC method used was evaluated by calculating the percent relative standard deviation (RSD%) of stigmasterol peak area obtained on five different days (table 9). The RSD increased with the dilution of the stigmasterol standard solution. Low RSD (6.5%) was obtained at the stigmasterol concentration of 2.5 mg/ml. At the intermediate concentrations, the RSD ranged between 11-14%, whereas a RSD of about 33% was obtained at the smallest concentration. The results from table 19 indicate that the method was better repeatable for higher than for lower stigmasterol concentrations.

Table 9 The RP-HPLC inter-day reproducibility

Concentration (mg/ml)	Peak area (mAU/10^3)		
	Mean (n= 5)	SD	RSD (%)
2.5	405.8	26.5	6.5
1.25	184.6	20.2	10.9
0.625	96.0	10.3	10.7
0.31	45.4	6.2	13.7
0.16	23.7	7.7	32.8

SD= standard deviation; RSD= relative standard deviation

4.5 Stability of stigmasterol, phytosteryl glycosides and phytosteryl ferulates under the stomach conditions

4.5.1 Stability of stigmasterol under the stomach conditions

To evaluate the possible degradation of stigmasterol under stomach conditions, samples were taken at different time points during digestion, analysed with RP-HPLC. The chromatograms of stigmasterol samples incubated with pepsin and those of enzyme blank are presented in figure 6.

The chromatographic profiles of stigmasterol samples incubated with pepsin did not differ from those obtained from the enzyme blank. In both samples with and without added pepsin, stigmasterol eluted at 29.9 min. The peak of stigmasterol was not detected in the substrate

blank. To further assess the stability of stigmasterol under stomach conditions, the recoveries at different time points during digestion were compared. The recoveries were calculated using a standard curve generated by analyzing different concentrations of stigmasterol standards. Table 10 shows the amount and percent recoveries of stigmasterol obtained at different time points of digestion. By comparing the recoveries of stigmasterol from digested samples and the blank, a similar trend was observed. In pepsin treated stigmasterol samples, the amount of stigmasterol recovered at different time points were almost the same. However, there was no significant difference between the amount obtained at different time points ($p= 0.45$). No statistical test was performed to compare the recoveries obtained from pepsin treated stigmasterol samples and enzyme blank at different time points because only one repetition was used for enzyme blank. However, the ranges of stigmasterol recoveries were closely comparable, being 59.5-71.3% and 63.1-71.9% in enzyme blank and stigmasterol digested samples, respectively. The stigmasterol recovery in enzyme blank at the beginning shows a slight deviation but this was considered as a random variation.

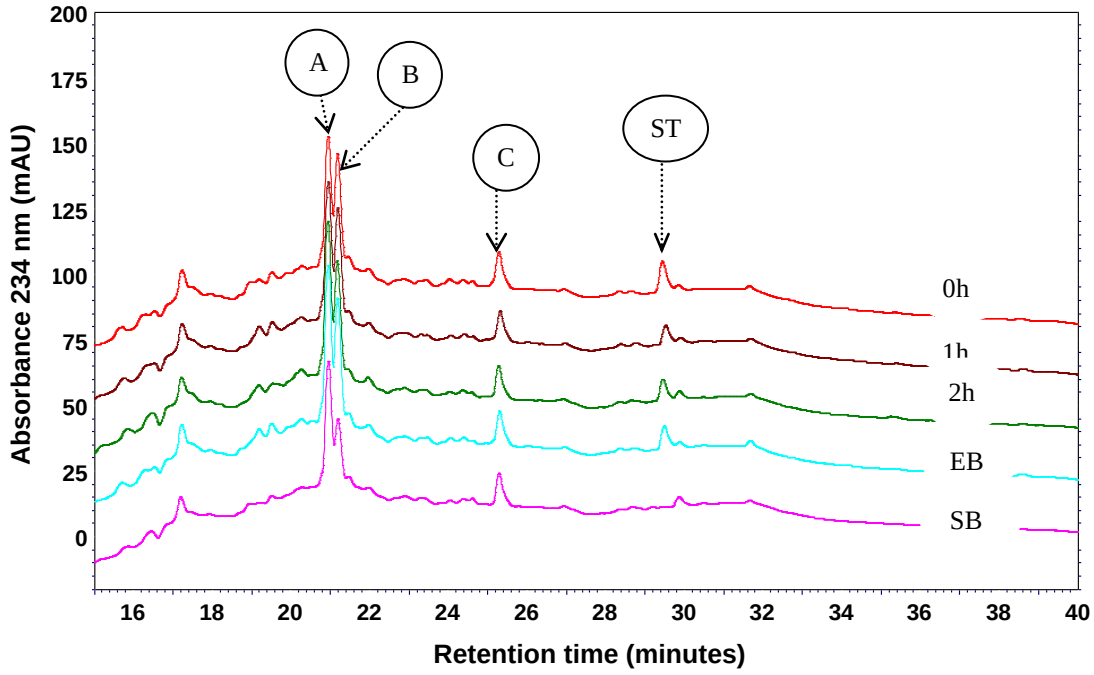

Figure 6 RP-HPLC chromatograms of stigmasterol and blanks at different time points during digestion under the stomach conditions. 0h, 1h, 2h= chromatograms obtained from samples incubated with pepsin at the beginning, after 1 hour and 2 hours; EB= enzyme blank; SB= substrate blank; ST= stigmasterol peak.

No new peaks were detected in all samples after 1 hour and 2 hours of digestion. However, a number of unknown peaks with retention times of 20.9 min (A), 21.1 min (B) and 25.2 min

(C) have been detected in samples with and without pepsin, as well as in the substrate blank. Table 11 shows the mean peak areas of unknown peaks detected in stigmasterol samples and enzyme blank. By comparing the mean peak areas of individual peak (i.e. A, B and C), the peak areas were found to remain almost constant over time except peak C (in enzyme blank) whose peak area seemed to have reduced after 2 hours compared to 0 hour and 1 hour. This is also supported by their presence in substrate blank. The origin of those peaks is not known but it is suggested that they originated from impurities present in the solutions used to adjust the pH. Those solutions were not freshly prepared. The peaks between 15-20 min were not considered as important peaks because they were also identified in methanol blanks.

Table 10 Stigmasterol recovery during digestion under the stomach conditions

Digestion time (h)	Pepsin treated stigmasterol samples		Stigmasterol enzyme blank	
	Amount of stigmasterol (mg/ml)	% recovery	Amount of stigmasterol (mg/ml)	% recovery
0	0.333±0.05[a]	68.1±12.1	0.291	59.5
1	0.351±0.01[a]	71.9±3.5	0.349	71.3
2	0.309±0.03[a]	63.1±7.7	0.320	65.5

The values are means (±SD) (n=3) for samples incubated with pepsin. The values followed by different letters are significantly different ($p \leq 0.05$). For the enzyme blank, (n= 1)

Table 11 Peak areas (mAU/10^5) of peaks other than stigmasterol peak detected at different time points during stigmasterol digestion under stomach conditions

	Digestion time (h)	Retention time (minutes)		
		20.9	21.1	25.2
Samples	0	56.4±6.4	42.3±0.8	16.4±0.6
	1	54.8±2.4	42.2±0.8	14.4±0.6
	2	61.2±3.4	43.1±0.2*	15.6±0.3
Enzyme blank	0	52.2	42.3	16.2
	1	49.1	40.7	13.4
	2	47.3	22.7	14.6

For samples, the values presented as means (±SD) (n= 3); for enzyme blank, n= 1; *= mean (±SD) calculated from two repetitions.

The degradation of free phytosterols in the stomach has not been reported in the literature. Moreover, stigmasterol was not expected to undergo enzymatic degradation as it is not a substrate of pepsin, which catalyzes the hydrolysis of proteins. But the pH effect was hypothesized based on the previous literature.

Kamal-Eldin *et al*. (1998) studied the stability of free phytosterols under acid conditions and reported that, among the free phytosterols studied, fucosterol and avenasterol underwent isomerization in their side chain, but stigmasterol was found to be stable (Kamal-Eldin *et al*., 1998). The peaks A, B, and C have also been detected in the chromatograms of other substrates, i.e. phytosteryl ferulates and phytosteryl glycosides, used during digestion under stomach conditions, suggesting that those peaks did not result from stigmasterol breakdown. Thus, based on the recoveries and on the fact that no new peaks were formed, it was concluded that stigmasterol was stable under stomach conditions.

4.5.2 Stability of phytosteryl glycosides under stomach conditions

The chromatograms of samples incubated with pepsin are shown in figure 7. They gave similar profiles. The peaks A, F and G were detected in all samples of phytosteryl glycosides incubated with pepsin as well as in the enzyme blank. Their retention times were 18.6, 28.1, 29.2, 31.3, 37.6 and 38.6 min. None of those peaks was detected in substrate blank, implying that they represent compounds present in phytosteryl glycosides extract. Table 12 presents their mean peak areas at different time points during digestion.

The results presented in table 12 indicate that the means peak area in the samples incubated with pepsin significantly reduced between 0 hour and 1 hour of digestion. The peak areas did not significantly decrease between 1 and 2 hours of digestion. One could presume that the observed reduction in peak areas between 0 hour and 1 hour of digestion was due to the enzymatic degradation of phytosteryl glycosides. However, the data also showed a reduction in the peak areas over time in the enzyme blank. Therefore, the reduction in peak areas observed in samples to which pepsin was added could not be attributed to the effect of enzyme activity. In addition, there was no formation of new peaks. The reduction in peak area could be explained by the adhesion of phytosteryl glycosides extract on the wall of the conical flasks used to perform the experiment.

Other peaks have also been detected in phytosteryl glycoside samples incubated with and without pepsin as well as in the substrate blank. Those peaks are indicated as B, C, D and E. Their respective retention times were 20.8, 21.1, 22.3, and 25.2 min. Their mean peak areas are shown in table 13. Based on data from this table, it can be seen that the mean peak areas changed over time but the variation did not show any specific trend either in the samples incubated with or without pepsin. The exact origin of peaks B, C, D and E is not known, but the same peaks were detected in other substrates digested under stomach conditions (except

some small differences in retention times). Their presence in substrate blank is an indication that they did not originate from the breakdown products of phytosteryl glycosides.

Notwithstanding all the changes that occurred in samples incubated with pepsin, all the peaks detected at the beginning could be detected after 2 hours of digestion. There was no formation of new peaks. If phytosteryl glycosides were hydrolysed, one could hypothesize that the point of hydrolysis would be at the glycosidic bond between free phytosterol and the sugar (see figure 4), and the latter two components would be the end products of hydrolysis (Kovganko & Kashkan, 1999; Moreau *et al*., 2002). In this experiment, stigmasterol and sitosterol standards were also injected and their retention times were found to be 29.9 and 30.1 min, respectively. No peak was detected at those retention times, suggesting that no stigmasterol/ sitosterol was released as a result of phytosteryl glycosides breakdown by pepsin.

No effect of pepsin on phytosteryl glycosides was expected, but the acidic conditions might influence the stability. Glycosidic bonds have been reported to be acid-labile (Panzeter *et al*., 1992). Acid hydrolysis has been previously suggested to be included in the sample preparation for phytosterol content analysis (Jonker *et al*., 1985). Toivo *et al*. (2000) analysed phytosterol content in whole wheat flour, and reported that the content increased by 20% when acid hydrolysis was included in the sample preprartion.

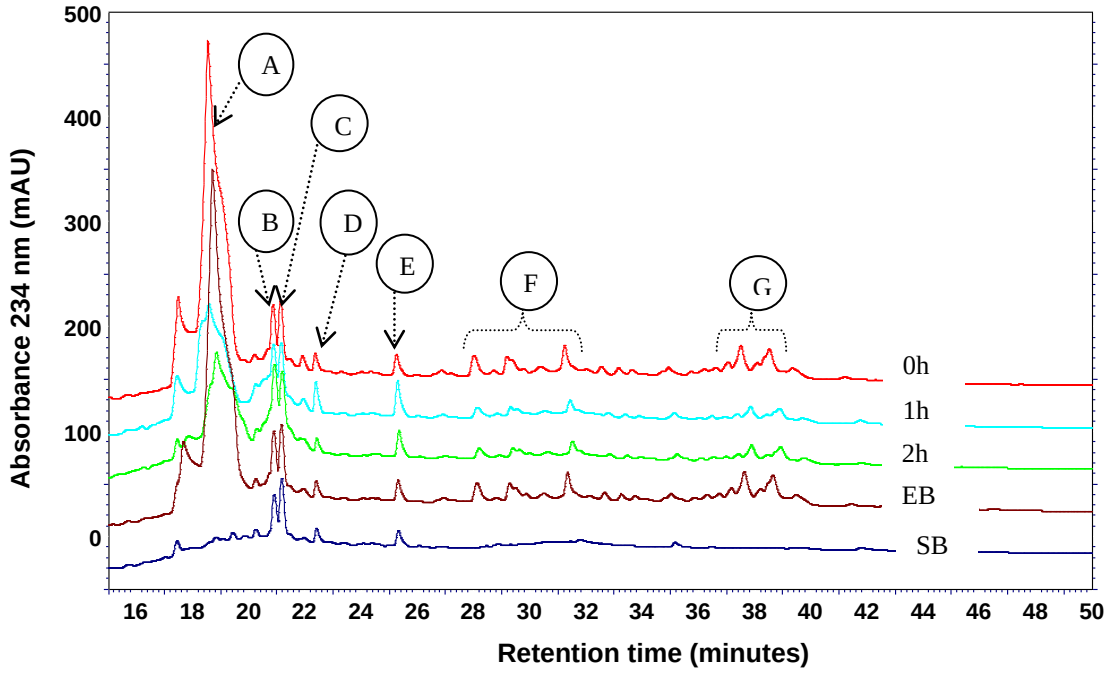

Figure 7 RP-HPLC chromatograms of phytosteryl glycosides and blanks at different time points during digestion under the stomach conditions. 0h, 1h, 2h= chromatograms obtained from samples incubated with pepsin at the beginning, after 1 hour and 2 hours; EB= enzyme blank; SB= substrate blank.

Table 12 Peak areas (mAU/10^4) of peaks detected at different time points during digestion of phytosteryl glycosides under stomach conditions

	Digestion time (h)	Retention time (minutes)					
		18.6	28.1	29.2	31.3	37.6	38.6
Samples	0	108.8±2.6[a]	2.7±0.3[a]	1.7±0.1[a]	2.4±0.4[a]	3.3±0.2[a]	2.9±0.2[a]
	1	43.8±12.7[b]	1.6±0.4[b]	0.8±0.3[b]	0.9±0.5[b]	1.2±1.0[b]	1.1±0.8[b]
	2	46.9±8.6[b]	1.8±0.5[b]	1.0±0.0[b]	1.1±0.2[b]	1.9±0.4[b]	1.5±0.3[b]
Enzyme blank	0	103.2	3.0	17.7	2.2	3.5	3.2
	1	67.6	2.2	12.8	1.3	2.4	2.4
	2	17.4	0.8	3.8	0.5	0.7	1.0

For samples incubated with pepsin, the values are means (±SD) (n=3); for each retention time, values followed by different letters are significantly different ($p \leq 0.05$); for enzyme blank, n= 1.

Table 13 Peak areas (mAU/10^4) of peaks indicated as B, C, D and E detected at different time points during digestion of phytosteryl glycosides under stomach conditions

	Digestion time (h)	Retention time (minutes)			
		20.8	21.1	22.3	25.2
Samples	0	46.4±2.9	54.1±1.3	12.9±1.7	39.1±28.6
	1	31.8±13.7	41.8±3.3	13.2±11.3	25.7±13.0
	2	43.3±9.4	45.0±1.8	12.2±2.4	25.4±4.7
Enzyme blank	0	43.1	55.8	13.0	24.6
	1	43.6	42.1	10.7	21.4
	2	41.5	46.1	9.8	22.9

For samples, the values are presented as means (±SD) (n= 3); for enzyme blank, n= 1

In these studies, it was suggested that the increase in phytosterol content resulted from the acid hydrolysis of phytosteryl glycosides (Jonker *et al*., 1985; Lin *et al*., 2009; Toivo *et al*., 2000). However, the hydrolysis of phytosteryl glycosides during the analysis of phytosterols in foods takes place under the pH and temperature conditions that do not represent those *in vivo*. In a study of the metabolism of [4-^{14}C]sitosteryl-β-D-glucoside in rats, it was reported that this compound was not hydrolyzed in the stomach (Weber, 1988). Recently, Lin *et al*. (2009) tested the effect of phytosteryl glycosides on cholesterol absorption. In their study, the cholesterol absorption was reduced by 37.6%, suggesting that phytosteryl glycosides have been hydrolysed in order to exhibt their hypocholesterolemic effect in a free form.

4.5.3 Stability of phytosteryl ferulates under the stomach conditions

To assess the stability of phytosteryl ferulates under stomach conditions, the chromatograms (figure 8) obtained from RP-HPLC analysis of samples to which pepsin was added and those of enzyme and substrate blanks were compared. The peaks indicated as E, with retention times of 36.5, 36.9, 37.4, 38.4, 39.2 and 42.2 min were detected in all phytosteryl ferulates samples incubated with and without pepsin. Among those peaks, none was detected in substrate blank, suggesting that they represent the compounds present in phytosteryl ferulates extract. Table 14 shows the means of the peak areas of the mentioned peaks. Based on the data from this table, the peak areas decreased over time during digestion. For all the peaks with retention time between 36 and 42.2 min, a significant decrease was observed between 0 hour and 1 hour of digestion. Although the statistical test did not reveal any significant reduction, a reduction of 38%-60% was observed between 1 hour and 2 hours of digestion. Just as with phytosteryl glycosides digestion under stomach conditions, the adhesion of materials on the wall of the conical flasks was also observed during digestion of phytosteryl ferulates under stomach conditions. This could explain the observed reduction in peak areas over time.

The chromatograms in figure 8 also show other peaks detected in the samples incubated with and without pepsin and in substrate blank. Those peaks are labeled as A, B, C and D and their retention times were 20.7, 21.0, 22.7 and 25.1 min, respectively. Their mean peak areas at different time points are presented in table 15. These peaks were also identified in the samples of stigmasterol and phytosteryl glycosides during digestion under stomach conditions. Therefore, they are not considered as the degradation products of phytosteryl ferulates

In regard to the effect of enzyme, the hydrolysis of phytosteryl ferulates by pepsin was not expected. Also, the sensitivity of ester bonds toward pepsin has not been reported in the literature. One *in vitro* digestibility study of γ-oryzanol, which is a mixture of phytosteryl ferulates isolated from rice (Xu & Godber, 1999), reported a decrease in concentration of γ-oryzanol when the latter was incubated with pepsin, which is a digestive enzyme in the stomach, and then pancreatin, which is a mixture of digestive enzymes in the small intesine (Huang, 2003).

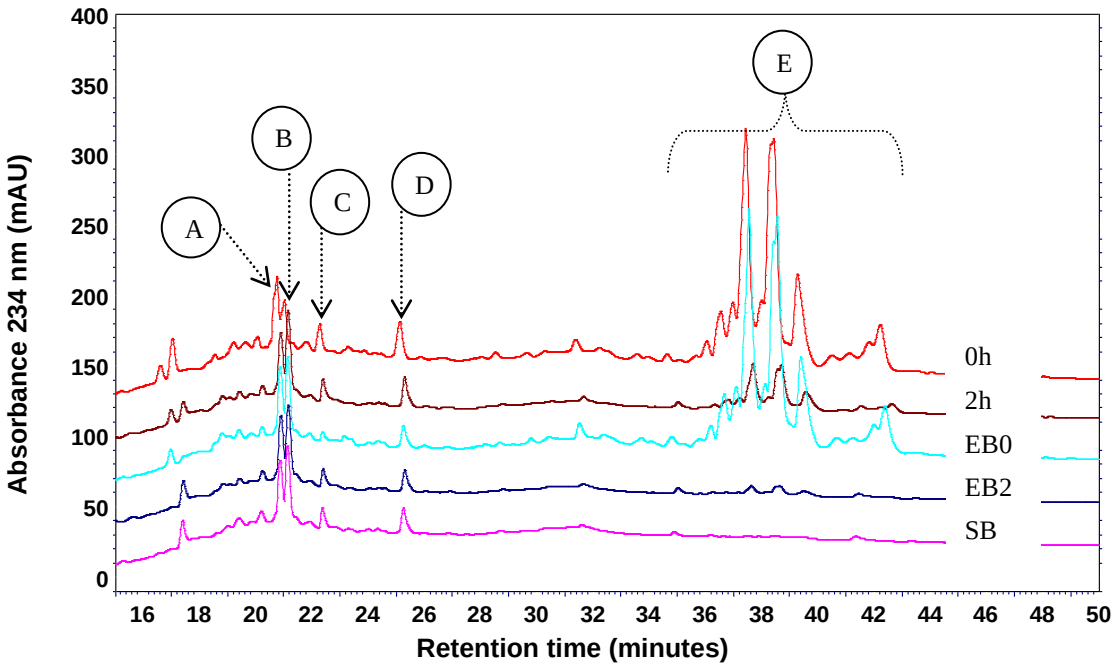

Figure 8 RP-HPLC chromatograms of phytosteryl ferulates and blanks at different time points during digestion under stomach conditions. 0h, 2h indicate the samples incubated with pepsin at the beginning and after 2 hours of digestion; EB_0 and EB_2 denote enzyme blanks at the beginning and after 2 hours; SB= substrate blank.

Table 14 Peak areas (mAU/10^4) of peaks E detected at different time points during digestion of phytosteryl ferulates under stomach conditions

	Digestion time (h)	Retention time (minutes)					
		36.5	36.9	37.4	38.4	39.2	42.2
Samples	0	33.1±4.1^a	18.9±2.2^a	224.9±11.7^a	282.7±33.0^a	98.8±16.0^a	35.8±4.3^a
	1	15.2±5.9^b	8.3±3.2^b	98.7±41.4^b	111.0±48.8^b	37.5±14.0^b	13.4±5.6^b
	2	6.0±1.4^b	3.5±1.0^b	41.7±13.6^b	49.3±16.6^b	17.6±5.3^b	8.2±4.9^b
Enzyme blank	0	35.3	19.5	234.8	253.4	84.1	22.3
	1	6.1	3.2	39.5	46.9	11.1	4.9
	2	1.2	0.7	8.0	9.9	3.6	1.3

For samples incubated with pepsin, the values are means (±SD) (n= 3); for each retention time, values followed by different letters are significantly different ($p \leq 0.05$); for enzyme blank, n= 1).

Table 15 Peak areas (mAU/10^4) of peaks indicated as A, B, C, and D detected at different time points during digestion of phytosteryl ferulates under stomach conditions

	Digestion time (h)	Retention time (minutes)			
		20.7	21.0	22.7	25.1
Samples	0	47.4±9.5	49.8±12.6a	14.2±5.1	24.9±8.8
	1	50.1±7.2	54.7±3.2a	10.8±1.1	22.7±0.7
	2	42.4±5.1	55.4±3.7a	11.4±0.8	23.1±4.9
Enzyme blank	0	43.9	53.0	5.1	19.0
	1	32.7	47.1	13.2	18.2
	2	39.4	49.5	10.8	19.0

For samples, the values are presented as means (±SD) (n= 3); for enzyme blank (n= 1)

Therefore, it was not clear whether the decrease was due to pepsin or pancreatin. Moreover, the author said that neither free phytosterols nor ferulic acid, which are the hydrolysis products of phytosteryl ferulates products of hydrolysis, was detected in the peptic digest. In our findings, there was no formation of new peaks, after 1 hour and 2 hours of digestion. Thus, there was no indication of degradation of compounds present in phytosteryl ferulates extract.

4.6 Stability of stigmasterol, phytosteryl glycosides and phytosteryl ferulates under small intestinal conditions

4.6.1 Stability of stigmasterol under small intestinal conditions

The stability of stigmasterol under small intestinal conditions was evaluated by comparing the chromatograms of samples with and without pancreatin, and those of substrate blanks at different time points of digestion. Figure 9 shows the chromatograms of stigmasterol samples with and without pancreatin and of the substrate blanks at the beginning (0 hour), after 1.5 hour and 3 hours of digestion. The stability of stigmasterol under small intestinal conditions was also assessed by comparing the stigmasterol recoveries at different times of digestion. Table 16 presents the amounts of stigmasterol recovered at the beginning (0 hour), after 1.5 hour and 3 hours of stigmasterol digestion under small intestinal conditions.

In all stigmasterol digested samples and in enzyme blanks, the peak of stigmasterol was detected, and its retention time varied between 29.7-29.9 min. The variation in retention time was probably due to the fluctuations in HPLC conditions (e.g. temperature, pressure). Stigmasterol was not detected in any of the substrate blanks. The peak of stigmasterol

obtained after small intestinal digestion coeluted with compound whose peak is labeled as C (figure 9). Its retention time was 30.1 min and its peak was detected in samples incubated with and without pancreatin and in substrate blanks. During the RP-HPLC analysis of substrates digested under small intestinal conditions, sitosterol standard solutions were also injected, and sitosterol eluted at 30.1 min. Thus, it is suggested that peak C did not result from stigmasterol breakdown. Rather, it could be sitosterol which originated from sitosterol standards. This is also supported by the fact that, in a RP-HPLC method, stigmasterol which is more polar compared to sitosterol, is expected to elute earlier.

The stability of stigmasterol was also evaluated by determining the recoveries (table 16). In stigmasterol digested samples, the amount of stigmasterol recovered at the beginning (0 hour), after 1.5 hour and 3 hours of digestion did not differ significantly ($p= 0.901$). In enzyme blanks, a statistical test did not reveal any significant difference in stigmasterol recovery ($p= 0.544$) between 0 hour and 3 hours of digestion.

Besides the peak of stigmasterol and the adjacent peak to it, three other important peaks indicated as A, B, and D were also detected in stigmasterol digested samples, in enzyme and substrate blanks. Their retention times were found to be 21.4 min (peak A), 25.8 min (peak B) and 39.3 min (peak D). The mean peak areas of peak A, B and D determined at different time points during digestion are presented in table 17. The peak areas of individual peak A, B and D at different time points showed variations but were almost the same in stigmasterol samples incubated with and without pancreatin. Three peaks eluted before peak A (between 17-21 min) and were found to be stable over time. These peaks were not considered as important because one of them (between 17-18 min) was also identified in stigmasterol samples digested under stomach conditions, and the other two were present in substrate blank. Peak D and the peaks eluting shortly before and after, are thought to come from the compounds that were present in phytosteryl glycosides samples which were run in the same sequence. The retention times of those peaks are the same in stigmasterol and phytosteryl glycosides samples digested under small intestinal conditions.

Pancreatin is a mixture of enzymes, principally amylase, lipase and trypsin (Mossner & Keim, 2011). Based on substrate specificity of these enzymes, stigmasterol breakdown by pancreatic enzymes was unexpected. The enzymatic degradation of free phytosterols under the small intestine has not been reported in the literature. Instead, free phytosterols, including stigmasterol, are found in the small intestine which implies that they are stable under stomach and small intestinal conditions.

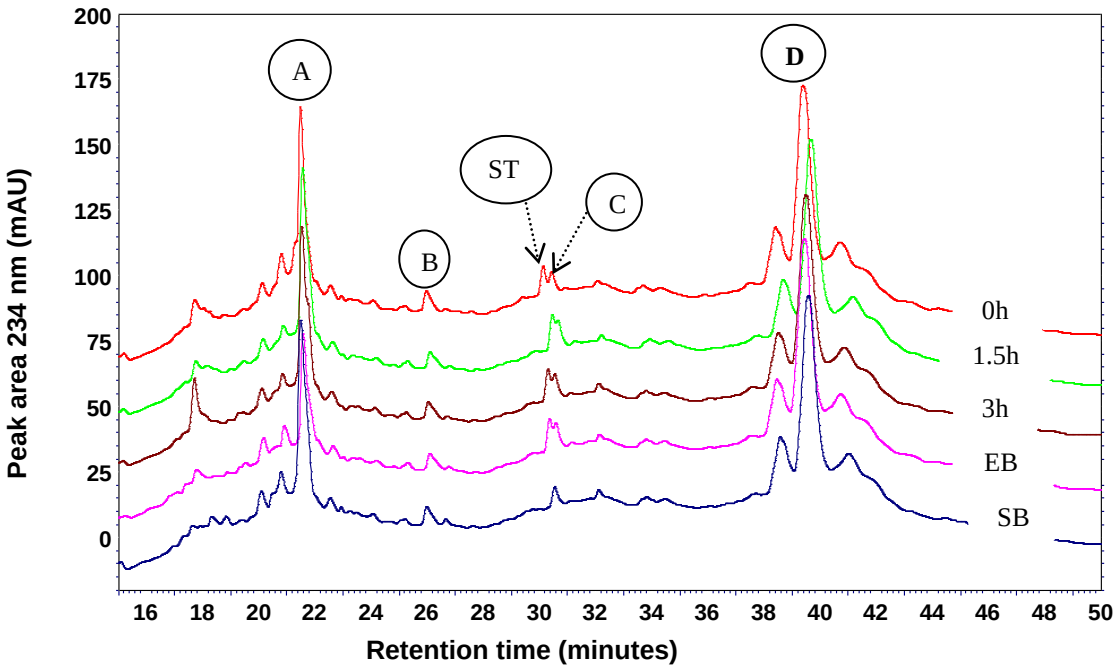

Figure 9 RP-HPLC chromatograms of stigmasterol and blanks at different time points during digestion under small intestinal conditions. 0h, 1.5h, 2h indicate the chromatograms obtained from samples incubated with pancreatin at the beginning, after 1h and 2 hours of digestion A, B, C and D are the peaks detected in the samples and in the blanks; ST= sigmasterol peak; EB= enzyme blank; SB= substrate blank.

Table 16 Stigmasterol recovered during digestion under small intestinal conditions

Digestion time (h)	Pancreatin treated stigmasterol samples (n=3)		Stigmasterol enzyme blanks (n=2)	
	Amount of stigmasterol (mg/ml)	% recovery	Amount of stigmasterol (mg/ml)	% recovery
0	0.520±0.06[a]	69.2±7.3	0.550±0.04[a]	72.7±5.4
1.5	0.543±0.04[a]	71.9±6.4	0.576*	72.4*
3	0.531±0.02[a]	70.3±3.5	0.557±0.01[a]	73.7±1.8

The values are means (±SD). In each column, the values followed by different letters are significantly different ($p \leq 0.05$); * recovery obtained from one repetition.

Table 17 Peak areas (mAU/10^4) of peaks other than stigmasterol peak detected at different time points during digesion under small intestinal conditions

	Digestion time (h)	Retention time (minutes)		
		21.4	25.8	39.3
Samples (n=3)	0	752.3±70.1	24.7±8.5	227.6±35.6
	1.5	843.0±168.3	21.0±6.0	205.1±2.4
	3	756.0±125.3	21.2±11.7	205.4±4.3
Enzyme blanks (n=2)	0	677.5±41.8	27.0±14.5	212.6±0.6
	1.5	735.9 ±5.5	29.5±2.8	211.0±2.1
	3	523.6±133.4	17.9±4.5	208.6±5.0

The values are presented as means (±SD).

4.6.2 Stability of phytosteryl glycosides under small intestinal conditions

To evaluate the stability of phytosteryl glycosides under small intestinal conditions, the RP-HPLC chromatograms (figure 10) derived from analysis of phytosteryl glycosides extract incubated with and without pancreatin, and those from substrate blanks were compared. The peaks indicated by A, B, D, E, F, G and H were detected in the phytosteryl glycoside samples incubated without pancreatin. Among them, the peaks A, B, E, F and G, with retention times 17.4, 18.2, 21.4, 28.1, 29.4, 31.1 and 37.2 min, are suggested to represent compounds that were present in phytosteryl glycosides extract because those peaks were not detected in the substrate blanks. On the contrary, the peaks D and H were detected in phytosteryl glycosides samples as well as in the substrate blanks. Their presence in the substrate blanks indicate that they did not derive from the compounds which were extracted during extraction of phytosteryl glycosides. The possible source of peaks D and H could be the nonenzymatic components from pancreatin. Tables 18 and 19 present the mean peak areas of peaks detected in the phytosteryl glycosides samples incubated without and with pancreatin, respectively.

Based on chromatograms shown in figure 10, several changes occurred when phytosteryl glycosides extract was incubated with pancreatin. For example, in samples incubated without pancreatin, the peak A (17.4 min), E (28.1 min) and G (37.2 min) which were present in the samples incubated without pancreatin, could not be detected in samples incubated with pancreatin. Additionally, the peak C (21.1 min) was formed in the samples incubated with pancreatin. However, its peak area decreased considerably ($p= 0.016$) between 0 hour and 1.5

hour of digestion. Between 1.5 hour and 3 hours, the peak area of peak C did not significantly change (p= 0.28).

Although their identity was not characterized, the disappearance of the peaks at 17.4 and at 37.2 min, and the formation of a peak at 21.1 min, was an indication that pancreatic enzymes have hydrolysed some compounds which were present in phytosteryl glycosides extract. However, no peaks corresponding to stigmasterol/sitosterol were identified. Thus, whether the hydrolysed compounds were phytosteryl glycosides or not still need to be evaluated.

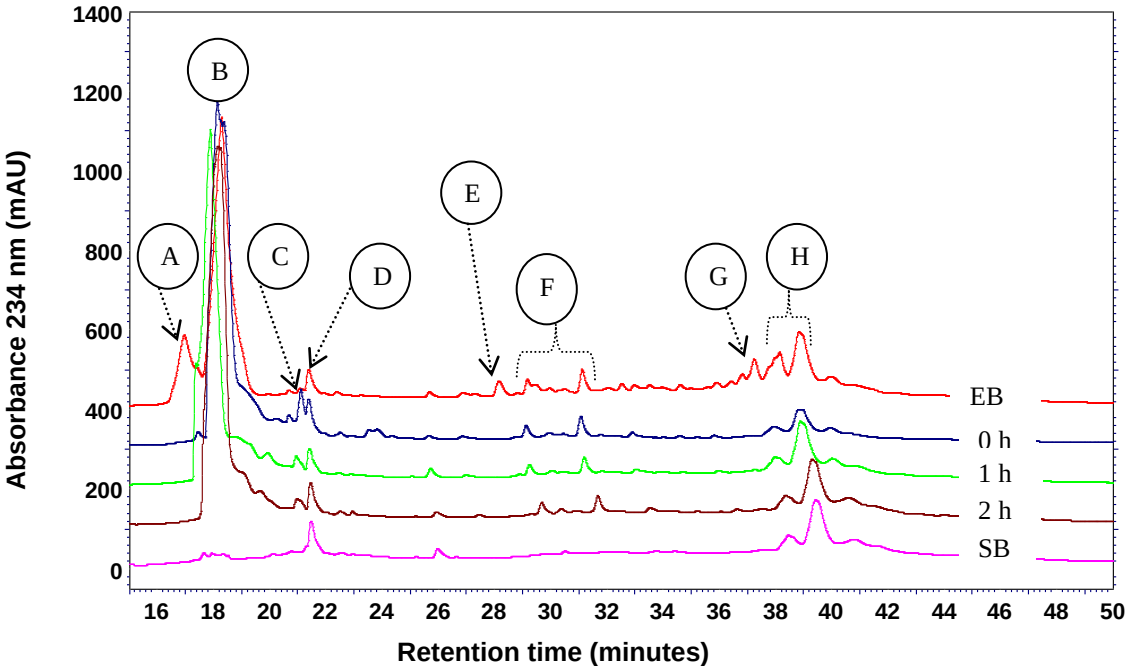

Figure 10 RP-HPLC chromatograms of phytosteryl glycosides and blanks at different time points during digestion under small intestinal conditions. 0h, 1h, 2h indicate the chromatograms obtained from the samples incubated with pancreatin at the beginning, after 1.5h and 3 hours; EB= enzyme blank; SB= substrate blank.

Table 18 Peak areas (mAU/10^4) of the peaks detected in phytosteryl glycosides samples incubated without pancreatin during digestion under stomach conditions

Digestion time (h)	Retention time (minutes)					
	17.4	18.2	28.1	29.4	31.1	37.2
0	59.0±31.9^{a}	254.5±24.2^{a}	7.1±0.9^{a}	3.4±0.4^{a}	7.4±0.8^{a}	7.5±0.8^{a}
1.5	17.9±16.8^{b}	294.1±2.4^{a}	7.7±0.7^{a}	3.7±0.5^{ab}	7.7±0.3^{a}	5.9±2.6^{a}
3	38.4±22.3^{a}	308.2±7.1^{a}	8.3±0.1^{a}	4.1±0.3^{b}	7.8±0.3^{a}	7.6±1.1^{a}

The values are means (±SD) (n= 3); for each retention time; the values with different letters are significantly different (p≤0.05).

Table 19 Peak areas (mAU/10^4) of the peaks detected in phytosteryl glycosides samples incubated with pancreatin during digestion of under stomach conditions

Digestion time (h)	Retention time (minutes)			
	18.0	21.1	29.2	31.2
0	170.2±40.5[a]	108.2±10.1[a]	4.4±0.2[a]	6.3±0.4[a]
1.5	177.8±29.1[a]	32.0±8.0[b]	3.0±0.6[a]	5.0±0.9[a]
3	221.5±54.8[a]	36.4±6.1[b]	3.9±0.6[a]	5.3±0.6[a]

The values are means (±SD) (n= 3); for each retention time, values with different letters are significantly different ($p \leq 0.05$).

Another observation which could be associated with the effect of pancreatic enzymes was the increase in peak area of peak A. Although the statistical difference was not revealed between the peak area at different time points of digestion ($p= 0.15$), the peak area of peak A increased over time, and this increase was considered as important. This peak increased by 13.8% between 0 hour and 1.5 hour, and by 14.3% between 1.5 hour and 3 hours of digestion. However, in enzyme blanks, the peak A also showed an increase of 15.5% between 0 and 1.5 hour and 4.7% between 1.5 and 3 hours of incubation. Therefore, the findings are still inconclusive as to whether this increase could be associated with the effect of peancreatin. Nevertheless, since phytosteryl glycosides are more polar than free phytosterols (Wewer *et al*., 2011), it was expected that they would elute before free phytosterols in a RP-HPLC system. Thus, the peak A with retention time of 18.2 min is thought to represent phytosteryl glycosides.

Previous studies have investigated the ability of mammalian digestive enzymes to hydrolyse phytosteryl glycosides. In a previously cited study by Weber (1988) on the metabolism of [4-^{14}C]sitosteryl-β-D-glucoside in rats, it was suggested that the hydrolysis of this compound took place in the small intestine, based on the presence of small amount of sitosterol. However, the author did not indicate which enzyme was responsible for this hydrolysis. On the contrary, a later *in vitro* study by (Moreau & Hicks, 2004) indicated that phytosteryl glycosides extracted from soy lecithin, oats oil and corn bran, were not hydrolysed by pancreatic enzymes. Instead, the authors indicated that incubation of phytosteryl glycosides extract with pancreatin resulted in an increase of peak area of phytosteryl glycosides (Moreau & Hicks, 2004). This increase was explained by the enzymatic hydrolysis of fatty acid moiety of acylated phytoteryl glycosides which were also present in the sample, and release of

phytosteryl glycosides (Moreau & Hicks, 2004). In a study by Nystrom *et al*. (2008), microbial and plant β-glycosidases were used to study their ability to hydrolyse phytosteryl glycosides extracted from wheat and rye. In their study, microbial β-glycosidases hydrolysed phytosteryl glycosides, but purified β-glycosidases from *A. niger* and from almonds were unable to hydrolyse phytosteryl glycosides. Rather, the latter enzymes increased the amount of phytosteryl glycosides by cleaving the fatty acid moiety of acylated phytosteryl glycosides, thus converting them into phytosteryl glycosides.

4.6.3 Stability of phytosteryl ferulates under small intestinal conditions

The stability of phytosteryl ferulates was assessed by comparing the chromatograms obtained from RP-HPLC analysis of phytosteryl ferulates extract digested under small intestinal conditions. Figure 11 shows the chromatograms resulting from phytosteryl ferulates extract incubated with and without pancreatin and substrate blank. The comparison of the chromatograms from samples incubated with and without pancreatin indicates that most of the peaks (between 35-42 min and between 28-30 min) which were detected in enzyme blanks could not detected in samples incubated with pancreatin. It is clear that the new peaks detected in samples to which pancreatin was added, resulted from the degradation of the compounds with the mentioned retention time ranges in the enzyme blanks. The newly formed peaks are indicated by A_1, A_2 and A_3, were formed at the beginning (0 hour), after 1.5 hour and 3 hours of digestion. Their retention times were 19.2, 18.5 and 17.6 min, respectively. Their mean peak areas were found to vary over time, being 361.7±39.7, 201.1±170.6, and 665.1±78.0 for A_1, A_2 and A_3, respectively. Apparently, the peaks A_1, A_2 and A_3 seemed to be related to each other but it is not known whether they are or not the same. It seems that the peak A_2 and peak A_3 are the same as peak A_1 which shifted in retention time. The peak B also seems to be the same as the peaks identified at around 22 min after 1.5 and 3 hours. However, the peak B had two adjacent peaks which could not be detected after 1.5 and 3 hours of digestion.

The information on the capability of pancreatic enzymes to hydrolyse phytosteryl ferulates from wheat germs is scarce; but some studies have provided evidence on the ability of pancreatic enzymes to hydrolyse phytosteryl ferulates from other sources. *In vitro* study by Moreau and Hicks (2004) indicated that 47.3% of purified sitostanly ferulates were hydrolysed by porcine pancreatin after 4 hours. However, the authors indicated that pancreatin was unable to hydrolyse oryzanol which is a mixture of phytosteryl ferulates extracted from rice bran.

Contrary to Moreau and Hicks (2004), Miller *et al*. (2004) and Huang (2003) studied the hydrolysis of individual components of oryzanol and concluded that among different types of phytosteryl ferulates present in oryzanol, only sitosteryl and campesteryl ferulates were hydrolysed by pancreatic enzymes. Cholesterol esterase which is also one of the pancreatic enzymes was found capable to hydrolyse phytosteryl ferulates (Miller *et al*., 2004; Nystrom *et al*., 2008)

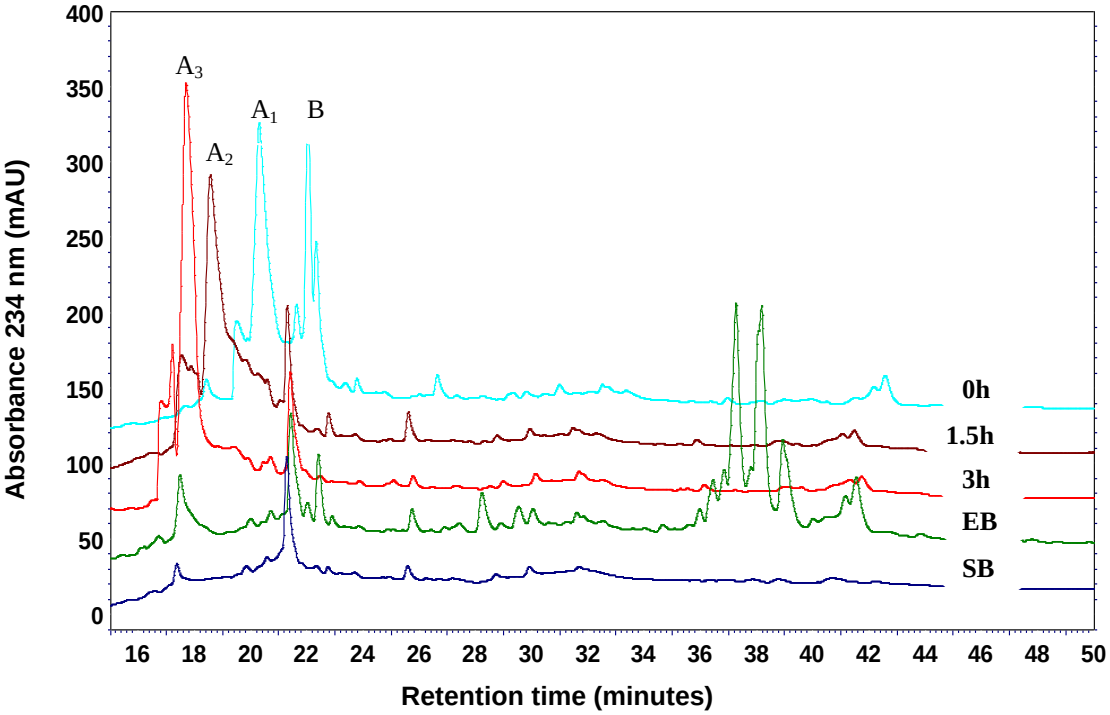

Figure 11 RP-HPLC chromatograms of phytosteryl ferulates and blanks at different time points during digestion under small intestinal conditions. 0h, 1h, 2h indicate the chromatograms obtained from samples incubated with pancreatin at the beginning, after 1h and 2 hours of digestion; SB= substrate blank; EB= enzyme blank.

The chromatograms shown in figure 11 indicate that most of the peaks which were present in the phytosteryl ferulates extract incubated without pancreatin could not be detected after addition of pancreatin. Comparing the chemical structures of free phytosterols and phytosteryl ferulates, the latter are relatively more polar than the former (Jiang & Wang, 2005). Therefore, in the RP-HPLC system, phytosteryl ferulates were expected to elute earlier than free phytosterols. Unfortunately, no peak was detected at the retention time of stigmasterol and sitosterol, which are among the most common free phytosterols in foods. As was evidenced by the results discussed in 4.3, phytosteryl ferulates elute before free phytosterols. Therefore,

it is still unkown whether the peaks detected between 35-40 min are phytosteryl ferulates or other compounds which were present in the extract, and which are also broken down when subjected to the small intestinal conditions. Phytosteryl ferulates extract from SPE fractionation was reported to contain some amounts of other polar lipids (Nystrom *et al*., 2008). Thus, there is need of further analysis to characterize the identity of the peaks in samples incubated without enzyme, and those which are formed when pancreatin is added.

Chapter 5 CONCLUSIONS

Phytosterols, in particular phytosteryl ferulates and phytosteryl glycosides have been shown to possess important physiological properties. The most studied is their ability to lower blood cholesterol and the underlying mechanisms, but more research is also needed in order to understand their metabolic fate. This thesis focused on the behavior of phytosteryl ferulates and phytosteryl glycosides from wheat germs under stomach and small intestinal conditions.

The results obtained indicate that phytosteryl ferulates and phytosteryl glycosides can be extracted from wheat germs, using Soxhlet extraction method and acetone as extraction solvent. The pH was found to influence the extractability of phytosteryl ferulates. The results also indicate that stigmasterol is not degraded neither under stomach nor small intestinal conditions. This implies that intact stigmasterol will be absorbed in the small intestine and that unabsorbed stigmasterol will be further metabolized in the colon or excreted as such.

For phytosterol conjugates, the qualitative analysis indicates that the extracts of both phytosteryl ferulates and phytosteryl glycosides are stable under stomach conditions. On the other hand, the results revealed that these extracts contain compounds which are unstable under small intestinal conditions.

Chapter 6 RECOMMENDATIONS

The digestion of phytosteryl ferulates and phytosteryl glycosides under stomach and small intestinal conditions was assessed based on the changes in the peak intensities. The use of this more qualitative, rather than quantitative approach, was due to the lack of suitable internal standards. Therefore, we suggest that pure standards are first obtained in order to quantitatively evaluate the stability of phytosteryl ferulates and phytosteryl glycosides under stomach and small intestinal conditions. In this regard, enzymatic or chemical synthesis of phytosteryl ferulates/ glycosides can be recommended.

Since phytosteryl ferulates and phytosteryl glycosides are heterogeneous compounds, their extracts contain different components. For example, ferulic acid or sugars may form different conjugates with different types of free phytosterols, and those conjugates may behave differently when subjected to certain conditions. Therefore, for metabolic studies, it would be necessary to evaluate individual components.

Finally, it should be kept in mind that the evaluation of the stability of compounds by mimicking stomach or small intestinal conditions may be impaired for example by the inhibition of enzymes by metabolic products. Therefore, well designed studies are needed for the metabolic studies of phytosteryl ferulates and phytosteryl glycosides. More UPLC-MS investigations are also important for the study and identification of the metabolites that are formed.

In view of the growing interest in using phytosterols, phytosteryl ferulates and phytosteryl glycosides may offer new opportunities to expand the benefits of phytosterols. Therefore, the studies of the stability of the phytosteryl ferulates and phytosteryl glycosides in the gastro-intestinal tract may be important for food industry.

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APPENDICES A: Chromatograms of selected ions of phytosteryl ferulates

Appendix A1

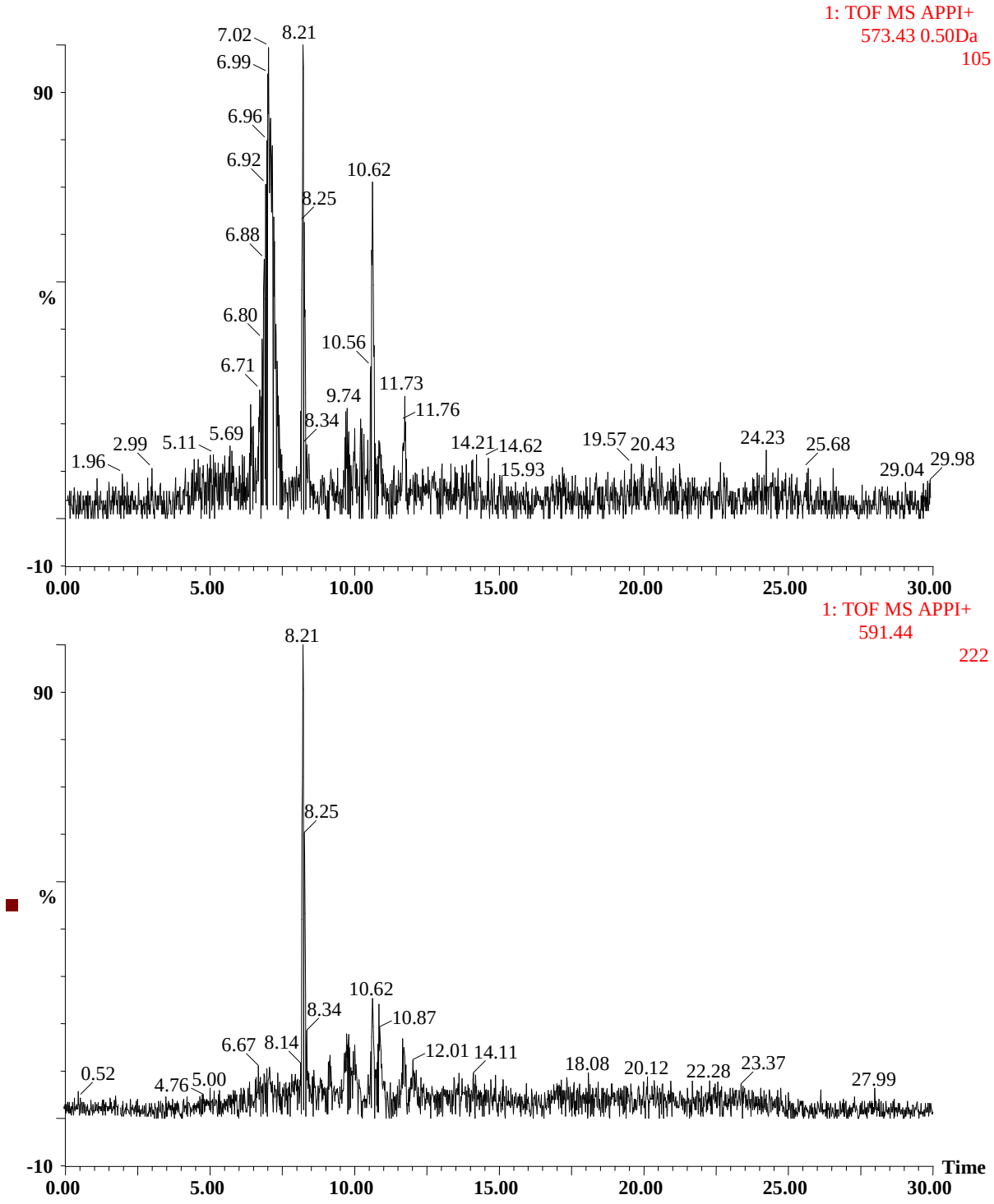

Figure 12 Selected ion chromatograms of sitosteryl ferulate; upper chromatogram *m/z*= 573.43 $[M\text{-}H_2O+H]^+$; lower chromatogram *m/z*= 591.44 $[M+H]^+$, in the phytosteryl ferulate 2 fraction.

Appendix A2

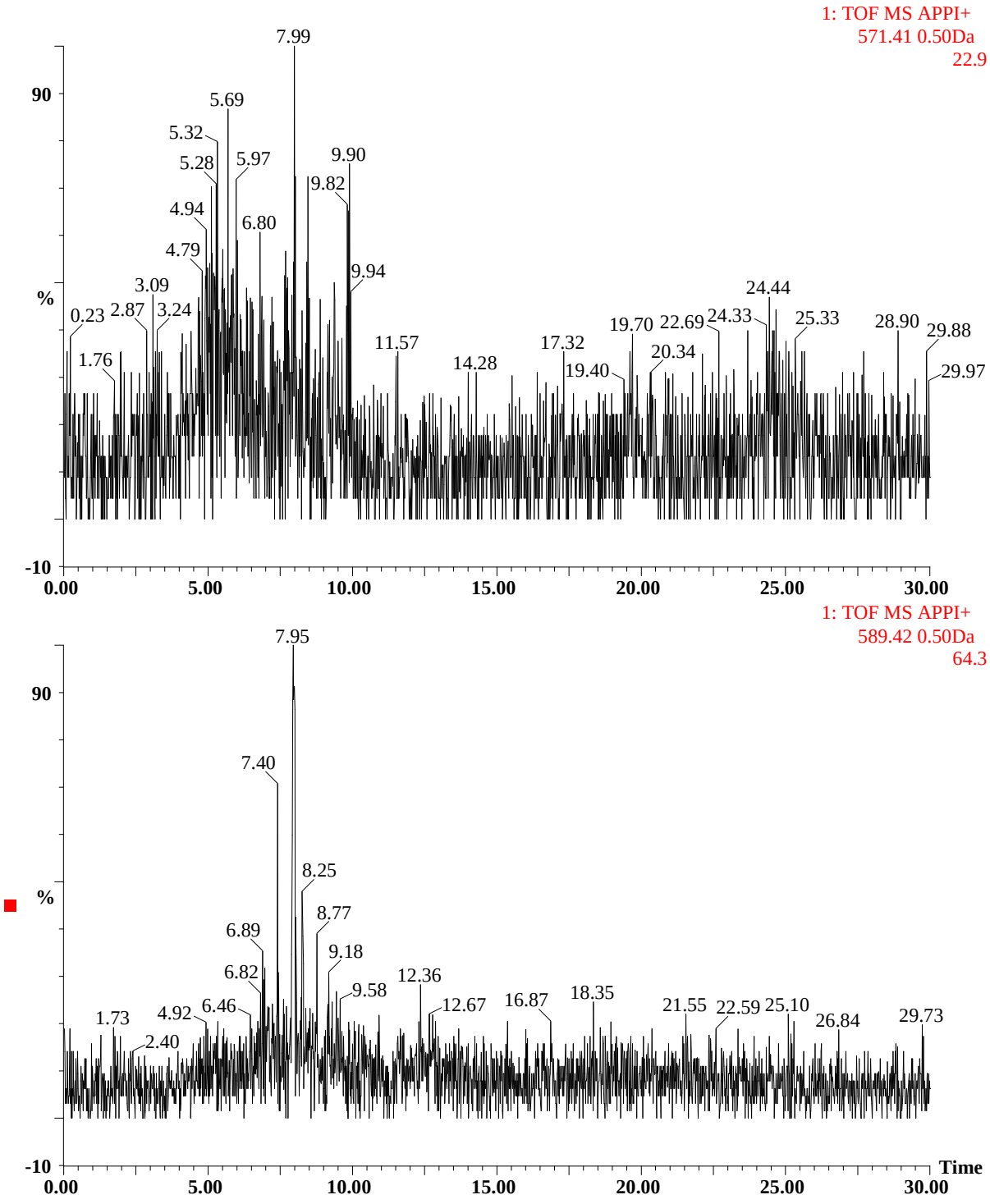

Figure 13 Selected ion chromatograms of stigmasteryl ferulate; upper chromatogram *m/z*= 571.41 $[M\text{-}H_2O+H]^+$; lower chromatogram *m/z*= 589.42 $[M+H]^+$, in the phytosteryl ferulate 2 fraction.

Appendix A3

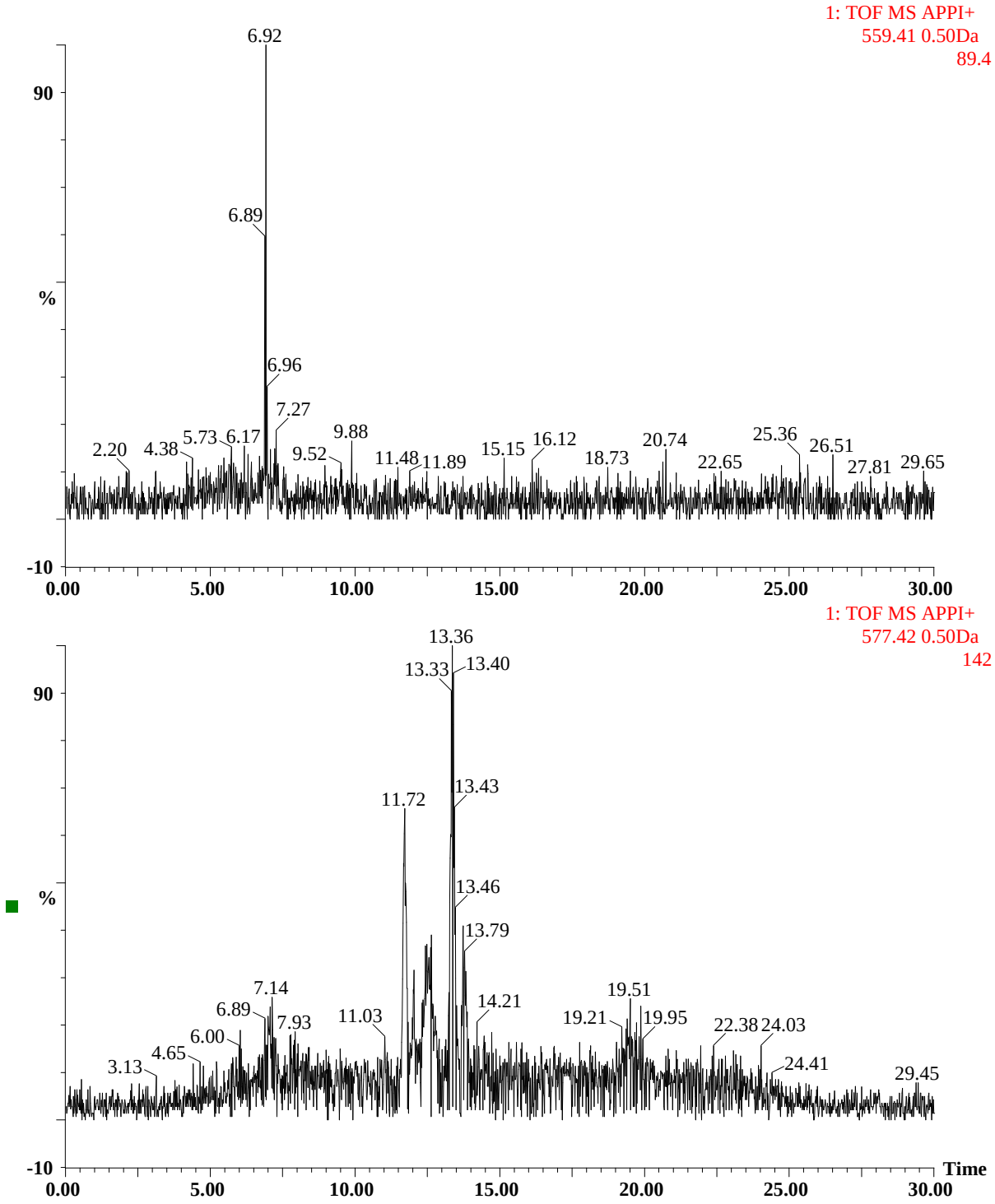

Figure 14 Selected ion chromatograms of campesteryl ferulate; upper chromatogram *m/z*= 559.41 $[M\text{-}H_2O+H]^+$; lower chromatogram *m/z*= 577.42 $[M+H]^+$, in the phytosteryl ferulate 2 fraction

APPENDICES B: Chromatograms of selected ions of phytosteryl glucosides

Appendix B1

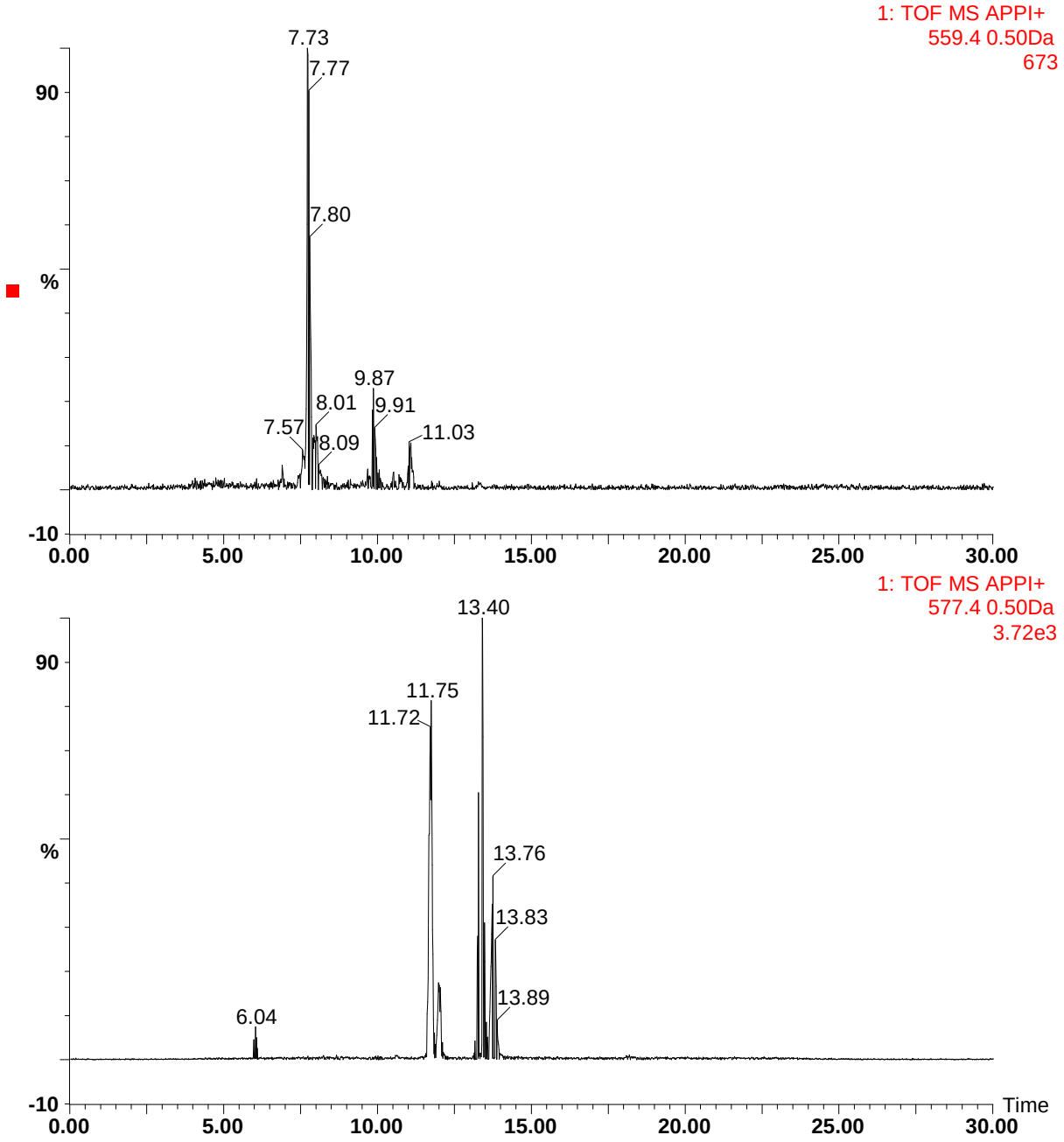

Figure 15 Selected ion chromatograms of sitosteryl glucoside (upper chromatogram *m/z* =559.4 $[M\text{-}H_2O+H]^+$, lower chromatogram *m/z* = 577.4 $[M+H]^+$) in the phytosteryl glycoside fraction.

Appendix B2

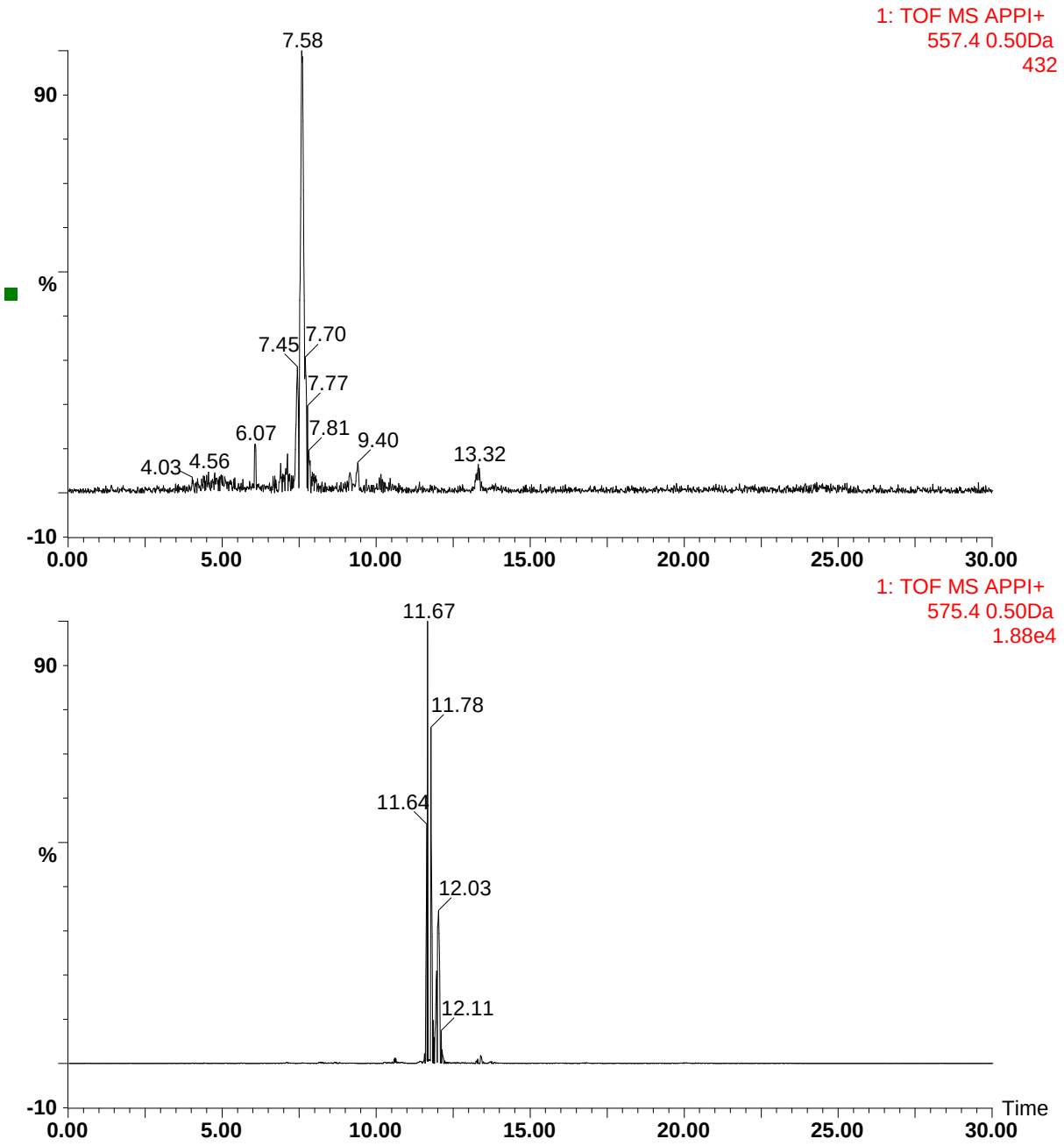

Figure 16 Selected ion chromatograms of stigmasteryl glucoside (upper chromatogram *m/z*= 557.4 $[M\text{-}H_2O+H]^+$, lower chromatogram *m/z*= 575.4 $[M+H]^+$) in the phytosteryl glycoside fraction.

Appendix B3

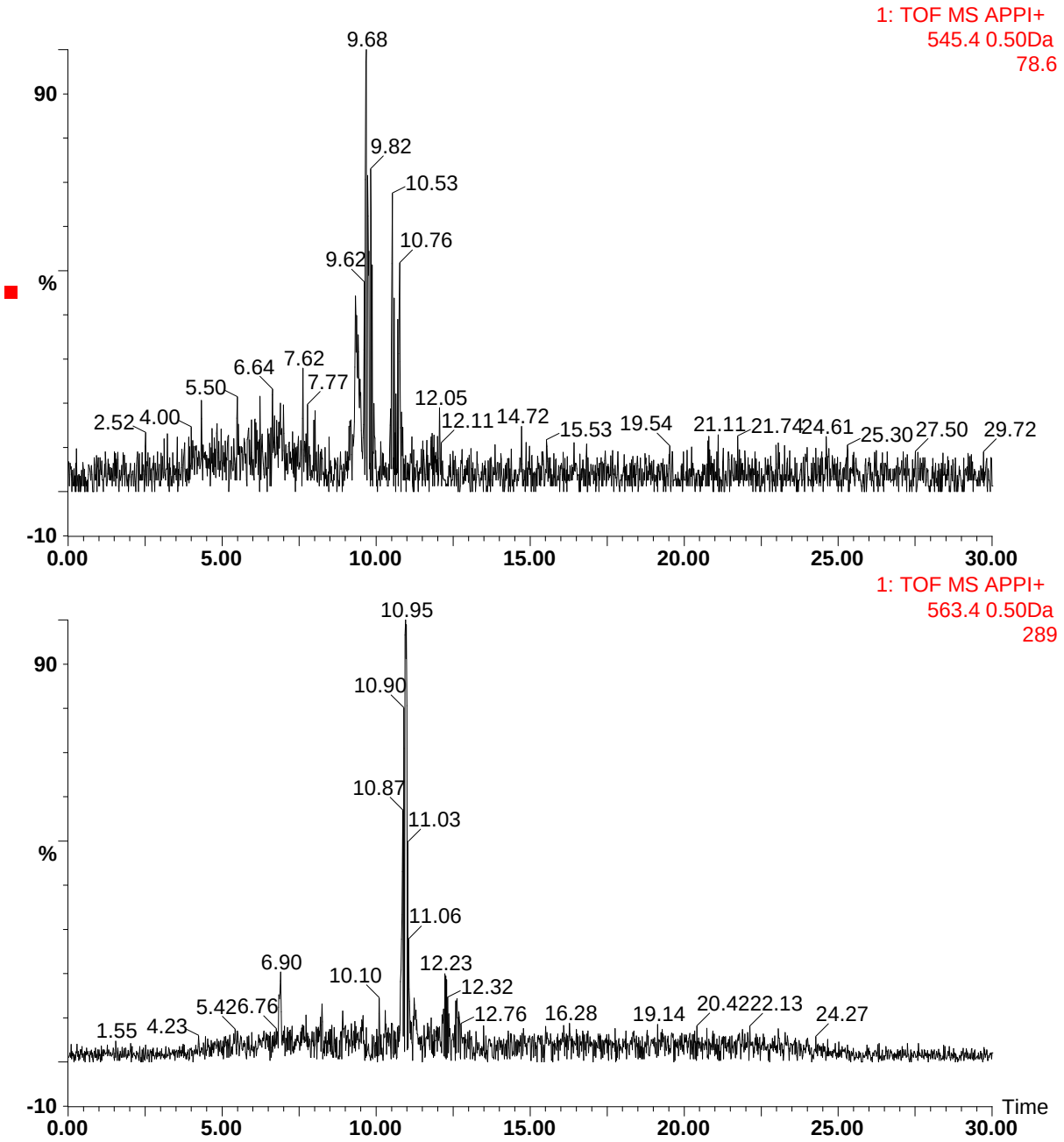

Figure 17 Selected ion chromatograms of campesteryl glucoside (upper chromatogram *m/z*= 545.4 $[M\text{-}H_2O+H]^+$, lower chromatogram *m/z*= 563.4 $[M+H]^+$) in the phytosteryl glycoside fraction.