

Occurrence and properties of steryl ferulates and glycosides in wheat and rye

Laura Nyström

ACADEMIC DISSERTATION

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To my family

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ABSTRACT

Cereal kernels are known to contain a number of minor components that possess beneficial health attributes. In this thesis rye and wheat were studied as sources of steryl ferulates and steryl glycosides and their behaviour in processing were evaluated. Further, enzymatic hydrolysis of these conjugates was studied, as well as the capacity of steryl ferulates to inhibit lipid oxidation at different temperatures.

Steryl ferulates were shown to have a strong positive correlation with dietary fibre contents in milling fractions from the outer parts of the kernels obtained from a commercial scale mill. Highest contents of steryl ferulates were found in the bran in both cereals, with the content decreasing once moving towards the inner parts of the kernel. Variation in the contents of steryl ferulates was higher in wheat fractions than rye fractions. Steryl glycosides, on the other hand, had either negative or no correlation with dietary fibre, and the range of the steryl glycoside contents was much narrower than that of steryl ferulates in both cereals. There were significant differences in the sterol compositions of these steryl conjugates when compared with each other or with the total plant sterols in the corresponding fractions.

Properties of steryl ferulates and steryl glycosides were evaluated after common processing methods and in enzymatic hydrolysis. Thermal and mechanical processing had only minor or no effects on the contents of steryl conjugates from rye and wheat bran. Enzymatic treatments on the other hand caused some changes, especially in the contents of glycosylated sterols. When steryl ferulates extracted from rye or wheat bran were subjected to enzymatic treatments by steryl esterase, significant differences in the rates of hydrolysis were observed between steryl ferulates from different sources with differing sterol compositions. Further, differences were also observed between enzymes from different sources. Steryl glycosides were shown to be hydrolysed by β -glucosidase (cellobiase) from *A. niger*, but less with β -glucosidases from other sources.

Steryl ferulates showed good antioxidant activity at both moderate and high temperatures. In bulk and emulsion systems of methyl linoleate at 40°C steryl ferulates extracted from rye and wheat bran inhibited hydroperoxide formation much more effectively than synthetic steryl ferulates or those extracted from rice (γ -oryzanol), demonstrating that the sterol composition has an effect on the activity. At cooking (100°C) and frying temperatures (180°C) sitostanyl ferulate was shown to inhibit polymer formation significantly and, especially at 100°C, comparably to α -tocopherol. The rate of antioxidant degradation was slower for sitostanyl ferulate, showing higher heat stability than α -tocopherol. When evaluated as a mixture, no synergistic effect was observed between these two antioxidants.

The data presented in this thesis provides information that may henceforth be applied when evaluating the intakes of steryl conjugates from cereal sources, as well as their possible influences as minor bioactive components. Wheat and rye both are good sources of steryl ferulates and steryl glycosides and, especially with steryl ferulates, what may be lost out to some other cereals on quantity is compensated with quality of the sterol composition.

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Sipoo, November 2007

A handwritten signature in black ink, appearing to read "Lina Myström". The signature is written in a cursive, flowing style with a long, sweeping tail on the final letter.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals:

- I** Nyström, L., Paasonen, A., Lampi, A.-M., Piironen V. 2007. Total plant sterols, steryl ferulates and steryl glycosides in milling fractions of wheat and rye. *Journal of Cereal Science*, 45, 106-115.
- II** Nyström, L., Lampi, A.-M., Rita, H., Aura, A.-M., Oksman-Caldentey, K.-M., Piironen, V. 2007. Effects of processing on availability of total plant sterols, steryl ferulates and steryl glycosides from wheat and rye bran. *Journal of Agricultural and Food Chemistry*, 55, 9059-9065.
- III** Nyström, L., Moreau, R.A., Lampi, A.-M., Hicks, K.B., Piironen V. Enzymatic hydrolysis of steryl ferulates and steryl glycosides. *European Food Research and Technology*, in press; DOI 10.1007/s00217-007-0780-z.
- IV** Nyström, L., Mäkinen, M., Lampi, A.-M., Piironen, V. 2005. Antioxidant activity of steryl ferulate extracts from rye and wheat bran. *Journal of Agricultural and Food Chemistry*, 53, 2503-2510.
- V** Nyström, L., Achrenius, T., Lampi, A.-M., Moreau, R.A., Piironen, V. 2007. A comparison of the antioxidant properties of steryl ferulates with tocopherol at high temperatures. *Food Chemistry*, 101, 947-954.

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Contribution of the author to papers I to V:

- I, II, V** Laura Nyström planned the study together with the other authors. She had the main responsibility for interpreting the results, and she acted as the corresponding author of the paper.
- III** Laura Nyström planned the study together with the other authors and she performed part of the experiments. She had the main responsibility for interpreting the results, and she acted as the corresponding author of the paper.
- IV** Laura Nyström planned the study together with the other authors and she performed the experiments. She had the main responsibility for interpreting the results, and she acted as the corresponding author of the paper.

LIST OF ABBREVIATIONS

AAPH	2,2'-azobis(2-methylpropionamidine)-dihydrochloride
α -T	α -tocopherol
AMVN	2,2'-azobis(2,4-dimethylvaleronitrile)
AOX	antioxidant
ASG	acylated steryl glycoside
BSTFA	<i>N,O</i> -bis-(trimethylsilyl)trifluoroacetamide
CAF	cycloartenyl ferulate
CAM	campesteryl ferulate
db	dry basis
DPPH	1,1-diphenyl-2-picrylhydrazyl
FAME	fatty acid methyl ester
FID	flame ionization detection
FS	free steryl alcohol(s)
GC	gas chromatograph(y)
HDL	high density lipoprotein
HOSO	high oleic sunflower oil
HPLC	high performance liquid chromatograph(y)
IC ₅₀	50% inhibition concentration
KOH	potassium hydroxide
LDL	low density lipoprotein
MeLo	methyl linoleate
24-met-CAF	24-methylenecycloartanyl ferulate
ORAC	oxygen radical absorbance capacity
ORY	γ -oryzanol
OSI	oxidative stability index
PC	phosphatidyl choline
RBO	rice bran oil
SE	steryl fatty acid ester(s)
SF	steryl ferulate(s)
SG	steryl glycoside(s)
SPE	solid phase extraction
SSta	sitostanyl ferulate
SSte	sitosteryl ferulate
TBARS	thiobarbituric acid reactive substances
TLC	thin layer chromatography
TMCS	trimethylchlorosilane
wb	wet basis
WOF	warmed over flavour

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1 INTRODUCTION

Plant sterols (aka. phytosterols) have gained a vast and increasing interest among researchers, food industry and consumers over the past decade. Functional foods enriched with sterols are marketed as means to decrease serum cholesterol levels and increase the proportion of HDL-cholesterol, thus decreasing the risks of cardiovascular disease. Sterol-enriched margarine has been on the market for over ten years, and the product range of sterol-enriched products has broadened to cover milk, yoghurt, pasta, bread etc. In addition to the cholesterol lowering properties, sterols are suggested to inhibit certain forms of cancer, though there are still less studies on this aspect. A daily dose of two grams is the recommended intake to accomplish 10-15% decrease in the total serum cholesterol content (Ostlund, 2002). However, recent studies have shown that cholesterol absorption from the intestine can be significantly reduced at levels as low as 150 mg per dose, and that supplemental doses of 800 mg reduce LDL-cholesterol levels significantly (Ostlund et al., 2002). Hence it is possible that also the natural dietary sources of plant sterols with moderate contents of plant sterols can positively affect the cholesterol levels in humans, and may therefore play an important role as a part of a healthy diet.

The range of sterol contents in cereals is on average 350-1200 µg/g, whereas the sterol content in vegetable oils can be 5-15 fold higher compared to that of cereals (Piironen and Lampi, 2004). Cereals and vegetable oils are the most important sources of dietary plant sterols, the order of importance depending on the country. In Finnish diets, for example, cereals are the primary source of plant sterols, contributing about 40% of the daily intake (Valsta et al., 2004). The average daily intakes of sterols in Finland are 305 mg for men and 237 mg for women (Valsta et al., 2004), whereas in the U.K. the average intakes are 310 mg and 303 mg for men and women, respectively (Andersson et al., 2004). Plant sterols in cereals belong to the group of so-called co-passengers of dietary fibre and thus are a part of the *dietary fibre complex*, which is a concept used to describe the dietary fibre and associated minor bioactive substances (Trowell, 1976). In addition to sterols the minor components include e.g. minerals, lignans, tocopherols, folates, phenolic acids and alkylresorcinols, which all add to the healthy nutritional effects of fibre rich foods.

In cereals significant proportion of sterols are found as glycosides and esters of phenolic acids, mainly ferulic acid, whereas in oilseeds and vegetables sterols are mainly found as unconjugated free sterols or as esters with fatty acids. Steryl glycosides can be found from various plant materials, whereas steryl ferulates are thought to be unique to seeds only. These two conjugates are far less studied than free sterols or their fatty acid esters, partly because the

methodologies for plant sterol analysis are based on cholesterol analysis, which might not take into consideration these compounds that are characteristic to cereals. However, they may each contribute about 10-15 % of total sterols in cereals and therefore should not be neglected. So far, studies on steryl ferulates have heavily focused on rice kernels and products, though significant quantities are found in other cereals as well (Seitz, 1989; Moreau et al., 1999; Hakala et al., 2002). Steryl glycosides, on the other hand have been documented from plant leaves, tubers and non-cereal seeds. However, information on the contents of steryl ferulates and glycosides in cereals like wheat and rye is still scarce and deserves further studies.

In addition to the inhibition of cholesterol absorption steryl ferulates and steryl glycosides are thought to possess some additional health promoting properties, and both compounds can be found in the market as nutraceutical preparations. Antioxidant activity of free ferulic acid is well established (Graf, 1992), and as a sterol ester this antioxidant is combined with a compound that has an additional health effect as a cholesterol absorption inhibitor. Esterification of ferulic acid to sterol also increases its hydrophobic character and therefore increases its solubility in less polar environments. The capacity of steryl ferulates to inhibit oxidation is thought to be responsible for e.g. anti-inflammatory, antiviral and anticancer properties. Steryl glycosides have been shown to have positive effects on for example the treatment of benign prostate hyperplasia (Pegel, 1997).

This thesis reviews the literature on the occurrence of steryl ferulates and steryl glycosides in cereals, as well as their properties as components in foods and diet. The experimental part of this thesis is a summary of the data published in the attached papers **I-V**, in which steryl ferulates and glycosides of rye and wheat are characterised, and their properties in processing are evaluated. Further, antioxidant activity of steryl ferulates is studied. The significance of the results is discussed and based on the described observations, suggestions for further research are offered.

2 REVIEW OF THE LITERATURE

2.1 Steryl ferulates and steryl glycosides in cereals

2.1.1 Steryl ferulates

Ferulic acid esters of sterols were first reported in 1955, when oryzanol was extracted and characterised from rice bran by Kaneko and Tsuchiya (1955). Oryzanol has later been characterised as a mixture of ferulic acid esters of several plant sterols and triterpene alcohols (Figure 1).

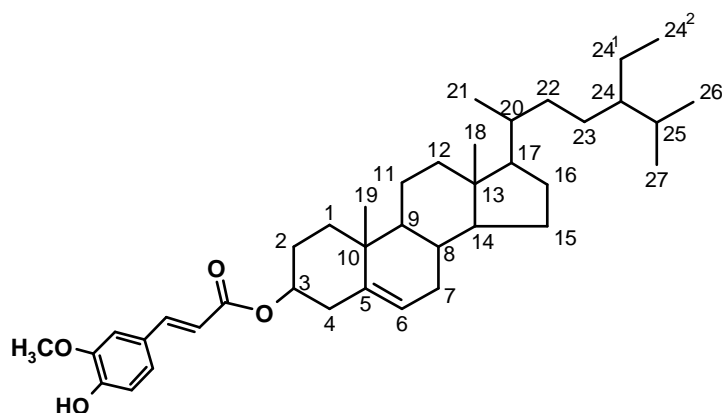


Figure 1. Structural formula of sitosteryl ferulate (for more common SF structures see Table 2).

The sterol moieties of steryl ferulates differ principally in three different aspects: 1) presence or absence of a double bond between C-5 and C-6 (the saturated sterols without the double bond are also called stanols), 2) the number of methyl groups attached to C-4 (thus grouped as desmethyl-, 4-monomethyl and 4,4'-dimethylsterols), and 3) structure of the side chain attached to the tetracyclic ring system. The 4,4'-dimethylsterols (triterpene alcohols) often also have a cyclopropane structure in position C-9/C-10, and a methyl group in C-14.

2.1.1.1 Rice

Steryl ferulates of rice (*Oryza sativa* L.) are by far the most extensively studied and reported. The mixture of steryl ferulates from rice is commonly called γ -oryzanol, in which at least 16 different ferulic acid esters of sterols and triterpene alcohols have been identified (Table 2). The content of oryzanol varies between different rice varieties, and highest content of

oryzanol is found in bran layers of the kernels (Table 1).¹ The content of oryzanol in brown rice kernels varies approximately between 200-750 µg/g, whereas the range of oryzanol content in rice bran is roughly ten times higher.

Table 1. Content of oryzanol in rice kernels and rice kernel fractions in recent studies. Long (L), medium (M) and short (S) grain cultivars are denoted when defined.

Sample (number of varieties)	Kernel length	Oryzanol content (µg/g)	db/wb ¹	Reference
Brown rice (4)	L	324-544	wb	Miller and Engel, 2006
Brown rice (8)	S	262-627	wb	ibid.
Brown rice (3)		201-388	db	Aguilar-Garcia et al., 2007
Brown rice		482		Ohtsubo et al., 2005
Brown rice (3)		500-720		Khatoon and Gopala Krishna, 2004
Rice grains (3)		310-627		Miller et al., 2003
Rice bran (7)	L, M	2510-6860		Bergman and Xu, 2003
Rice bran		3101		Shin and Godber, 1996
Rice bran (3)		1550-2720	db	Aguilar-Garcia et al., 2007
Rice bran		2490		Collins et al., 2002
Rice bran	L	4740		Lloyd et al., 2000
Rice bran	M	4000		ibid.
Rice bran (2)	L	6730-8400		Rohrer and Siebenmorgen, 2004
Polished rice		61		Ohtsubo et al., 2005
Milled (polished) rice (3)		70-120		Khatoon and Gopala Krishna, 2004

¹ See the footnote.

Natural variation of oryzanol in rice

The effects of genetic and environmental factors on oryzanol contents in rice have been reported recently in two studies and they show enormous variation in oryzanol contents. Bergman and Xu (2003) studied the oryzanol contents of seven different rice cultivars grown in four locations and in two consecutive seasons. They showed that the oryzanol content of rice bran (averaged across cultivars, years and growing locations) ranged from 2510-6860 µg/g with mean content at 4260 µg/g. The greatest effect on oryzanol content were ranked in the following order: year-by-growing location interaction effect, cultivar and year, and in general growing location was shown to have a greater effect on oryzanol content than genotype of the samples. The study of Miller and Engel (2006) showed similar effects of growing location and cultivar on the oryzanol content of European brown rice. For example one cultivar (Balilla) grown in Italy had about 20% higher content of oryzanol than the same cultivar grown in Spain in the same year. Even greater difference (27%) in oryzanol content in that study was seen when the same cultivar was grown in the same location in two different years. On the other hand, no significant differences were observed, when long-grain and

¹ **NOTE:** In all the tables presented in the literature review the values for the contents of steryl conjugates are given as the values presented in the references, without amendments with the moisture contents. Specifications on the presentation format, db for dry basis and wb for wet basis, are given, when unambiguously presented in the reference.

short-grain rice cultivars were compared, as they both had fairly wide ranges of oryzanol contents (Table 1).

Processing effects on oryzanol content

Most of oryzanol is removed in milling (polishing) of rice, as the oryzanol content in the remaining rice kernels after milling is about 60-120 $\mu\text{g/g}$. Localization of oryzanol in outer pericarp, seed coat and nucellus layers of grains was demonstrated by Lloyd et al. (2000), who studied the effects of commercial milling process on contents of oryzanol in rice bran. The oryzanol content in the bran from first mill break, which removed the outermost bran layers, was 51% higher than the bran from the second break. The content of oryzanol in long-grain rice from the first mill break (6420 $\mu\text{g/g}$) was significantly higher than that of medium-grain rice (5170 $\mu\text{g/g}$), but differences in oryzanol contents between different rice types were no longer seen in bran samples from later mill breaks.

Localization of oryzanol in outer layers of two long-grain rice varieties (cv. Cypress and Drew) was further studied by Rohrer and Siebenmorgen (2004), who demonstrated that oryzanol content was significantly reduced in bran fractions obtained by successive millings of rice grains with a dehulling apparatus. They separated the kernels by size before milling, and showed that the overall content of oryzanol was higher in bran from thicker kernels (>1.84 mm) than bran from thinner kernels of the same cultivar. As shown in Table 1 the contents of oryzanol in rice bran vary greatly, the range between different studies being 1550-8400 $\mu\text{g/g}$. In addition to natural causes of variation (e.g. cultivar, place and year of cultivation discussed above) and differences in the analytical procedures, it is likely that significant variation is caused by the milling procedure. Removal of 5.6% of the kernel weight by milling can decrease the content of oryzanol to half of the original content. Therefore, if unequal percentage of kernel is removed as the bran, the resulting milling products are not directly comparable in terms of their oryzanol content.

In addition to milling practises also other processing affects the oryzanol content in rice products. The effects of milling cause differences in the actual content of steryl ferulates in the original product, but the original content may also be a subject to change during subsequent processing of the cereal product. Shin et al. (1997) studied the effects of extrusion of rice bran on the content of oryzanol, and demonstrated that the content was significantly decreased by longer hold times (5% decrease) and higher extrusion temperatures (3% decrease). Much higher decreases in oryzanol content were, however, observed in the same study when rice bran was stored at ambient temperature (22-26°C) for one year (Shin et al., 1997). The content of steryl ferulates in both raw rice bran (non-extruded) and extruded rice bran decreased about 63% during storage of 375 days. A much higher decrease in the

oryzanol content caused by extrusion was observed when germinated brown rice was extruded (Ohtsubo et al., 2005). The germination procedure before extrusion did not cause significant increase or decrease in the oryzanol content of rice bran, but extrusion of the germinated brown rice decreased the oryzanol content by 60%. It is thus possible that oryzanol of brown rice is more susceptible to degradation than oryzanol of rice bran.

Shin and Godber (1996) studied the effects of irradiation on steryl ferulate content of rice bran and demonstrated that both the γ -irradiation (5-15 kGy) procedure and subsequent storage (up to 52 weeks) decreased the content of steryl ferulates of rice bran. Irradiation at 5 kGy, 10 kGy and 15 kGy resulted in oryzanol contents that were 11%, 18% and 22% lower than the original oryzanol content, respectively. Also with the irradiated rice bran more significant decrease in the sterol content was observed only after the bran was stored at an ambient temperature for one year.

Sterol composition of oryzanol

Oryzanol is composed of several different esters of sterols and triterpene alcohols. The most abundant compounds are 24-methylenecycloartanol, cycloartenol and campesterol esters of ferulic acid (for structures see Table 2). The composition of oryzanol in different studies varies to some extent likely owing to both natural variation and analytical method (Table 2). Esterification to ferulic acid is selective, as the composition of sterols as ferulic acid esters is significantly different from that of total sterols (Ha et al., 2006). Sitosterol, campesterol and stigmasterol are the most abundant sterols of total plant sterols in rice, but as ferulic acid esters 24-methylenecycloartanol and cycloartenol predominate.

The sterol composition of oryzanol varies quite significantly in different studies, but also in different rice materials. Miller and Engel (2006) studied the composition of brown rice steryl ferulates and found significant differences between rice cultivars (ranges given in Table 2). However, they also observed that the ratio of desmethylsteryl ferulates (campesteryl ferulate, campestanyl ferulate and sitosteryl ferulate) to 4,4'-dimethylsteryl ferulates (24-methylenecycloartanyl ferulate and cycloartenyl ferulate) was relatively constant. In a few recent studies of genetically modified rice varieties with improved insect tolerance were studied and no changes in the content or sterol composition of oryzanol were seen as a result of the genetic modification (Poulsen et al., 2007a; Poulsen et al., 2007b; Schröder et al., 2007).

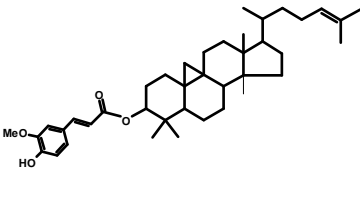
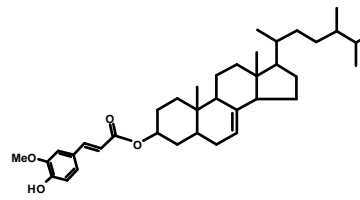
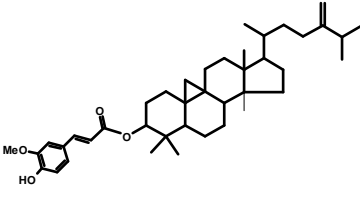
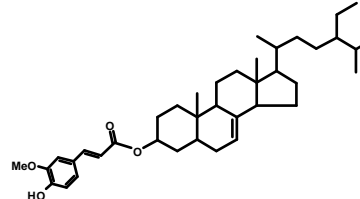
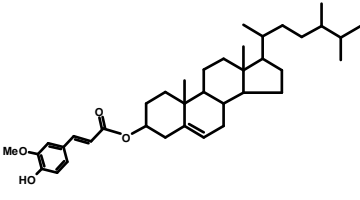
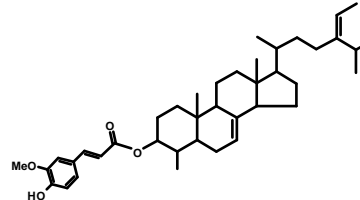
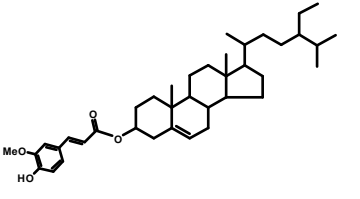
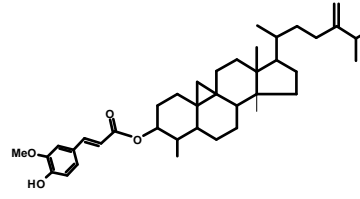
Rice bran oil

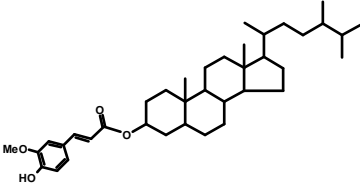
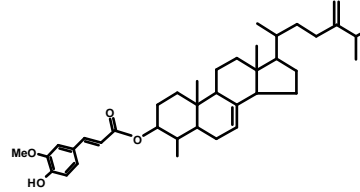
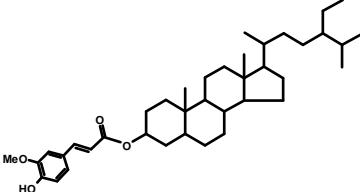
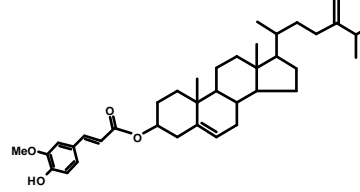
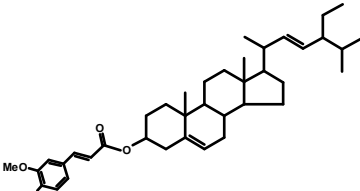
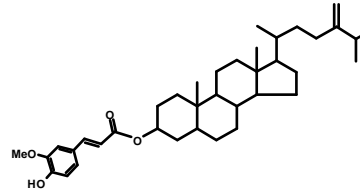
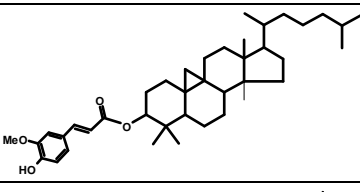
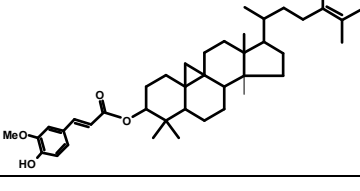
Rice bran oil (RBO) is extracted from rice bran produced in rice milling industry as a co-product of the polished rice. As has been demonstrated above the oryzanol content of rice bran is significantly higher than in whole rice kernels. In addition to this, the lipid content of rice bran is exceptionally high (14-22%) among cereal bran sources (Sayre and Saunders, 1990; Kahlon et al., 1992; Lloyd et al., 2000; Cicero and Derosa, 2005). Thus oil yield from rice bran is very good and the oil contains high concentration of oryzanol as well as other bioactive components like tocopherols and tocotrienols. The oryzanol content of crude RBO is highly dependent on the rice cultivar and ranges between 1.5-2.7% (Norton, 1995; Gopala Krishna et al., 2001; Van Hoed et al., 2006). Further, the oryzanol content of RBO is significantly affected by processing. Physical refining methods were shown to decrease the content of oryzanol less (28% decrease) than chemical methods (89% decrease) (Gopala Krishna et al., 2006b). In another study neutralization, bleaching, dewaxing and deodorisation each were shown to decrease the oryzanol content of rice bran oil from 1.8 % to 0.3-0.4% (Van Hoed et al., 2006).

2.1.1.2 Corn

Steryl ferulates (SF) of corn (*Zea mays*) have also reached a great deal of interest in the recent decades. First report on isolation and characterisation was by Evershed et al. (1988), who also demonstrated that the sterol composition of corn SF was different from oryzanol from rice. The corn SF consisted mainly of stigmastanyl (= sitostanyl, as the structure of fully saturated stigmasterol is the same as the structure of sitostanol) and campestanlyl ferulates. In later years interest in corn SF has increased further, when new uses for by-products of corn milling have been searched for. The use of corn in production of bioethanol has increased very rapidly, thus increasing also the by-products from the process that are not converted to ethanol. These by-products include various types of corn fractions, which have a high content of lipids and bioactive components like SF. Hence, the studies on corn SF are mainly focused on corn fractions and oils extracted from these fractions, and not so much on whole grains.

Table 2. Composition of sterols and triterpene alcohols as ferulates in oryzanol (Nakayama et al., 1987; Xu and Godber, 1999; Akihisa et al., 2000; Collins et al., 2002; Parrado et al., 2003; Gopala Krishna, A. G. et al., 2006a; Miller and Engel, 2006) Different compounds have been detected in different studies.

Structural formula	Common name (in bold) CAS Index name CAS number	% of oryzanol in rice bran	% of oryzanol in brown rice	Structural formula	Common name (qualitatively determined in rice)
	Cycloartenyl ferulate 9,19-cyclolanostan-24-en-3-ol, 3-(4-hydroxy-3- methoxyphenyl)-2-propenoate CAS# 21238-33-5	14-35	35-51		24-Epifungisteryl ferulate
	24-Methylenecycloartanyl ferulate 9,19-cyclolanostan-3-ol, 24- methylene-, 3-(4-hydroxy-3- methoxyphenyl)-2-propenoate CAS# 469-36-3	25-46	23-37		22- Dihydrospinasteryl (schottenyl) ferulate
	Campesteryl ferulate Ergost-5-en-3-ol, 3-(4- hydroxy-3-methoxyphenyl)-2- propenoate CAS# 20972-07-0	23	5-18		Citrostadienyl ferulate
	Sitosteryl ferulate Stigmast-5-en-3-ol, 3-(4- hydroxy-3-methoxyphenyl)-2- propenoate CAS# 4952-28-7	10-13	5-10		Cycloeucalenyl ferulate

	Campestanoyl ferulate	10	3-13		Gramisteryl ferulate
	Sitostanoyl (stigmastanoyl) ferulate	6			24-Methylenecholesteryl ferulate
	Stigmasteryl ferulate	20			24-Methylenecholestanyl ferulate
	Cycloartanoyl ferulate	17			
	Cyclobranyl ferulate	10			

The content of SF in corn kernels is generally much lower than in brown rice kernels (Table 3). The range of SF contents found in the literature is 30-220 µg/g, and in a similar manner as with rice, most of the SF are located in the corn bran layers. Corn fibre obtained by a wet-milling contained higher content of SF than corn bran from dry milling. Owing to the high content of SF in the starting material, also high contents of steryl ferulates (up to 6.75 %) were found in oils extracted from corn fibre (Moreau et al., 1996). As demonstrated by the values in Table 3 of the contents of SF in corn kernel parts, the highest content of SF within the outer kernel layers are found in the aleurone layer. This was further supported by the study of Singh et al. (2001), who studied bran layers in more detail by dissecting kernel layers by hand, and found that 64% of SF in coarse corn fibre were in the aleurone and the remaining part in the pericarp fraction without aleurone. Another study by the same authors showed that the content of SF in corn is also significantly affected by the corn hybrid and growing location (Singh et al., 2000).

Table 3. Contents of SF in corn kernels, corn fractions and oils extracted from corn fractions.

Sample	Content of SF (µg/g)		db/wb ^d	Reference
Corn kernels	31-70			Seitz, 1989
Corn kernels	98-113			Moreau et al., 1999
Corn grains	80-105			Singh et al., 1999
Corn grains (49 varieties)	30-220			Moreau et al., 2001
Teosinte ^a seeds (9 varieties)	14-31			ibid.
Corn bran oil	5.6	mg/g oil		Iwatsuki et al., 2003
Corn fibre oil	2.95	mg/g oil ^b	db	Jiang and Wang, 2005
Corn fibre oil	4.68-6.75	wt%		Moreau et al., 1996
Corn fibre ^c	1800			Moreau et al., 1996
Corn bran ^c	200-250			Moreau et al., 1999
Corn fibre	440-1530			ibid.
Corn fibre	1810		db	Wu and Norton, 2001
Corn kernel parts				
Aleurone	2001			Moreau et al., 2000
Fibre	861			ibid.
Germ	78			ibid.
Pericarp	105			ibid.

^a ancestors to corn, also belonging to *Zea* species

^b calculated as free sterols contributed by steryl ferulates

^c corn fibre and bran are the pericarp enriched fractions from wet-milling and dry-milling, respectively

^d See the note on p. 16.

The composition of SF in corn is significantly different from that of rice. As was already demonstrated in the early study by Evershed et al. (1988) sitostanyl and campestanol ferulates were the predominating compounds in corn. Later studies have shown that the average composition of sterols as SF in corn is 63-85% sitostanol, 8-29% campestanol, 5-14%

sitosterol, 3-9% campesterol 1% stigmasterol and traces of other minor sterols (Seitz, 1989; Norton, 1995; Iwatsuki et al., 2003; Jiang and Wang, 2005). In addition to SF also esters of another hydroxycinnamic acid, *p*-coumaric acid, have been reported found in corn bran and corn oil (Norton, 1995). However, their proportion of cinnamic acid sterol esters was generally below 3%.

2.1.1.3 Wheat

There are only a few studies that have reported SF in wheat (*Triticum aestivum* L.). The content of total SF in whole grain winter wheat varies at range 62-94 µg/g, whereas the value for spring wheat is slightly higher (123 µg/g) (Table 4). However, it should be noted that these are results from two studies only, and thus they are inadequate for making far-reaching conclusions.

Table 4. Contents of SF in wheat grains and grain fractions.

Sample	Content of SF (µg/g)	db/wb ^a	Reference
Winter wheat varieties (6 varieties)	62-94		Seitz, 1989
Spring wheat	123		ibid.
Durum wheat	68		ibid.
Wheat (mixed varieties)	62-63	wb	Hakala et al., 2002
Wheat enriched flour (4.5% ash)	194-216	wb	ibid.
Wheat bran	297-390	wb	ibid.
Wheat bran	584		Collins et al., 2002
Durum wheat (mixture of germ and bran)	1.47 mg/g lipids ^b		Jiang and Wang, 2005
Wheat bran	2.05 mg /g lipids ^b		ibid.
Wheat bran oil	2.0 mg/ g oil		Iwatsuki et al., 2003

^a See the note on p. 16.

^b calculated as free sterols contributed by steryl ferulates

Like in rice and corn, also in wheat the content of SF is higher in the bran than whole grain flour, demonstrating that SF are localised in the outer layers of the kernel (Table 4). The content of SF in four wheat bran samples varied approximately between 300-400 µg/g in one study (Hakala et al., 2002), whereas in the other study a significantly higher content of 584 µg/g was reported (Collins et al., 2002).

The composition of sterols as ferulic acid esters in wheat is different from that of rice and corn. In wheat the sterol composition is comprised merely of desmethylsterols, campestanol being the most abundant. The percentage composition of different sterols in whole grain wheat and wheat bran SF is as follows: campestanol 42-60%, sitostanol 28-36%, campesterol 8-21% and sitosterol 7-9% (Seitz, 1989; Collins et al., 2002; Jiang and Wang, 2005). There is no data available on the variation of SF in different wheat varieties.

2.1.1.4 Rye

The studies on SF in rye (*Secale cereale* L.) are even fewer than those for wheat. The content of SF in whole grain rye samples ranged between 29 and 65 $\mu\text{g/g}$ (Table 5), which is somewhat lower compared to wheat whole grains. The content of SF in rye bran was higher than in the whole grain samples, again indicating localisation of SF in the outer parts of the kernel. Further, Werner et al. (2002) demonstrated that increasing ash content in milling fractions correlated positively with increased SF contents in the same fractions.

Table 5. Contents of sterol ferulates in rye grains and grain fractions..

Sample	Content of SF ($\mu\text{g/g}$)	db/wb ^a	Reference
Rye whole grain flour	29		Seitz, 1989
Rye whole grain flour	40		Werner et al., 2002
Rye milling fractions with 0.56-2.0% ash	6-70		ibid.
Rye whole grain flour	55-64	wb	Hakala et al., 2002
Rye bran	150-251	wb	ibid.
Rye bran	180		Werner et al., 2002
Rye bran oil	5.9 mg/ g oil		Iwatsuki et al., 2003

^a See the note on p. 16

Detailed sterol composition of SF has been reported in two studies (Seitz, 1989; Iwatsuki et al., 2003). The sterol composition of rye SF is much like that of wheat SF, with no dimethylsterols (like in rice), but merely desmethylsterols. The composition of rye sterols, based on the rye bran oil of Iwazuki et al. and the whole grain sample reported by Seitz, is the following: campestanol 42-54%, sitostanol 31-36%, campesterol 14-15% and less of sitosterol and possible other minor sterols. There is no data available on the variation of SF contents of different rye cultivars.

2.1.1.5 Other cereals

Steryl ferulates are thought to be characteristic to different kind of seeds, and in addition to the cereals discussed above, a few other sources of SF have been reported. Seitz (1989) reported that the content of SF of triticale was 52 $\mu\text{g/g}$, which was higher than that of rye, but smaller than of wheat in the same study. The content of SF in barley and oat have been studied less. In oat bran oil the content of SF was 0.4%, corresponding to 270 $\mu\text{g/g}$ of oat bran (Moreau et al., 1996), though SF was not detected in oat kernels (Moreau et al., 1998). In barley kernels the content of SF was 4 $\mu\text{g/g}$ (Moreau et al., 1998). On another study by Hakala et al. (2002) SF were not detected in oat bran or barley pearling dust samples.

2.1.2 Steryl glycosides and acylated steryl glycosides

Plant sterols occur also as glycosylated conjugates, in which a carbohydrate is connected with a β -glycosidic bond to the hydroxyl group in C-3 of the sterol. The carbohydrate moiety can be esterified with a fatty acid, most commonly in C-6, thus forming an acylated steryl glycoside (ASG) (Figure 2). SG was first reported from tall morning-glory (*Ipomoea purpurea* Roth) in 1913 (Power and Salway, 1913), and ASG was reported for the first time in 1964 from potato tubers (Lepage, 1964). The most common carbohydrate in steryl glycosides is D-glucose, but also conjugation to other carbohydrates like galactose, mannose, xylose and gentiobiose has been reported (reviewed by Eichenberger, 1977) (for general structures see Figure 2). The fatty acid in ASG is most often palmitate, stearate, oleate, linoleate or linolenate (Wojciechowski, 1983). Structures of the most common SG and ASG are given in Table 6.

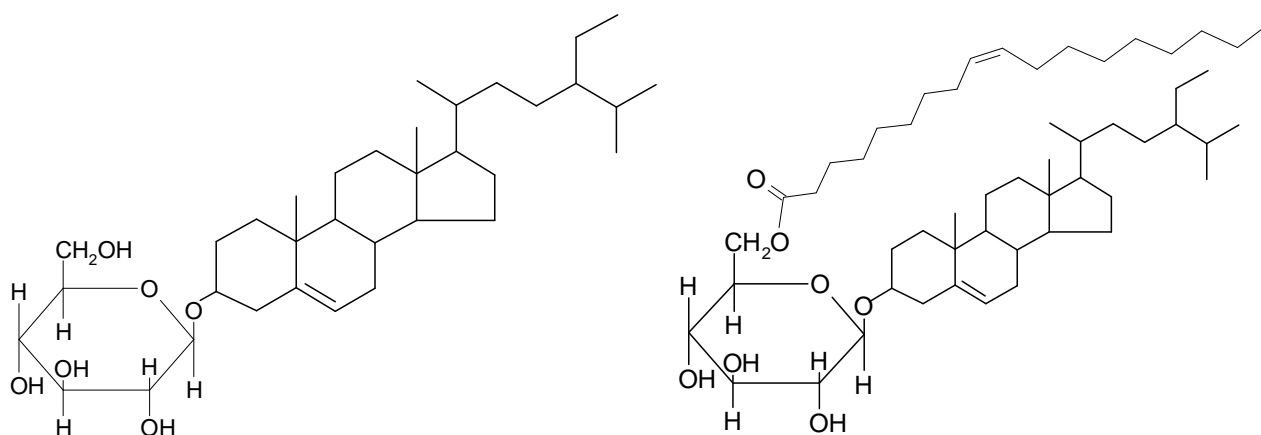
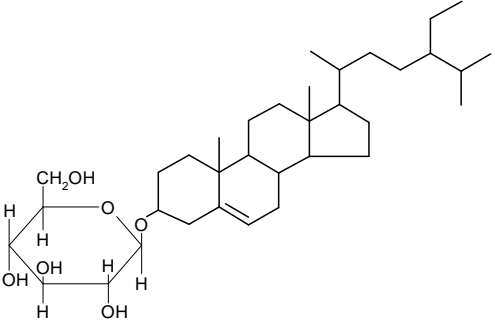
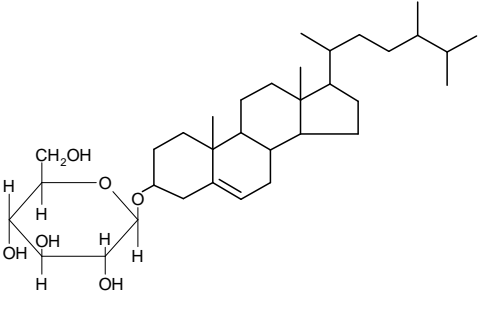
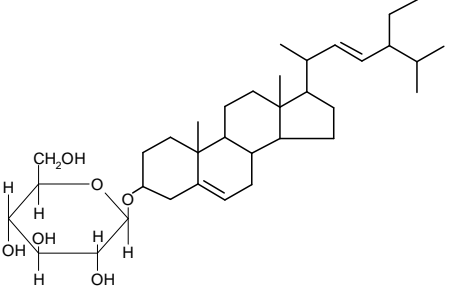
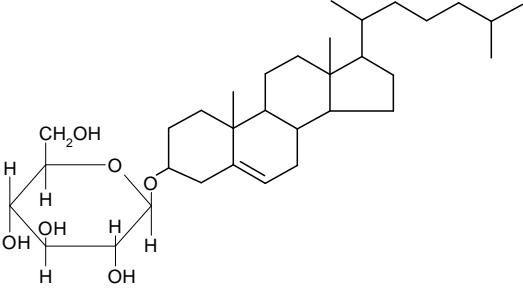
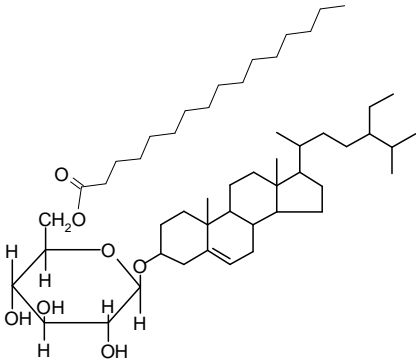


Figure 2. Example structures of SG (sitosteryl β -glucoside) and ASG (sitosteryl β -glucoside oleate)

Cholesterol is not ubiquitously found as a glycoside, or is found in only small amounts. Cholesteryl glycosides have been reported to be indicators of heat related stress in human cultured cells (Kunimoto et al., 2000), and to occur in tissues like chicken epidermis (Wertz et al., 1986) and snake epidermis (Abraham et al., 1987). Many of the commonly used methods for analysis of plant sterols are based on procedures for cholesterol analysis, and include only saponification (alkaline hydrolysis) to hydrolyse SE to FS. These methods only include SE and FS, but do not take SG into consideration, as SG would need acid hydrolysis. Therefore, much less information is available on SG compared to FS and SE.

Table 6. Structural formulae of the most common SG and ASG

Structural formula	Common name (in bold), CAS Index name CAS number	Structural formula	Common name (in bold), CAS Index name CAS number
	<p>Sitosteryl glucoside (aka. daucosterol) β-D-glucopyranoside, (3β)- stigmaste-5-en-3-yl</p> <p>CAS# 474-58-8</p>		<p>Campesteryl glucoside β-D-Glucopyranoside, (3 β,24R)-ergost-5-en-3-yl</p> <p>CAS# 32214-82-7</p>
	<p>Stigmasteryl glucoside β-D-glucopyranoside, (3β, 22E)- stigmasta-5,22-dien-3-yl</p> <p>CAS# 19716-26-8</p>		<p>Cholesteryl glucoside β-D-Glucopyranoside, (3β)- cholest-5-en-3-yl</p> <p>CAS# 7073-61-2</p>
	<p>β-sitosteryl β-D-glucoside-6- monopalmitate β-D-glucopyranoside, (3β)- stigmaste-5-en-3-yl, 6- hexadecanoate</p> <p>CAS# 18749-71-8</p>		

SG from various plant materials is in some cases referred to as *saponin* and in some literature *daucosterol* is used as a synonym for sitosteryl glucoside. Glycosylated sterols are often a minor part (up to 20%) of total sterols in plant materials with the exception of plant leaves in the *Solanum* genus (e.g. tomato and potato), in which glycosylated sterols can contribute majority of sterols (Wojciechowski, 1991). SG and ASG have been reported from e.g. potato tuber (Galliard et al., 1975), apple pulp (Takakuwa et al., 2005), oranges (Nagy and Nordby, 1971), eggplant (Zimowski and Wojciechowski, 1996), tomatoes (Whitaker, 1991), bell pepper (Whitaker and Lusby, 1989; Yamauchi et al., 2001) and a number of herbal or medicinal plants like *Mentha longifolia* (Shaiq Ali et al., 2002), *Adenophora tetraphylla* (Yao et al., 2007) and *Santolina chamaecyparissus* (Rios et al., 1989).

Literature of the occurrence of SG and ASG in cereals is scarce. Most of the studies on the subject are qualitative and actual contents in different cereals are not systematically reported (Table 7). The contents of SG in common cereals (barley, wheat, corn and rice) are about 100 µg/g, and the content of ASG is higher than the content of SG. The amount of plant sterols contributed by these conjugates is naturally dependent on the carbohydrate(s) and possible fatty acid attached to them, and thus the actual content of sterols in these conjugates cannot be accurately calculated. The contents of SG and ASG in common cereals are much lower than in other seeds and products derived of the seeds. Lecithin preparations from soybean and rapeseed contain about 3-4% and 2% of glycosylated sterols (ASG+SG) (Breinhölder et al., 2002), which is probably one of the highest contents of sterol glycosides reported from any plant material.

Table 7. Content of SG and ASG ($\mu\text{g/g}$) in cereals kernels and plant parts, pseudo-cereals and other seeds

Sample origin	SG	ASG	db/wb ^a	Reference
Barley flour	66	395	wb	Sugawara and Miyazawa, 1999
Corn flour	34	154	wb	ibid.
Rice flour	19	236	wb	ibid.
Wheat flour	54	256	wb	ibid.
Wheat flour	116 ^b			Phillips et al., 2005
Wheat flours with 50-76% extraction rates		190-220		Morrison et al., 1975
Winter wheat (5 varieties)	93-127 ^b			Ruibal-Mendieta et al., 2004
Spelt (16 varieties)	106-142 ^b			ibid.
Buckwheat	10			Takakuwa et al., 2005
Tatary buckwheat	320			ibid.
Grain amaranth	640			ibid.
Rye seedlings	15 mol% ^c	4.3 mol% ^c		Lynch and Steponkus, 1987
Flaxseed	351 ^b			Phillips et al., 2005
Peanut butter	685 ^b			ibid.
Almonds	839 ^b			ibid.
Soybeans	551 ^b			ibid.
Soybean lecithin	12600-14300	19000-28000		Breinhölder et al., 2002
Rapeseed lecithin	18700	2800		ibid.

^a See the note on p. 16.

^b $\mu\text{g/g}$ free sterol contributed by the SG and ASG

^c of total lipids

Sterol composition of SG and ASG between different plant materials varies to some extent (Table 8). In common cereals sitosterol, campesterol and stigmasterol are the most common sterols species found in the glycosylated form. In all of the cereal samples sitosterol contributes over half of the sterols in SG and ASG. The ranges of the proportions of campesterol and stigmasterol are 10-35% and 2-11%, respectively. There is some more variation in the sterol compositions of other seeds: the dominance of sitosterol is emphasised in almonds and ASG of rapeseed lecithin. Furthermore, the percentage of Δ^5 -avenasterol is higher in almonds and peanut butter than other seeds. The plant parts (wheat roots and rye seedlings) were also reported to contain 0.4-10% cholesteryl glycosides.

Though the sterol composition of SG and ASG is fairly similar to that of total sterols in the same plants, some preferential glycosylation has been reported. Palmer and Bowden (1975) suggested that the synthesis of SG is mediated by enzymes that are specific for the structure of the sterol side chain, and that cholesterol was a preferred sterol substrate for glycosylation. It was further noted that tetracyclic triterpenes (like cycloartenol and 24-methylenecycloartanol) were not found in the glycosylated form, which was also supported by Pegel (1980).

Table 8. Composition of SG and ASG in cereals kernels and plant parts, pseudo-cereals and other seeds

Sample origin	Sterol composition (%)					Reference	
	Sitosterol	Campesterol	Stigmasterol	Cholesterol	Other ^a		
Wheat flour	SG+ASG	57.1	11.9	1.7		Δ^5 -A 29.3	Phillips et al., 2005
Wheat flour	SG	77.4-84.0					McKillican, 1964
	ASG	76.4-85.2					
Winter wheat (5 varieties)	SG+ASG	73-76	23-26	0.4-0.5		Δ^7 -A 0.5-1.7	Ruibal-Mendieta et al., 2004
Spelt (16 varieties)	SG+ASG	73-77	21-25	0.2-0.5		Δ^7 -A 0.6-1.6	ibid.
Sorghum grains	SG	58.8	23.0	10.6	7.3		Palmer and Bowden, 1975
Oat flour	SG	~60	~10	<5		Δ^5 -A ~20, Ch + Δ^7 -A <5	Kesselmeier et al., 1985
Rice bran	SG	77.2	9.3	11.8		Δ^7 -St 1.0	Kuroda et al., 1977
	ASG	80.2	8.0	11.3		Δ^7 -St 0.5	
Wheat aleurone	SG	50.3	34.8	10.0	5.0		Singh and Paleg, 1985
Winter wheat crowns and roots	SG	68.9	26.5	4.3	0.4		Willemot, 1980
	ASG	65.7	30.6	2.9	0.8		
Wheat roots	SG	62.5	23.4	8.9	4.8		Quartacci et al., 2001
	ASG	63.4	25.8	6.3	4.5		
Rye seedlings	SG	59.7	23.0	3.8	7.9		Lynch and Steponkus, 1987
	ASG	56.3	23.3	3.4	9.9		
Flaxseed	SG+ASG	57.3	31.4	8.2		Δ^5 -A 3.1	Phillips et al., 2005
Peanut butter	SG+ASG	68.5	11.0	9.5		Δ^5 -A 11.0	ibid.
Almonds	SG+ASG	83.9	3.0	3.0		Δ^5 -A 10.1	ibid.
Soybeans	SG+ASG	55.1	28.1	15.0		Δ^5 -A 1.9	ibid.
Soybean lecithin	SG	66				C+St 27, Δ^5 -A 7	Breinhölder et al., 2002
	ASG	66				C+St 33, Δ^5 -A 0.6	
Rapeseed lecithin	SG	65				C+St 22, B 13	ibid.
	ASG	79				C+St 21	

^a C= campesterol, St= stigmasterol, Ch= cholesterol, A= avenasterol, B= brassicasterol

2.2 Enzymatic hydrolysis and metabolism in mammals

2.2.1 Hydrolysis of steryl ferulates

Enzymatic hydrolysis of SF has not been studied extensively and there are only few papers concerning the capability of various enzymes to hydrolyse SF. Though the metabolism of SF in mammals is not well understood, it is generally agreed that the ester bond of SF must be hydrolysed and separate the sterol and ferulic acid moieties, before they may affect cholesterol absorption or be absorbed in the intestinal tract.

Steryl esterase (aka. cholesteryl ester hydrolase)

Steryl fatty acid esters are effectively hydrolysed by steryl esterase (EC 3.1.1.13) generally of pancreatic or bacterial origin. Different enzymes have shown specificity towards different sterol and fatty acid moieties (Swell et al., 1954; Goller and Sgoutas, 1970; Sgoutas, 1971), and thus it is obvious that the activity of these enzymes towards different sterols as ferulic acid esters should vary as well. Recent studies have shown that steryl ferulates of oryzanol are partially hydrolysed by steryl esterases, but that there are great differences between different sterol moieties. Miller et al. (2004) examined steryl esterases from porcine pancreas, bovine pancreas, *Pseudomonas* sp. and *Candida cylindracea*, and stated that irrespective of the origin of the enzyme the specificity of the enzymes was the same i.e. ferulic acid esters of desmethylsterols (sitosterol and campesterol) were accepted as substrates, whereas esters of 4,4'-dimethylsterols (cycloartenol and 24-methylenecycloartanol) were not. Other studies have suggested that 4,4'-dimethylsteryl ferulates are hydrolysed to some extent, but that in the γ -oryzanol mixture desmethylsteryl ferulates are preferred substrates (Huang, 2003; Moreau and Hicks, 2004).

Pancreatic enzymes

The contents of the four main γ -oryzanol components were shown to decrease as a result of consecutive incubation with pepsin and pancreatin, though the rate of hydrolysis was not quantified (Huang, 2003). In another study pancreatin alone was shown to hydrolyse sitostanyl ferulate (after 4 hour incubation 56% hydrolysed), when no hydrolysis was observed with γ -oryzanol (Moreau and Hicks, 2004). The ability of pancreatin to hydrolyse SF is likely a result of the steryl esterase enzyme present in the enzyme mixture. Further, the crude preparations of bovine and porcine pancreas exhibited similar sterol specificity as purified steryl esterases, thus supporting the view that hydrolysis is caused by the same enzymes (Miller et al., 2004). Pancreatin contains also numerous other enzymatic activities that theoretically could hydrolyse SF. However, lipases alone could not effectively hydrolyse SF (Miller et al., 2004), and thus it is considered that the active enzyme in SF hydrolysis by pancreatin is steryl esterase.

2.2.2 Hydrolysis of steryl glycosides

Like the enzymatic hydrolysis of SF, also the hydrolysis of SG is not well understood. Papers on this topic are scarce, and thus far very little is known about the capabilities of various enzymes to hydrolyse the glycosidic bond of SG.

Steryl- β -glucosidase

Steryl- β -glucosidase (EC 3.2.1.104) has been isolated from *Sinapis alba* seedlings and there is only one reported study on its activity by Kalinowska and Wojciechowski (1978). They demonstrated that the enzyme had similar activity towards sitosteryl and cholesteryl glucosides, but no other SG were analysed.

β -glucosidase

Hydrolysis of SG by β -glucosidase (EC 3.2.1.21) has been utilized in the analytical procedure to replace acid hydrolysis of SG in two studies (Kesselmeier et al., 1985; Moreau et al., 1994), but neither of these studies systematically evaluated the capability of the enzyme preparation to hydrolyse SG. Both et al. (2006) stated that SG in lecithin preparations could be partially converted to FS by β -glucosidase preparations such as almond β -glucosidase, Depol® 40L (from *Aspergillus* sp.) and Cellubrix® (from *Trichoderma longi* or *Aspergillus niger*), though detailed data was not given in the report. Further, no hydrolysis of SG was detected by β -glucosidase Novozym® 188 (from *Aspergillus niger*). The conversion of SG to FS was nearly complete if β -glucosidase was used together with α -amylase, though α -amylase alone had only partial activity (Depol® 200L) or no activity (α -amylase from porcine pancreas) (Both et al., 2006). It is possible that the activity in the α -amylase preparations originates from side activity rather than the primary activity, as the glycosidic bond of SG is in β -configuration.

β -galactosidase

Hydrolysis of synthetic cholesteryl glycoside by a crude enzymatic extract from *Helix pomatia* in the presence of taurocholic acid was reported by Predescu and co-workers (2006). The enzyme responsible for the hydrolysis was named to be β -galactosidase, though no specific description of the enzyme or quantitative data on its hydrolytic activity was given in the paper.

2.2.3 Metabolism in mammals

There are only a few studies on the metabolism of SF and SG in mammals. Absorption and metabolism of SF was studied in rabbits (Fujiwara et al., 1980) and rats (Fujiwara et al., 1983). When the rats were fed a dose of 50 mg/kg of ^{14}C -labelled γ -oryzanol, less than 10%

of radioactivity was detected in the urine in the 72 hours following administration, and about 85% of the radioactive dose was found in the faeces. This demonstrates that a vast majority of γ -oryzanol was not absorbed from the intestine. Further, most of the absorbed radioactivity was detected in the blood, partially as intact γ -oryzanol, and was excreted to the urine as various metabolites and not in the intact form. Also in rabbits the absorption of γ -oryzanol was low, but unlike in rats, in rabbits γ -oryzanol was found in the intact form (Fujiwara et al., 1980). In a more recent study on the γ -oryzanol uptake by human intestinal cell model *in vitro*, no uptake of γ -oryzanol was observed during two hours incubation, whereas some cholesterol taken up by the cells under the same conditions (Huang, 2003).

Umehara et al. (2004) cited two papers, in which it was stated that an oral dose of 600 mg of γ -oryzanol in healthy volunteers resulted in peak plasma concentrations from 21 ng/ml to 106.8 ng/ml, and a steady-state peak plasma concentration of 117 ng/ml after repetitive administration of 100 mg of γ -oryzanol three times a day for 10 days. Using these values for a rough estimation, and estimating further that the volume of plasma in average adult is about 5000 ml, it can be calculated that at the peak concentration level in humans less than 1% of the administered dose can be detected in the plasma. Similar suggestions are given in other papers as well, but with citations to papers unavailable in English (Fry et al., 1997; Cicero and Gaddi, 2001). To conclude, one may say that it is generally agreed that SF are poorly absorbed in the intestinal tract of mammals, but the number of studies with a valid scientific setup is scarce, and they are mostly reported in languages other than English, which makes it difficult to evaluate the topic as a whole.

Studies on the metabolism of SG are, if possible, even more scarce than those of SF metabolism. In his review Pegel (1997) stated that in humans about 1-2.5% of ingested SG is absorbed, and then converted to FS, SE and ASG. It was claimed by Stohler that glycosylation of sterols and subsequent dispersion with a solubilising macromolecule (like lecithin) would result in a readily absorbable SG product, but no absorption data was presented (Stohler, 1999).

2.3 Properties of steryl ferulates and steryl glycosides

2.3.1 Cholesterol lowering properties

2.3.1.1 Steryl ferulates

The ability of SF to lower serum cholesterol levels is probably the most studied property of SF. Though many of the studies have been done using rice bran or rice bran oil, not merely SF, it is generally agreed that γ -oryzanol is the active constituent in the oil. It was

demonstrated by Most and co-workers that the oil in rice bran is responsible for the cholesterol lowering in humans, not the fibre (Most et al., 2005). Further, it has been demonstrated that rice bran oil and γ -oryzanol have better activity in reduction of the cholesterol concentrations than free ferulic acid (Wilson et al., 2007). On the other hand Yokoyama (2004) showed that a much lower dose of γ -oryzanol (0.09%) compared to phytosterol mixture (0.25%) given to hamsters resulted in a similar reduction in total cholesterol level and enhancement in the LDL/HDL-ratio. These results suggest that SF are active components in cholesterol reduction, and may possess better activity than other types of sterols or free ferulic acid. Studies on the effects of SF and rice bran oil on plasma lipids are summarized in Table 9. Rice bran oil naturally contains also other components in addition to SF, which may also affect the cholesterol levels.

Table 9. Studies on the effects of steryl ferulates (γ -oryzanol) and rice bran oil on serum lipids.

Addition ^a	Dose or proportion of the diet	Duration of the study	Test organism	Effect ^b				Reference
				Total chol.	LDL	HDL	Total lipids	
γ-Oryzanol in animal studies								
ORY*	2%	12 weeks	rat	↓	n.e.	n.e.	n.e.	Shinomiya et al., 1983
ORY*	0.5%	7 weeks	rat	↓	↓	n.e.	↓	Seetharamaiah and Chandrasekhara, 1988
ORY*	0.5%	7 weeks	rat	↓	↑	↑	n.e.	Seetharamaiah and Chandrasekhara, 1989
ORY*	0.1g/kg b.w. ^c	12 days	rat	n.e.		n.e.	n.e.	Sakamoto et al., 1987
ORY*	1%*	7 weeks	hamster	↓	↓	n.e.	n.e.	Rong et al., 1997
ORY*	1 g/ kg b.w.	12 days	rat	n.e.		n.e.	n.e.	Nakayama et al., 1987
ORY	0.09%	10 weeks	hamster		↓	n.e.		Yokoyama, 2004
ORY	0.5%	10 weeks	hamster	↓	↓	↑	↓	Wilson et al., 2007
Rice bran oil in animal studies								
RBO*	10%	8 weeks	rat	↓	↓	↑		Sharma and Rukmini, 1986
RBO	0-168 g/kg	8 weeks	monkey	↓	↓	n.e.	n.e.	Nicolosi et al., 1991
RBO	20% of energy	4 weeks	monkey	↓	↓	n.e.	↓	Wilson et al., 2000
RBO	15 g/kg	4 weeks	rat	n.e.		↑		Ha et al., 2005
RBO	10%	10 weeks	hamster	↓	↓	↑	↓	Wilson et al., 2007
γ-Oryzanol and rice bran oil in human studies								
ORY	300 mg/d	3 months	hyperlipidemic subjects	↓	↓	↑	↓	Yoshino et al., 1989
RBO+ORY	0.05 or 0.8 g/d of ORY	4 weeks	mildly hypercholesterolemic	↓	↓	n.e.	↓	Berger et al., 2005
RBO	1/3 of total dietary fat	10 weeks	healthy subjects	↓	↓	n.e.	n.e.	Most et al., 2005

^a Diets in studies marked with an asterisk (*) also had an addition of dietary cholesterol

^b ↑= increase, ↓= decrease, n.e.= no effect

^c b.w.= body weight

2.3.1.2 Steryl glycosides

Though it is often mentioned in the literature that SG has the same ability to lower blood cholesterol levels like FS and SE, there are no studies available with humans that would show this to be the case. However, the ability of soybean SG to inhibit cholesterol absorption from the intestine was demonstrated along with a decrease in the level of plasma triacylglycerols in rats. Further, the addition of 5% SG in the diet was also accompanied with a reduced body and liver weights (Tateo et al., 1994). As SG nutraceutical products are available on the market, and are marketed as cholesterol lowering agents, there is a strong demand for scientific studies to demonstrate the capability of SG (not only SE, FS and SF) to positively affect cholesterol levels in humans.

2.3.2 Antioxidant activity of steryl ferulates

2.3.2.1 In vitro-systems and food models

Steryl ferulates have been shown to prevent oxidation in various systems. The activity is thought to be based on the capability of ferulic acid to donate hydrogen from the phenolic hydroxyl group to a radical. The resulting SF radical formed is resonance stabilized, and the SF radicals may still effectively interfere with the chain reaction of lipid oxidation as alkyl radicals (Kochhar, 2000). In studies of oxidation, inhibition with other antioxidant mechanisms like metal chelation, ferulic acid has proven to be ineffective (Graf, 1992). Most of the studies on antioxidant activity of SF have been performed using γ -oryzanol or addition of rice bran oil (containing also other components with antioxidant activity), and from those studies it is not possible to evaluate activities of single compounds. Furthermore, there is a broad spectrum of methods used to evaluate the capability of SF to prevent oxidation. Thus many of the studies are difficult or impossible to compare, or they give contradictory results. Studies on SF as oxidation inhibitors in various model systems are summarised in Table 10.

Table 10. Antioxidant activities of steryl ferulates in various studies.

Monitored factor representing oxidation	Substrate	Accelerator or radical initiator (other than heat)	Temp. (°C)	Time	SF evaluated (in order of activity) ^a	Compared to α -T (+/-)	Reference
Diene formation	linoleic acid		30°C	0-30 h	CAM>24-met-CAF> CAF	n.a. ^b	Yagi and Ohishi, 1979
Scavenging of superoxide radicals (decrease in the superoxide ESR signal)			ambient		ORY	–	Tajima et al., 1983
Formation of lipid dimers and polymers	sunflower oil and rapeseed oil		170°C	2 h	ORY	+	Gertz et al., 2000
Hydroperoxide formation in linoleic acid model	linoleic acid	air flow	37°C	0-200 min	24-met-CAF=CAM=CAF	–	Xu and Godber, 2001
Formation of cholesterol oxidation products	cholesterol in emulsion	AAPH	37°C	0-24 h	24-met-CAF>CAM≥CAF	+	Xu et al., 2001
Oxidative stability index and increase in oil viscosity	1) soybean oil 2) soybean oil FAME		1) 100°C 2) 90°C	1) 20-25 h 2) 17.5-18 h	SSta>ORY	n.a.	Wang et al., 2002
Oxidation in multiple different systems	1) MeLo-bulk 2) OSI 3) ethanol-buffer mixture 4) PC liposomes	3) ferrous chloride 4) AAPH	1) 40°C 2) 90°C 3) 40°C 4) 37°C		1) CAF=ORY=24-met-CAF 2) CAF=ORY>24-met-CAF 3) n.a. 4) no inhibition by SF	1) – 2) SF ≤ α -T 3) n.a.	Kikuzaki et al., 2002
Modified ORAC for lipophilic compounds		AAPH	ambient	35 min	ORY	+	Huang et al., 2002
Stability of beef patties during storage (formation of TBARS, WOF, hydroperoxides, hexanal and cholesterol 7-oxides)			stored at 4°C	20 d	ORY	+	Kim et al., 2003
Protein and lipid oxidation (formation of carbonyl groups)	brain cell homogenate	AAPH	37°C	30 min	water soluble oryzanol (enzymatic extract from rice bran)	n.a.	Parrado et al., 2003
Oxidation in multiple different systems	1) Scavenging of OH [•] radicals 2) Scavenging of O ₂ ^{•-} radicals 3) Lipid peroxidation in PC liposomes	3) AMVN	1) ambient 2) ambient 3) 41°C		1) no inhibition by SF 2) no inhibition by SF 3) ORY	1) n.a. 2) n.a. 3) –	Juliano et al., 2005

^a CAM= campesteryl ferulate, CAF= cycloartenyl ferulate, 24-met-CAF= 24-methylenecycloartanyl ferulate, ORY= γ -oryzanol, SSta= sitostanyl ferulate, SSt= sitosteryl ferulate

^bn.a.= not applicable/ not analysed

A number of studies have evaluated the capability of SF as a radical scavenger i.e. the capacity to donate a hydrogen to a radical such as the stable radical DPPH used in the experiments (Table 11). In all the reported studies the DPPH-scavenging activity has been less than that of α -tocopherol. To study the activity of SF in scavenging other types of radicals Juliano and co-workers demonstrated that in addition to the stable DPPH \cdot radicals, γ -oryzanol could inhibit AMVN initiated peroxidation (hydrogen donation to peroxy radicals), but did not scavenge OH \cdot or O $_2^{\cdot-}$ radicals (Juliano et al., 2005). On the other hand in an earlier study by Tajima et al. (1983) γ -oryzanol was shown to scavenge also superoxide radicals (O $_2^{\cdot-}$).

Table 11. Radical scavenging activity studies with DPPH

Final AOX concentration in sample	SF evaluated	Compared to α -T (+/-)	Reference
1 g/l	SSte	–	Koski et al., 2000
20 μ M	CAF = ORY = 24-met-CAF	–	Kikuzaki et al., 2002
0.12-0.36 mM	ORY	–	Yunoki et al., 2004
0.5- ~8 mM	SSte=CAM=CAF=24-met-CAF	n.a.	Akiyama et al., 2001
0-238 μ M	ORY	–	Juliano et al., 2005

In many studies that evaluate the capacity to inhibit oxidation, activity is compared to the activity of α -tocopherol, which is readily available and well known for its antioxidant activity. The order of activity of different compounds varies in different systems and a compound that is more active in one system may be less active in another, as seen in the activities of SF and α -tocopherol in Table 10. In antioxidant chemistry a phenomenon called “polar paradox” is well known. It suggests that polar antioxidants are more active in non-polar environments and vice versa (Frankel, 1998). This means that in various studies of antioxidants found in the literature, in non-polar systems (bulk lipid matrices), where other factors (temperature, oxygen availability etc.) are equal, polar antioxidants possess greater activity to inhibit oxidation than less polar ones (Porter, 1993). And similarly, in more polar environments (emulsions, micelles) the nonpolar, lipophilic antioxidants are more potent than polar ones. SF and α -tocopherol are both lipid-soluble, and thus relatively non-polar and active in similar environments. Differences in their activities cannot be explained well with polar paradox, but is rather a result of other differences in the experimental setup.

2.3.2.2 Addition of rice bran oil

Many of the studies concerning the capacity of steryl ferulates to inhibit oxidation are actually evaluations of the capacity of RBO to retard oxidation. The ability of RBO to inhibit oxidation is indeed attributed to the high SF content in the oil. However, in addition to SF,

RBO contains a number of other components like tocopherols and tocotrienols that may also affect oxidation, though their content in RBO is about one tenth compared to that of SF. Even if studying effects of RBO addition does not give information about antioxidant activity of individual compounds in the oil, studying the effects of RBO addition to oxidation is still relevant, as RBO is the valuable co-product from rice industry and applicable as such to various food systems. It has been suggested that addition of 0.5-10% of RBO to oils that are high in linolenic acid (e.g. soybean oil and canola oil) effectively inhibits oxidation, but does not negatively affect sensory attributes like colour and taste of the product (Taylor et al., 1996).

Table 12. Inhibition of oxidation by addition of rice bran oil (RBO) in food models.

Food model system	RBO addition ^a	Storage temp.	Storage time	Monitored factor	Reference
Frying performance of HOSO	mixture of 2% RBO + 4% sesame oil			frying performance (increased from 35 h to 65 h)	Kamal-Eldin et al., 1998
Beef roasts	2% + 3% rice fibre	4°C	0, 4, 8 d	TBARS, 7-ketochol. ^b , SFA/UFA-ratio	Kim et al., 2000
Beef roasts	0, 1 or 2 %	4°C	1, 7, 14 d	TBARS, 7-ketochol.	Kim and Godber, 2001
Whole milk powder	0.1 or 0.2%	45°C	0, 10, 20, 30, 40 d	TBARS	Nanua et al., 2000

^a= Concentrations that are active in inhibition of monitored oxidation factors compared to control samples without RBO addition are given in bold

^b=7-ketocholesterol

A few studies have been done to see the effects of RBO addition to oxidative stability of various food model systems (Table 12). It can be seen from these studies that addition of 0.1-2% RBO to food systems like beef patties or milk powder enhance the oxidative stability of these foods during processing and subsequent storage. It has been mentioned in all of these studies that RBO addition has not negatively affected sensory attributes such as pleasantness of the product.

2.3.3 Other properties

There are a number of other biological properties that have been associated with steryl ferulates and steryl glycosides from various sources. Many, though not all, of these have not yet been adequately proven, and are given without sufficient data to prove the stated functions. Therefore, the studies reviewed in this section need to be considered as preliminary indications of biological activities that require more research.

2.3.3.1 Steryl ferulates

Anti-inflammatory activity

Different SF showed good activity against induced inflammation in mice ears with ID₅₀ (50% inhibition dose) being 0.1-0.8 mg/ear depending on the sterol moiety of SF (Akihisa et al., 2000). The activity of all sterols was higher as ferulic acid esters than corresponding free sterols (ID₅₀ 0.7-2.7 mg/ear). Further, the inhibition effect on induced inflammation by SF was shown to be dose-dependent (0-1 mg/ear) (Yasukawa et al., 1998). Potential anti-inflammatory activity was also demonstrated by Nagasaka and co-workers (2007), who showed that cycloartenyl ferulate inhibited the nuclear factor kappa B (NF-κB), which is thought to be associated with cellular functions like proliferation and inflammation, and thus its inhibition could be a way to treat inflammatory disorders. Further, it was demonstrated that free ferulic acid was unable to inhibit NF-κB activation, thus suggesting that activity is dependent on the sterol region of the molecule.

Anti-viral activity

Synthetic cholesteryl esters of various cinnamic acids (including cholesterol ferulate) showed some anti-viral activity against a poliovirus, but not other types of viruses, like influenza virus A, Newcastle disease virus or pseudorabies virus (Galabov et al., 1998). Cycloartenyl ferulate and 24-methylenecycloartanyl ferulate demonstrated inhibitory activity against HIV-1 reverse transcriptase with IC₅₀ values at 2.2 μM and 1.9 μM, respectively (Akihisa et al., 2001). SF from wheat, rye and corn bran oils, especially campesteryl, campestanil and sitostanyl ferulates, were shown to inhibit Epstein-Barr virus activation, thought to be an indication of antitumour properties (Iwatsuki et al., 2003). Antitumour properties of SF (cycloartenyl ferulate) in mouse skin was also reported by Yasukawa et al. (1998).

2.3.3.2 Steryl glycosides

Benign prostate hyperplasia

SG are thought to have positive effects on prostate disorders, though most of the published papers on this issue have in fact evaluated the effects of FS, not SG. When fed a daily dose of 60 mg sitosterol, patients with symptomatic prostate hyperplasia (benign enlargement of the prostate) experienced improvement in the symptoms, though no relevant reduction in the volume of the prostate was observed (Berges et al., 1995). Many of the nutraceutical products for prostate disorders available on the market are extracts of pumpkin seeds, which are known for their relatively high contents of SG (Breinhölder et al., 2002). Thus it is possible that SG may be the active component. A daily dose of 0.45 mg SG was said to decrease the size of the prostate, and significant decrease in for example the residual urine and bladder pressure was observed by Pegel (1980).

Modulation of the immune system

Studies on the effects of SG on the immune system have generally been done with a mixture of sitosterol and its glycoside in the ratio of 100:1, and thus it is difficult to estimate the contribution of SG in this mixture. However, it has been suggested that they have apparently synergistic effect, and for example FS have been stated to be protective against damage caused by free radicals especially in the presence of SG (van Rensburger et al., 2002). Further, Bouic et al. (1996) studied the effects of SG and FS on proliferation of lymphocytes, and stated that both compounds were active in enhancing the proliferation of T-cells, but that the best result was gained with the mixture of FS and SG in the ratio of 100:1. Positive effects on the T-cells was also observed in HIV-positive patients, when fed a daily dose of 20 mg FS and 0.2 mg SG for three months (Breytenbach et al., 2001).

Absorption enhancement

The ability of soybean SG to enhance nasal absorption of drugs administered to rabbits has been evaluated in a number of studies. Soybean SG addition (1%) in buffer suspensions was shown to enhance the nasal absorption of insulin, and it was shown that SG were more effective in enhancement than FS. Further, there were differences between various sterols: the more lipophilic sterol moiety (sitosterol) in SG had higher activity than the slightly less lipophilic (campesterol and stigmasterol) (Ando et al., 1998). Similar absorption enhancements of insulin have been demonstrated also when the insulin dosage has been given in powder form or in a peanut oil suspension, without significant changes in the morphological properties of the nasal epithelium (Yamamoto et al., 1995; Yamamoto et al., 1998). Furthermore, significant enhancement in the absorption of verapamil (used as a model drug due to its fluorescence enabling detection of small concentrations) was seen, when SG was added to the powder mixture administered nasally to rabbits (Maitani et al., 2000).

Neurotoxicity

Though most of the studies on properties of SG are associated on positive effects and enhancements of desirable effects, there are also a few studies that suggest undesirable effects of SG. Various SG from cycad seeds (*Cycas circinalis*) have been suggested to be potential neurotoxins and to have a role in neurodegenerative disorders like ALS-parkinsonism dementia complex (Khabazian et al., 2002; Wilson et al., 2002). However, these studies have been done using *in vitro*-systems and seed extracts that contain other compounds in addition to SG, and thus further research is required to confirm these results. In other studies of toxicity no acute effects have been seen after oral administration 1-2 g SG/ kg body weight), nor toxic effects after chronic dose of 100-200 mg/kg body weight (Pegel, 1980).

Other properties

In addition to the properties described above, SG given to mice was shown to be analgesic (157% increase in pain tolerance), though higher activity was obtained with free sitosterol (Villaseñor et al., 2002). Further, SG have been suggested to be used as tissue specific targeting of drugs. SG introduced together in a cationic liposome with a modified polyethylene glycol was shown to be an alternative carrier for targeted hepatic cell delivery for an antisense for the hepatic B virus (Shi et al., 2005). Furthermore, SG was shown to stimulate insulin secretion in normoglycemic rats (Ivorra et al., 1988), and diabetic rats (Ivorra et al., 1990). Finally, SG isolated from *Santolina chamaecyparissus* exhibited anti-inflammatory properties in mice (Rios et al., 1989).

3 AIMS OF THE STUDY

The overall aim of this thesis was to study occurrence and properties of steryl ferulates and steryl glycosides of wheat and rye. The studies were carried out to obtain information of rye and wheat as sources of these steryl conjugates, and to evaluate factors that may affect their action as health-promoting compounds in foods.

The main objectives of the individual studies were:

1. To analyse the content of steryl ferulates and steryl glycosides in milling fractions of rye and wheat and to relate their occurrence to fibre and ash contents (**I**).
2. To study the effects of thermal, mechanical and enzymatic processing on the extractability and availability of total sterols, as well as characteristic cereal steryl conjugates SF, SG and ASG from rye and wheat bran (**II**).
3. To study enzymatic hydrolysis of SF and SG from metabolic and analytical viewpoints (**III**).
4. To study the antioxidant activity of steryl ferulates at moderate temperatures (**IV**) and high temperatures (**V**).

4 MATERIALS AND METHODS

This section summarises the materials and methods presented in more detail in the original papers I-V.

4.1 Materials

4.1.1 Cereal materials

Milling fractions (I) from industrial scale rye (*Secale cereale*) and wheat (*Triticum aestivum*) milling processes were obtained from Raisio plc. (Raisio, Finland). More details of the samples are given in Table 13. Milling fractions that were chosen as samples are those that originate from the outer parts of the kernels. As the mill flow of rye milling contains only break rolls, and is thus shorter than that of wheat containing also smooth rolls, samples from roughly the same position in the rye kernel and with a similar ash content are obtained from earlier rolls.

Table 13. Names and ash contents of the milling fractions in Study I.

W H E A T		R Y E	
Sample name	Ash (%)	Sample name	Ash (%)
C6	1.4	Special light flour	1.4
Dark wheat flour	1.9	B3	1.4
DIV III	2.3	B2	1.9
DBR	2.8	B1	2.8
C9	2.9	Special dark flour	3.0
C11	3.0	B5	3.7
C10	3.8	B4 throughs	4.2
Feed flour	4.1	B4 overs	5.0
Germ	4.8	B5	5.2
Fine bran	6.3	Bran	6.5
Coarse bran	7.5		
Whole grain	1.8	Whole grain	1.7

The rye and wheat brans for processing (II) and for preparation of steryl ferulate and steryl glycoside extracts for enzymatic hydrolyses (III), and steryl ferulate extracts for antioxidant analyses (IV) were obtained from Fazer Company, Finland and Melia Company, Finland. Moisture contents of the milling fractions (I) and processed bran samples (II) were determined by drying the samples overnight in pre-weighed dishes at 103°C. Ash content (I) was determined gravimetrically by incinerating samples in the presence of nitric acid in a furnace oven at 550°C. Dietary fibre content of milling fractions (I) was analysed using the AOAC Official Method 985.29.

4.1.2 Standards, reagents and enzymes

Dihydrocholesterol (95% purity) used as an internal standard in gas chromatographic sterol analyses (**I**, **II**) was obtained from Sigma (St. Louis, MO, USA). Steryl glycoside standard (purity >98%) and acylated steryl glycosides (purity >99%) were obtained from Matreya Inc. (Pleasant Gap, PA, United States). Sitostanyl ferulate (purity >99%) used in studies **III** and **V** was a gift from Dr. Robert Moreau (United States Department of Agriculture/ Agricultural Research Service/ Eastern Regional Research Center, USA). γ -oryzanol (study **III**) was from CTS Organics (Atlanta, GA, USA). Cycloartenyl ferulate and γ -oryzanol for antioxidant analyses in study **IV** were provided by Dr. Parkash Kochhar (Good-Fry International, Rotterdam, The Netherlands). Sitosteryl ferulate and cholesteryl ferulate (study **IV**) were synthesised by Dr. Kristiina Wähälä (Department of Chemistry, University of Helsinki, Finland).

Ferulic acid, pyrogallol, and diphenylpicrylhydrazyl (DPPH) for study **IV** were obtained from Sigma Chemicals (St. Louis, MO, USA), and α -tocopherol (studies **IV-V**) was from Merck (Darmstadt, Germany). Methyl linoleate (**IV**) (purity >99%) was obtained from Nu Chek Prep, Inc. (Elysian, MI, USA) and each lot used was checked not to contain tocopherols at a level that could lead to tocopherol content equal to or greater than 1 $\mu\text{g/g}$ in a final sample. High oleic sunflower oil (**V**) was from Raisio plc. (Raisio, Finland).

The enzymes used for processing cereal brans (**II**) were Multifect Xylanase (398100 nkat/ml, Genencor International B.V. Leiden, the Netherlands) and β -glucanase Cereflo 200L (22000 nkat/ml, Novozymes A/S, Bagsvaerd, Denmark). In study **III** steryl ferulates were hydrolysed with bovine pancreatic steryl esterase (Sigma C-3766, 211 units/g, Sigma, St. Louis, MO, USA) and porcine pancreatic steryl esterase (CEPM, 950 units/gram, Worthington Biochemicals, Lakewood, NJ, USA), and steryl glycosides were hydrolysed with a purified microbial β -glucosidase (Megazyme E-BGLUC, 1 unit/25 μl , Wicklow, Ireland), β -glucosidase from almonds (BioChimica 49290, 7.55 U/mg, Fluka Chimie GmbH, Buchs, Switzerland) and microbial β -glucosidase (cellobiase from *Aspergillus niger*, ≥ 250 U/mg, Novozym 188, Sigma C-6105).

4.1.3 Preparation of steryl ferulate and steryl glycoside extracts

For the studies on enzymatic hydrolysis of SG and SF (**III**) and antioxidant activity of SF (**IV**) steryl ferulate extracts were prepared from rye and wheat bran. In study (**III**) total lipids were extracted from cereal bran by hot acetone and fractionated by silica-SPE-cartridges. The SG fraction was concentrated and used without further purification. The fraction containing SF was further purified from other lipids with an acid-base wash and preparative-HPLC with

a silica column (Supelco PLC-Si, 250 × 21.2 mm, 12 µm) and a mixture of heptane, isopropanol and acetic acid (99:1:0.1, v/v/v) as a mobile phase. UV-detection was set at 315 nm and cis- and trans-SF were collected, pooled and concentrated prior to use. In study **IV** SF were extracted with acetone at ambient temperature and the extract was purified with C18-SPE-columns (Supelco LC18, 20 ml, 5 g) using 96% methanol to wash the column and 100% methanol to elute the SF. The collected fractions were analysed with RP-HPLC (details given in section 4.2.1.3), and the ones containing merely SF were pooled and concentrated.

4.2 Experimental

4.2.1 Analyses of total plant sterols and individual steryl conjugates (I-V)

4.2.1.1 Lipid extraction and SPE fractionation of steryl conjugates (I, III)

Lipids of rye and wheat milling fractions (2 g samples) were extracted with hot acetone using Soxtec instrument (Soxtec Avanti 2050, Foss Tecator, Hillerød, Denmark) and fractionated using silica SPE-cartridges. The SE fraction was eluted with heptane:diethyl ether (9:1, v/v), FS fraction with heptane:diethyl ether (1:1, v/v) and SG fraction with acetone. As steryl ferulates (SF) are more polar than SE, but less polar than FS, they could not be accurately fractionated into any one single fraction. Thus the SE and FS fractions were combined and SF were analysed from the combined extract. The correct elution of SG and ASG with acetone was confirmed with silica-TLC comparing the extracts to standard solutions. TLC-plates were eluted with a mixture of chloroform:acetone:water (30:60:2, v/v/v). The plates were sprayed with 10% sulphuric acid in methanol and heated in an oven at 100°C to visualise the spots.

4.2.1.2 Analysis of total plant sterols by gas chromatography (I-II)

Cereal samples were analysed for their total sterol contents hydrolysed to free sterols with direct acid and alkaline hydrolyses with a procedure reported by Piironen and co-workers (Piironen et al., 2002). A 1-gram sample was first hydrolysed with hydrochloric acid to cleave the glycosidic bond of steryl glycosides, and after that with potassium hydroxide to saponify lipids and hydrolyse the esterified sterols. After hydrolyses non-saponifiable lipids were extracted and the extract was then purified using silica SPE-cartridges. Free sterols were derivatized to trimethylsilyl (TMS) ethers using BSTFA/TMCS (99:1 v/v) as reagent in pyridine. After silylation samples were evaporated, redissolved to heptane and determined using GC-FID. GC instrument model Hewlett Packard was HP5890 Series II used for the analysis of total plant sterols and sterol composition (**I**, **II**, **III**). The capillary column used was RTX-5w/Integra-Guard (crossbond diphenyl 5%-dimethylpolysiloxane 95%, 60 m × 0.32 mm, film thickness 0.10 µm, Restek Corp. USA). Quantification of sterols was performed

using dihydrocholesterol (purity 95%, from Sigma) as an internal standard (added before acid hydrolysis).

4.2.1.3 Analysis of steryl ferulates (I-V)

The content of steryl ferulates and composition of different sterol moieties in rye and wheat milling fractions was analysed after SPE-fractionation (I). The fractions containing SE, SF and FS were combined and purified from interfering lipids with an acid-base wash. After purification the SF extract was dissolved to 98% methanol and was analysed with RP-HPLC. The instrument was a Hewlett Packard HP1090 II liquid chromatograph with a diode array detector at 325 nm with a Waters Spherisorb ODS-2 RP-HPLC column (250 × 4.6 mm, 5 µm, Waters, Milford, MA, USA) operated at 50°C, and the mobile phase methanol:water:acetic acid (97:2:1, v/v/v) at a flow rate 1.5 ml/min.

Total steryl ferulates in processed bran samples (II), after enzymatic hydrolysis (III) and in antioxidant systems (V) were determined using NP-HPLC. The instrument was again HP1090 II liquid chromatograph, but the diode array detector was operated at 315 nm. The column was a Lichrosorb 100 Diol 5 µm (100 × 3.0 mm, VDS Optilab, Berlin, Germany) and the mobile phase a mixture of heptane: isopropanol:acetic acid (99:1:0.1, v/v/v).

4.2.1.4 Analysis of steryl glycosides (I-III)

In study I steryl glycosides were analysed from the SPE fraction containing both SG and ASG (obtained as described in earlier section). The internal standard (dihydrocholesterol) was added to this fraction, and the fraction was subjected to acid hydrolysis to hydrolyse the glycosidic bond of SG and ASG thus obtaining FS. After this the sample was further hydrolysed with KOH to remove possible interfering saponifiable lipids. FS were then derivatized to TMS-ethers and analysed with GD-FID with the same method as for total sterols (section 4.2.1.2).

In liquid chromatographic analysis of steryl glycosides and acylated steryl glycosides from extract of sequential extraction (II) the instrument was HP1090 II with an evaporative light scattering detector (ELSD) (Cunow DDL21, Cunow Department DMS, France). The column was a Lichrosorb 100 Diol 5 µm (100 × 3.0 mm, VDS Optilab, Berlin, Germany) and the mobile phase a mixture of A=heptane and B=isopropanol. The gradient program was 0-8 min 100% A, 8-10 min 100-99% A, 10-30 min 99% A, 30-40 min 99-75% A, 45-55 min 75% A. The flow rate was 0.5 ml/min and column temperature was set to 25°C. In the study III steryl glycosides before and after enzymatic hydrolysis were analysed using a HP1100 liquid chromatograph with ELSD detector (Model 55, Richard Scientific, Novato, CA, United States) with a diol column (LiChrosorb Diol 5 µm, 100 × 3.0 mm, Chrompak, Raritan, NJC

USA). Mobile phase was an isocratic mixture of A:B 85:15 with A=hexane-acetic acid (1000:1) and B=isopropanol at 0.5 ml/min flow rate and column heating at 30°C.

4.2.2 Processing of rye and wheat bran (II)

To analyse the possible effects of processing on cereal bran sterols, rye and wheat bran were subjected to different thermal, mechanical and enzymatic processes described in Table 14 below.

Table 14. Processing methods for rye and wheat bran samples (II)

Processing method	Process description
<i>Mechanical</i>	
Milling	milling to particle size <0.5 mm with an ultracentrifugal mill
Cryogenic grinding	grinding in liquid nitrogen using a Waring laboratory blender
<i>Thermal</i>	
Roasting	roasting in open dishes at 120°C for 1 hour
Microwave heating	heating in a microwave oven for 3 × 2 min, 630 W output, used at 90 % power
<i>Enzymatic</i>	
Control (No enzyme)	Incubation of bran samples in water at 40°C for 1 hour (control for endogenous enzyme activity)
Xylanase	Incubation with food-grade xylanase, conditions as for the control
β-glucanase	Incubation with food-grade β-glucanase, conditions as for the control
Both enzymes	Incubation with a mixture of food-grade xylanase and β-glucanase, conditions as for the control

Sample size of rye or wheat bran was 10 g for the mechanical and thermal processes and 2.5 g for the enzymatic processes. Each process was repeated four times and a sub-sample of each processed lot was taken for further analysis. These samples were subjected to sequential extraction to determine effects of processing on apparent sterol content, ease of extraction of sterols and the effects of processing in the contents of SF, SG, and ASG. The apparent sterol content was defined as the content of sterols available for analysis after processing, as the processing was not thought to affect the intrinsic content of sterols, but rather the amount available from the matrix. Further, the ease of extraction from the matrix was analysed using sequential extraction, in which free, unbound sterols were extracted with heptane and moderately bound sterols with acetone, both using Soxtec Avanti extraction apparatus. Tightly bound sterols were analysed from the remaining residue after acid and alkaline hydrolyses.

4.2.3 Enzymatic hydrolyses of steryl conjugates (III)

Steryl ferulates and steryl glycosides extracted from rye and wheat bran were subjected to enzymatic hydrolysis by various mammalian, plant and microbial enzymes. Substrate stock solutions (0.005 mol/L) were prepared in either heptane or isopropanol. In the hydrolysis of steryl ferulates 0.15 mg of substrate solution was transferred into a test tube and evaporated to dryness with nitrogen stream. Sample was re-dissolved to 5 ml of Trizma buffer (0.05 mol/L, pH 7.0) with 35 mmol/L of taurocholate as a detergent. Steryl esterases (EC 3.1.1.13) from bovine and porcine pancreas were used to hydrolyse different steryl ferulate substrates.

Steryl glycoside substrate (0.10 mg) was transferred into a test tube, evaporated to dryness with nitrogen stream and dispersed to 50 μ l of dimethylsulphoxide (DMSO) before the enzyme was added in 500 μ l of buffer solution (acetate buffer, pH 4.0, 0.05 mol/L or citrate buffer 0.1 mol/L, pH 5.0). β -glucosidases (EC 3.2.1.21) from almonds and *Aspergillus niger* were tested for their capability to hydrolyse steryl glycosides.

Steryl conjugates were hydrolysed for 0-18 hours in a shaking water bath at 37 °C for steryl glycosides and 40 °C for steryl ferulates. After the incubation lipids were extracted with the Bligh-Dyer –method and the sterol conjugates were analyzed with NP-HPLC. Hydrolyses were performed generally twice in triplicate.

4.2.4 Antioxidant activity of steryl ferulates (IV-V)

4.2.4.1 Antioxidant activity at moderate temperatures (IV)

The antioxidant activity of steryl ferulates from different origins was studied (IV). The different types of steryl ferulates analysed can be grouped as follows:

- 1) synthetic steryl ferulates, namely sitosteryl and cholesteryl ferulates
- 2) mixture of extracted steryl ferulates from wheat or rye bran
- 3) steryl ferulates of rice, namely cycloartenyl ferulate and γ -oryzanol (the latter being a mixture of several ferulates)

The activities of steryl ferulates were compared to ferulic acid and α -tocopherol at same concentrations. The ability of these compounds to inhibit hydroperoxide formation was studied both in bulk lipid and emulsion systems at two concentration levels 0.52 mM and 2.57 mM, corresponding to 100 μ g/g and 500 μ g/g of ferulic acid, respectively. The compounds in analysis were added to 0.5 g of methyl linoleate in glass vials. Emulsions were prepared by sonicating the samples and using Tween 20 as an emulsifying agent. All samples were oxidised in a dark oven at +40°C, and oxidation was monitored by taking aliquot samples at

different time points. Bulk lipid oxidation was performed in open vials, whereas the emulsion samples were kept under magnetic stirring in vials closed with screw caps (to prevent water evaporation). The surface areas of the bulk and emulsified lipid samples were 1.1 and 4.9 cm², respectively. Lipids of the emulsion samples were extracted with heptane before inserting the sample to HPLC, whereas the bulk lipid samples were merely dissolved to heptane and the content of hydroperoxides was analysed with HPLC.

The methyl linoleate hydroperoxides were analysed using a liquid chromatographic system previously reported by Mäkinen et al. (2000) containing a Waters 700 Satellite WISP autosampler, a Waters 510 pump and a Waters 996 diode array detector, and a silica column Supelcosil LC-SI 57930 (250 × 2.1 mm, 5 µm particle size) preceded by a Supelcosil precolumn (20 × 2.1 mm). The mobile phase was an isocratic system of heptane and diethyl ether (88:12, v/v) with flow rate of 0.4 ml/min. Detection of hydroperoxides was performed at 234 nm and the peak areas of the four hydroperoxide isomers were combined to get the value for total hydroperoxides in the sample.

A sample of uninhibited oxidation was used as a control to calculate the percentage of inhibition of hydroperoxide formation, which was calculated with the following formula (A= peak area of methyl linoleate hydroperoxides):

$$\text{Percentage of inhibition} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

4.2.4.2 Radical scavenging activity (IV)

Radical scavenging activity was measured by the method outlined by Malterud et al. (1993). Antioxidant solutions were prepared at two concentrations, 0.1 and 1.0 mM in methanol resulting in final concentrations of 1.67 and 16.67 µM in the sample. Absorbance of the DPPH-solution (0.45 mg/L in methanol) was recorded at 517 nm. After the sample addition the absorbance recorded immediately (A_0) and further at 15-second intervals up to 5 min. Pyrogallol (4.0 mM) was used as a positive control to determine 100 % scavenging activity. The percentage for radical scavenging activity (RSA %) was calculated with the following formula: $\text{RSA \%} = (A_0 - A_t) / (A_0 - A_p) \times 100$, where A_0 = absorbance at the beginning of the measurement time zero, A_t = absorbance of the sample after 5 minutes and A_p = absorbance of the pyrogallol sample after 5 minutes.

4.2.4.3 Antioxidant activity at high temperatures (V)

The ability of steryl ferulates to inhibit lipid polymerisation at high temperatures alone or with tocopherol was analysed in high oleic sunflower oil (HOSO), which is recommended for high temperature applications owing to its good heat stability as a result of the high oleic acid

content. The oil was stripped to remove natural pro- and antioxidants using the method reported by Lampi et al. (1999). Sitostanyl ferulate and α -tocopherol were added to 1 g of HOSO at two levels, 0.5% and 1.0%, and as a mixture containing 0.5% of both antioxidants. Samples in open vials were heated in a dark oven at 100 °C up to 96 hours or at 180 °C up to 6 hours. The surface area of the samples was 3 cm².

HOSO polymers formed during heating were analysed with a HP1090 II liquid chromatograph equipped with a A/D-converter (Hewlett Packard model 35900) and a refractive index detector (Hewlett Packard model 1047A) using three size exclusion chromatography columns (100 Å and 2 × 50 Å, 5 µm, 300 × 7.5 mm, Polymer Laboratories Inc., Amherst, MA, USA) connected in series, with dichloromethane as the eluent at 0.6 ml/min and column heating at 35 °C. Further, the rate of degradation of the antioxidants was monitored using the NP-HPLC method for steryl ferulates described above, using additional fluorescence detection for α -tocopherol (fluorescence detector HP1046A, using an excitation wavelength 290 nm and emission wavelength 325 nm).

4.2.5 Quality assurance

Method validation

Validation for the performance of the analytical methods had been previously reported for the method of total sterol analysis (**I**, **II**) by Piironen et al. (2002) and for method of steryl ferulate analysis (**I**) by Hakala et al. (2002). For other methods data on method performance is presented in papers **I-V**.

Sample storage

Milling fractions and processed bran samples (**I**, **II**) were stored at -20°C in plastic bags, if not analysed directly after processing. In-house reference samples were also stored at -20°C, taking a sub-sample to +4°C for the time of use. Storage at +4°C was at a maximum two weeks. Samples were allowed to temper at room temperature in a desiccator for 1 hour before weighing.

In-house reference samples for sterols and steryl conjugates

In all analyses of total sterols and steryl conjugates at least one sample of in-house reference material was studied in the same series. Wholemeal wheat flour was used as a reference sample for total sterol analyses (**I** and **II**), and in the analyses of steryl conjugates wholemeal wheat flour was used as a reference for wheat samples and rye bran as a reference for rye samples (**I**).

In-house reference samples for chromatographic performance

To test the daily performance of the chromatographic systems (peak intensities and separation) an in-house reference was analysed at the beginning of each new series. In GC-analysis of sterols (**I**, **II**, **III**) the sample consisted of equal masses of dihydrocholesterol, cholesterol and stigmasterol, and a sample of this mixture was silylated as TMS-ethers before analysis. In the HPLC-analysis of sterol conjugates (**II**) a sample containing each standard (SE, FS, SF, SG and ASG) was analysed. In the HPLC-analysis of hydroperoxides (**IV**) a sample of purified MeLo-hydroperoxides, and for the polymerised lipids (**V**) a HOSO sample that had been heated for 3 hours at 180°C was used as a daily chromatographic control.

Number of samples

All analyses of sterol contents were run at least in triplicate, and generally results with a coefficient of variation <5% were accepted. If the content of sterols or sterol conjugates was very low resulting in a higher variation in analytical result, greater variation was accepted, but the higher variation was also mentioned in the papers **I-V**. Processing of the cereal bran samples (**III**) was performed in quadruplicate and one sample from each processed lot was analysed. Enzymatic hydrolyses (**III**) were performed generally twice in triplicate. At moderate temperatures (**IV**) the antioxidant series were performed in triplicate, repeating ferulic acid and α -tocopherol in all series. Oxidation at high temperatures (**V**) was performed three times with each antioxidant presented once in a heating experiment.

4.2.6 Data analysis

In studies **I-V** analyses were performed at least in triplicate and differences between results were analysed using one-way analysis of variance (ANOVA) with Tukey's test or Fisher's LSD-test. Outliers were not removed in studies **IV** and **V**, in which variation was likely a result of the phenomenon, rather than the analytical method. Normal distribution of the data was tested with Bartlett's test. Some of the data was not normally distributed, in which case non-parametric Kruskal-Wallis test was used. In study **II** differences in the apparent sterol contents were analysed using ANOVA and pre-stated contrasts. Similarities between sterol content averages after different processing were summarised as endpoints of the 90% confidence intervals (corresponding to 95% certainty) for the corresponding contrasts with a method described in detail by Rita and Ekholm (2007). In all analyses 95% confidence level was used. Statistical analyses were performed using Statgraphics Plus 4.0 software (Manugistics, Inc. Rockville, MD, USA), Statistix 8.0 (Analytical Software, Tallahassee, FL) and SPSS 13.0 software (SPSS Inc., Chicago IL). All data on the contents of sterols and sterol conjugates in later sections of this study are given on dry weight basis, unless otherwise stated.

5 RESULTS

5.1 Contents of steryl ferulates and steryl glycosides in rye and wheat milling fractions (I)

5.1.1 Steryl ferulates

The ranges of sterol contents contributed by steryl ferulates were 12-308 $\mu\text{g/g}$ and 26-199 $\mu\text{g/g}$ in wheat and rye milling fractions, respectively (Figure 3 and **I**: Table 3). Variation in the content of SF was greater in wheat than rye milling fractions. In flourlike wheat samples with an ash content below 4% the content of SF was relatively low (below 60 $\mu\text{g/g}$), whereas the fine and coarse brans contained approximately fivefold contents when compared to the richest flourlike fraction. Wheat germ, on the other hand, contained very little SF (17 $\mu\text{g/g}$). In rye milling fractions the differences between SF contents were smaller between the different fractions, and the rye bran was not distinctively different from the flour fractions.

The content of SF in both rye and wheat milling fractions was highly correlated with the fibre content in the corresponding samples (Figure 3). The correlation coefficients (R) for the correlations with the fibre content were 0.9988 and 0.9809 for rye and wheat samples, respectively. Wheat germ was distinctively different from all other wheat fractions having lower fibre content than feed flour with a roughly equal ash content. In other samples increasing ash was accompanied with higher contents of fibre and SF.

5.1.2 Steryl glycosides

The content of sterols as SG (originating from SG and ASG) in wheat milling fractions ranged from 92 to 170 $\mu\text{g/g}$, with highest content being in the feed flour (shorts) (Figure 3). Compared to wheat samples the range of SG in rye samples was slightly narrower from 64 to 103 $\mu\text{g/g}$. The coarse wheat bran and rye bran both contained 65 $\mu\text{g/g}$ sterols as SG, which corresponded to 4 % of total sterols in the corresponding fractions in both cereals. The highest proportions of SG of total sterols were found in the flour fractions with the lowest ash contents. In wheat flour from the 6th smooth roll (C6) 11% of sterols were glycosylated, and in rye samples the special light rye flour contained the highest percentage (9%) of SG. There was a positive correlation between fibre and SG in wheat flour fractions, but not in the wheat bran. Further, a negative correlation between SG and fibre was observed in rye milling fractions (correlation coefficient $R=0.8338$ for the correlation curve with a gradient coefficient -1.5).

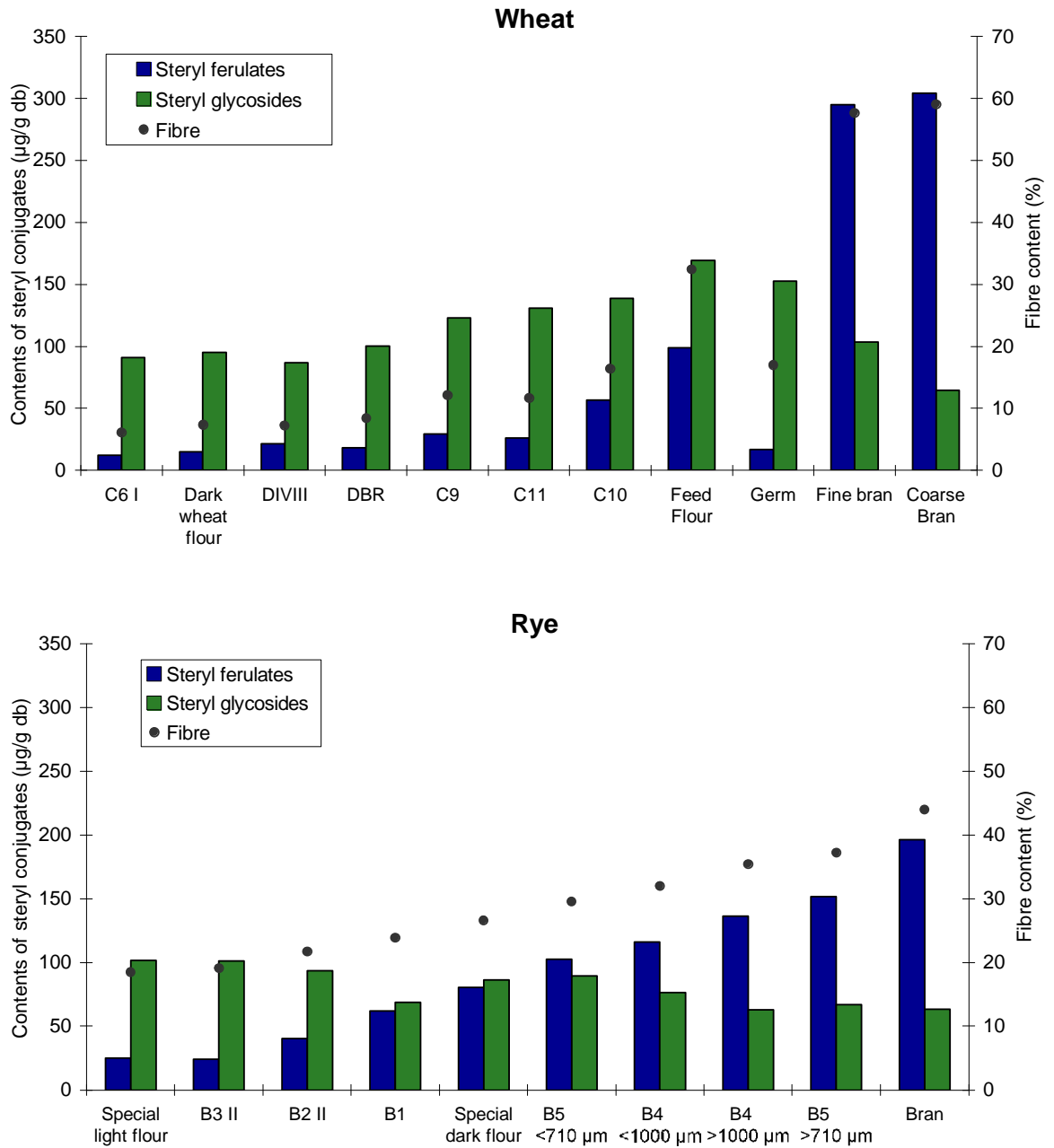


Figure 3. Contents of fibre (%), steryl ferulates and steryl glycosides ($\mu\text{g/g db}$) in wheat (upper graph) and rye (lower graph) milling fractions given in the order of increasing ash content.

5.1.3 Sterol composition

The sterol composition of SF and SG were different from that of total sterols, thus suggesting a preferential conjugation of certain sterols as different conjugates (Figure 4). Of the total sterols, sitosterol was the most abundant sterol in both wheat and rye, contributing about half of the overall sterol content, followed by campesterol (~17%). Total stanols (sum of sitostanol and campestanol) was 18% and 22% in rye and wheat, respectively. As glycosylated sterols, the proportion of sitosterol was even higher (~70%), whereas the proportion of stanols was less than 10% in both cereals. The proportion of stanols was emphasised in the composition of steryl ferulates, in which stanols contributed approximately 70% of sterols, and only 12% of sterols as SF was sitosterol. The proportion of campesterol was nearly equal (16-18%) in both steryl ferulates and glycosides.

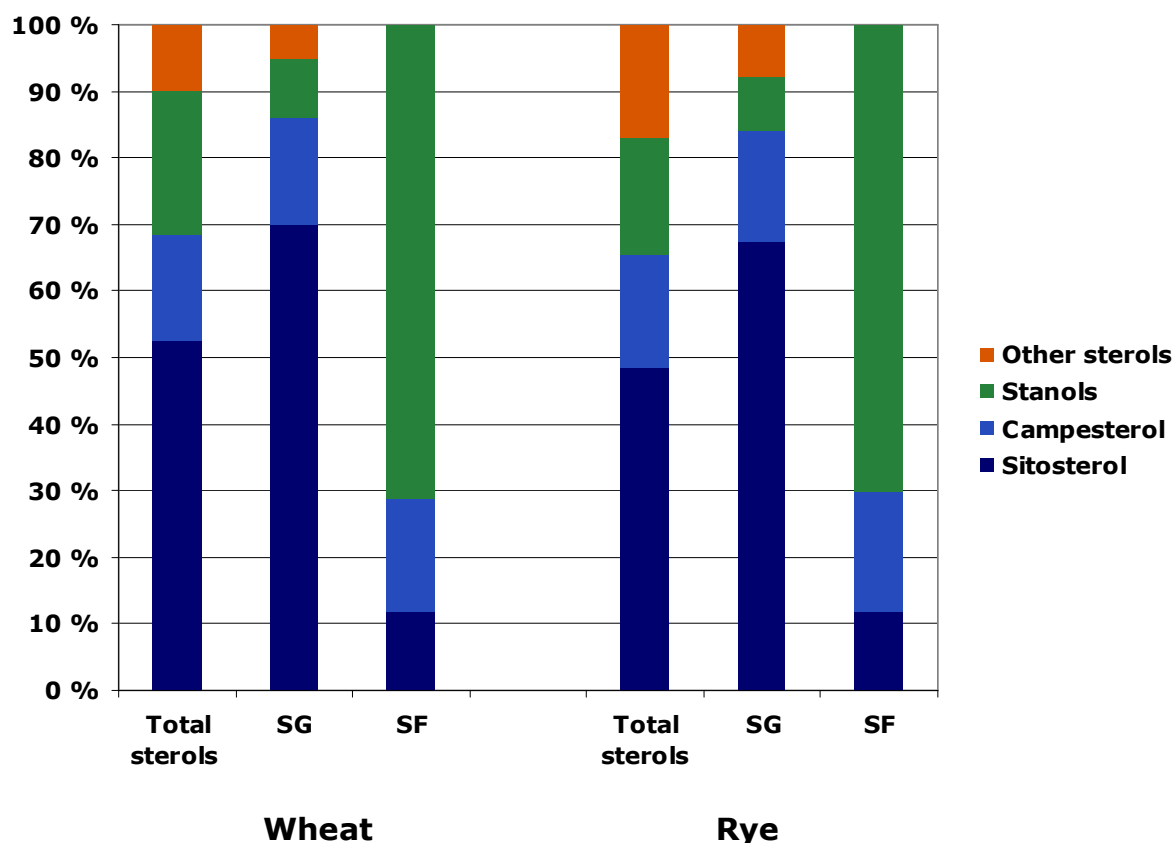


Figure 4. Proportions of different sterols in whole grain wheat and rye.

The percentages of campestanol and sitosterol as SF are calculated with the assumption that 77% of the coeluting peak of sitosterol and campestanol in wheat is campestanol (Hakala et al., 2002), and the same value is used to estimate the proportions in rye.

5.2 Effects of processing on steryl ferulates and steryl glycosides of rye and wheat bran (II)

Processing affected only moderately the apparent sterol content in rye and wheat brans (II: Table 1). Greatest effects were observed with the mechanical methods (milling and cryogenic grinding), which ground the bran material into a finer particle size. Thermal processing (roasting or microwave heating) had virtually no effect on apparent sterol contents, whereas varying effects (depending on the cereal) were seen with enzymatic treatments of rye and wheat bran with xylanase, β -glucanase or their mixture. More significant effects were observed when evaluating the extractability of the sterols, especially after aqueous treatments (incubation in water with or without enzymes). When rye bran was incubated in water without enzyme addition, the proportion of easily extractable sterols decreased significantly (II: Figure 3). The effects of aqueous treatments on apparent sterol content and extractability of sterols can be seen in Figure 5.

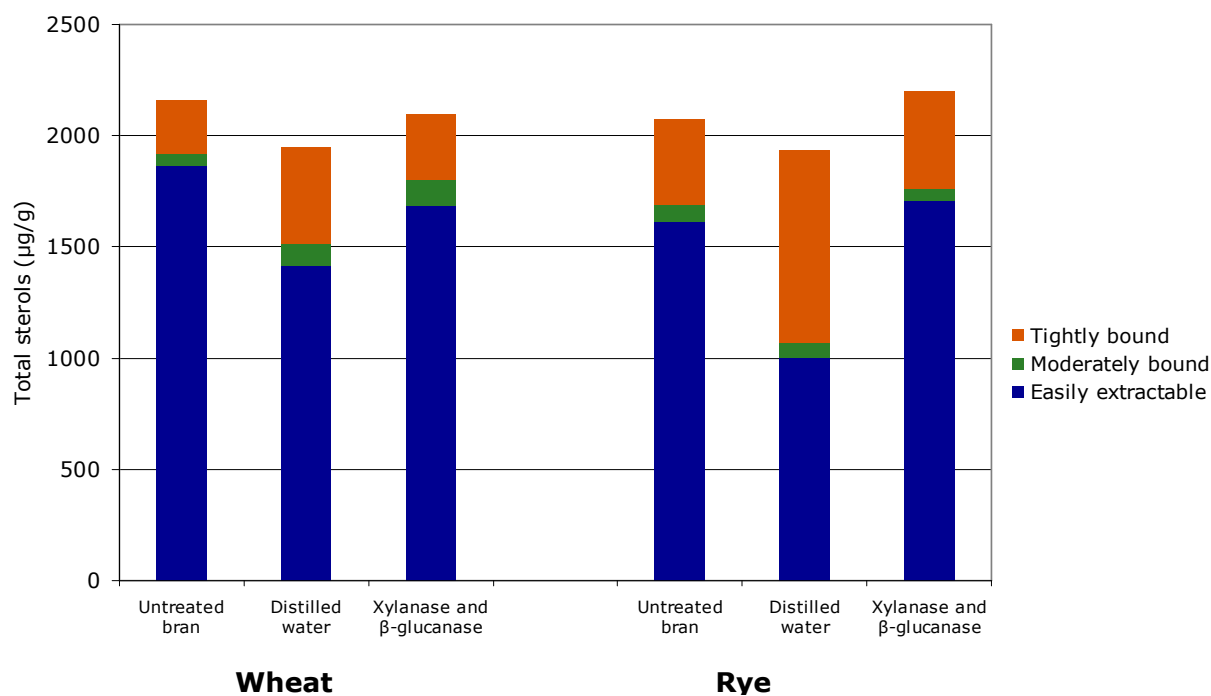


Figure 5. The apparent sterol contents and extractability of total sterols from rye and wheat bran ($\mu\text{g/g db}$) by sequential extraction. Easily extractable and moderately bound lipids were extracted with heptane and acetone, respectively, and tightly bound lipids were analysed from the residue.

The treatments had only minor effects or no effect on the contents of steryl conjugates in rye and wheat bran (II: Table 2). The contents of SG were generally reduced as a result of the aqueous enzymatic treatments, whereas the addition of enzymes increased the contents of ASG in rye bran, but decreased the content if water was added without enzymes. Similar

changes in the contents of ASG were not seen in processed wheat bran samples. The content of SF remained virtually unchanged during processing.

5.3 Enzymatic hydrolysis of steryl ferulates and steryl glycosides (III)

A mixture of SF from rye and wheat bran was hydrolysed with bovine and porcine steryl esterases (III: Table 1). The percent of hydrolysis was increased, when incubation was extended from 4 hours to 18 hours. Further, the percent of hydrolysis was significantly higher for the mixture of rye and wheat SF than synthetic sitostanyl ferulate or the mixture of SF from rice (γ -oryzanol). The most effective hydrolysis (66%) was obtained with SF mixture from rye and wheat bran after 18 hours incubation. Overall higher percentages of hydrolysis were gained with bovine than porcine steryl esterases.

A mixture of SG from wheat bran was not hydrolysed with highly purified β -glucosidase from *Aspergillus niger* or a β -glucosidase from almonds, but was on the other hand hydrolysed with a less pure preparation of β -glucosidase from *Aspergillus niger*, which has a primary activity in the hydrolysis of cellobiase (III: Table 2). The hydrolysis of SG was evaluated using various amounts of enzyme (5-90 units), two pH-values (pH 4.0 and pH 5.0) and using various detergents or dispersants. Highest percentage of hydrolysis, 57% (substrate amount 0.10 mg) was gained at pH 5.0 with a 30 U addition of enzyme, and with taurocholate as the detergent.

5.4 Antioxidant properties of steryl ferulates (IV-V)

5.4.1 Radical scavenging activity of steryl ferulates (IV)

Steryl ferulates were shown to possess radical scavenging activity i.e. they are able to donate a hydrogen atom to a radical. At the lower concentration (1.67 μ M) no differences were seen in the activities of steryl ferulates, free ferulic acid and α -tocopherol (Figure 6, IV: Table 1).

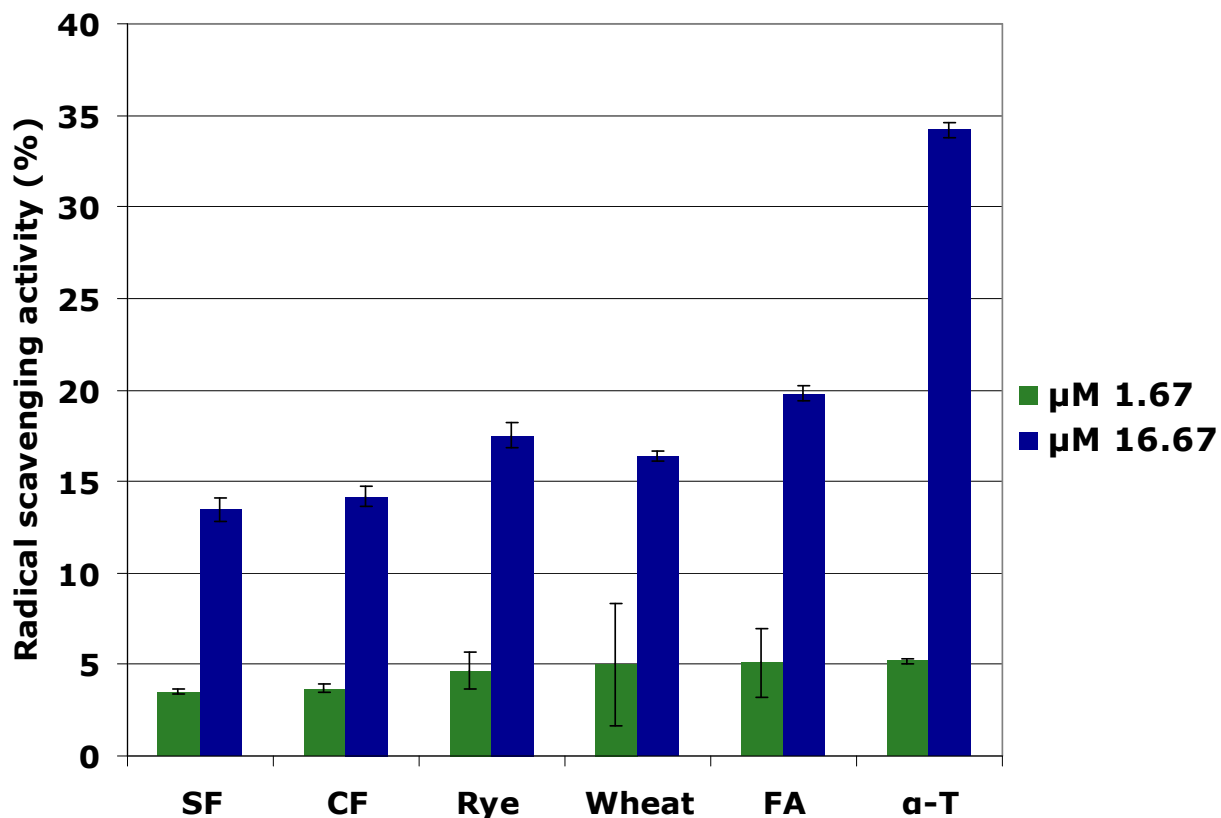


Figure 6. Radical scavenging activities of sitostanyl ferulate (SF), cholesteryl ferulate (CF) rye extract, wheat extract, ferulic acid (FA) and α -tocopherol (α -T) at final concentration of 1.67 μ M and 16.67 μ M.

At a tenfold concentration, however, free ferulic acid and α -tocopherol possessed a significantly higher activity than steryl ferulates. The radical scavenging activity of synthetic steryl ferulates was less than that of steryl ferulates extracted from rye or wheat bran.

5.4.2 Antioxidant activity at moderate temperatures (IV)

Steryl ferulates from rye and wheat bran inhibited lipid oxidation in bulk methyl linoleate system at 40°C much more effectively than free ferulic acid when added at two concentration levels (0.52 and 2.57 mM) corresponding to 100 and 500 μ g/g of ferulic acid (IV: Figure 2). The effect was concentration dependent, i.e. higher concentration inhibited the formation of methyl linoleate hydroperoxides more than the lower concentration. However, the capacity of steryl ferulates to inhibit hydroperoxide formation never exceeded that of α -tocopherol. Of the different types of steryl ferulates analysed, those extracted from rye or wheat bran had higher antioxidant activity than steryl ferulates from rice (γ -oryzanol or single compound cycloartenyl ferulate) or synthetic steryl ferulates (sitosteryl ferulate or cholesteryl ferulate).

In a system of emulsified methyl linoleate steryl ferulates extracted from rye or wheat bran again exerted substantial antioxidant activity (IV: Figure 3). Activity at the lower added concentration was equal to that of free ferulic acid. α -Tocopherol was added at the lower concentration, but was still the most effective antioxidant in the inhibition of hydroperoxide formation.

5.4.3 Antioxidant activity at high temperatures (V)

Sitostanyl ferulate significantly inhibited polymer formation of high oleic sunflower oil stripped of natural antioxidants at 100°C (Figure 7). At the lower level of addition (0.5%) the formation of polymers began to increase rapidly after 72 hours, whereas the higher concentration of sitostanyl ferulate (1.0%) inhibited polymer formation more effectively, and even after 96 hours of heating the concentration of polymers was only 4%. Sitostanyl ferulate was relatively stable under these heating conditions: after 72 hours about half of the higher addition still remained in the system. Of the lower addition about one-fifth was still left after 72 hours, but was fully degraded at the end of heating.

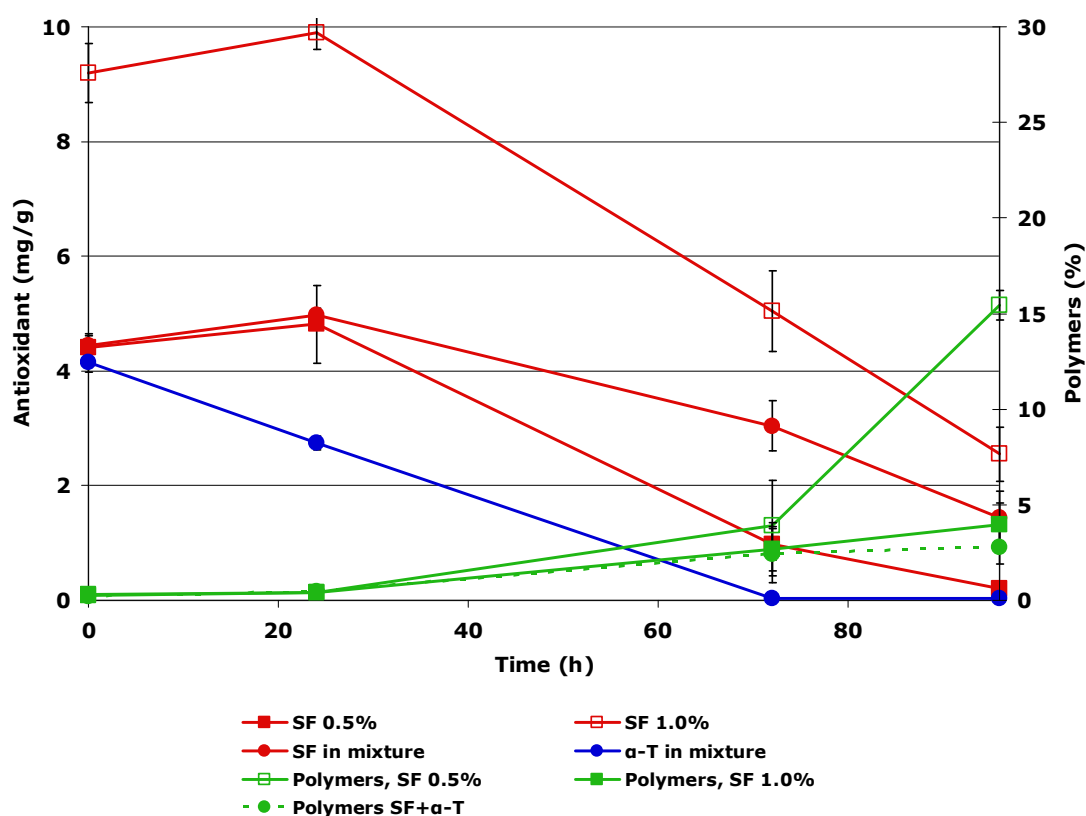


Figure 7. Formation of HOSO polymers and decrease in the antioxidant levels during heating at 100°C

At the higher temperature (180°C) polymer formation was much more rapid than at 100°C. The polymer formation began to rapidly increase after 1 hour heating (Figure 8). Sitostanyl ferulate again inhibited HOSO polymer formation effectively: after 3 hours heating the polymer contents in the samples with 0.5% and 1.0% additions of sitostanyl ferulate were 12.8% and 8.6%, respectively, whereas the polymer content of the sample without antioxidant addition was 21.3%. The content of sitostanyl ferulate in the system decreased significantly during heating. After 3 hours heating about half of the 1.0% addition and less than half of the 0.5% addition of sitostanyl ferulate remained in the sample, and at the end of heating all of the lower amount of addition was consumed, whereas some sitostanyl ferulate was left in the sample with the original 1.0% addition of sitostanyl ferulate.

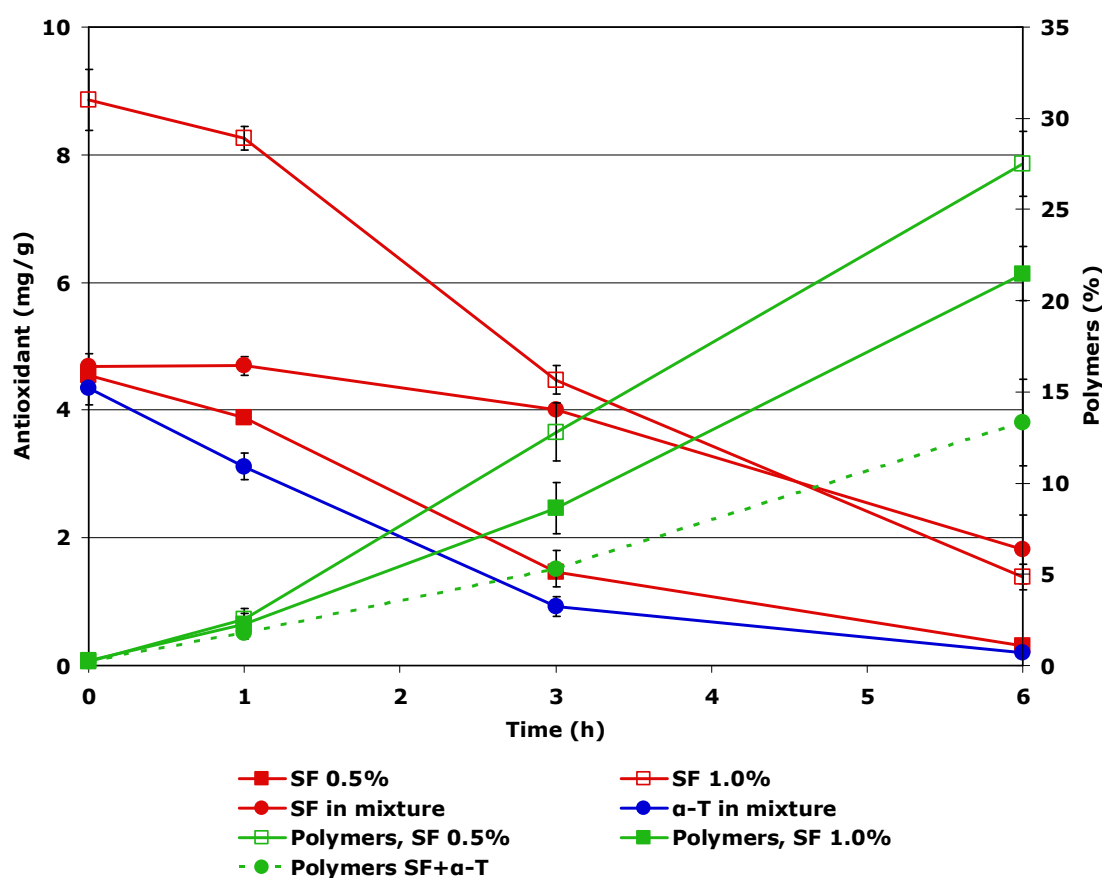


Figure 8. Formation of HOSO polymers and decrease in the antioxidant levels during heating at 180°C

5.4.4 Synergism with α -tocopherol (V)

No synergistic inhibition of polymer formation was seen in the HOSO systems with 0.5% additions of sitostanyl ferulate together with α -tocopherol at either 100°C or 180°C (V: Table 1). At the end of heating (96 h) at 100°C the inhibition of polymer formation in the sample with both antioxidants was comparable to that of 1.0% of sitostanyl ferulate. At 180°C, on the

other hand, after 6 hours heating the mixture of antioxidants was more effective in the inhibition of polymer formation than 1.0% of sitostanyl ferulate, but equal to 1.0% of α -tocopherol. The levels of sitostanyl ferulate decreased slower than α -tocopherol at both temperatures (Figures 7 and 8) and slower than in the samples with 0.5% of sitostanyl ferulate alone, suggesting that α -tocopherol to some extent protected sitostanyl ferulate in the mixture containing both antioxidants.

6 DISCUSSION

6.1 Steryl conjugates in rye and wheat

The contents of steryl ferulates and steryl glycosides were determined in milling fractions with ash contents varying from roughly 1.4 to 7.5%. The fractions were chosen from later rolls in the milling process to represent samples from the outer parts of the kernel, whereas pure endosperm samples were omitted. As sterols are regarded as “co-passengers of dietary fibre”, their occurrence was studied in relation to fibre contents in the corresponding fractions.

Steryl glycosides

As shown in Figure 3 the content of SG in both rye and wheat milling fractions varied much less than the content of SF, and further, between these two cereals variation in the SG content was smaller in rye fractions than wheat fractions. The content of SG (contributed by SG and ASG) in whole grain wheat was 75 $\mu\text{g/g}$, which is slightly lower than range, 93-127 $\mu\text{g/g}$, for winter wheat SG content reported by Ruibal-Mendieta and co-workers (2004). There is no prior literature available on the contents of SG in rye or distribution of SG in cereal kernels. Based on the data obtained in this study it can be concluded that the content of SG in rye is on the same level as that of wheat, and that variation in the SG content within the kernel is low. The contents of SG were negatively correlated with dietary fibre in rye samples, and no correlation was found in the wheat SG and fibre contents.

The role of SG in plants is not well understood, and thus it does not offer clear explanations as to why the content of SG is relatively equal in all parts of the kernel. However, SG is considered to be primarily a membrane-lipid and not to accumulate inside cells in any specific tissue (Wojciechowski, 1991). This likely affects the fact that variation between tissue types is small and that the content of SG is dependent on the amount of membranes in the sample, not the contents inside the cells. The differences between rye and wheat can partly be explained by the differences in the organization and sizes of the cells in the kernels. In rye, the number and size of the cells varies significantly less than in wheat. In wheat, the number of cells is smaller in the inner endosperm and the size of the cells is larger, and when moving outwards in the wheat kernel the number of cells increases and the size decreases. Thus compared to wheat kernels, rye kernels have more cell membranes in the inner parts of the kernels and variation in SG content is small. This applies to amount of cell membranes, but not necessarily on membranes of the organelles, as their contents are not known. Further, the

data in this study shows that though plant sterols generally have strong correlations with the fibre content, this is not the case with SG.

Steryl ferulates

As could be expected from earlier studies (Seitz, 1989; Hakala et al., 2002) bran was the best source of SF in both rye and wheat. With respect to the total sterol content some of the flourlike fractions were equally rich in sterols as the bran fractions, but not in the contents of steryl ferulates (I: Tables 2 and 3). Relatively little SF was found in the flour fractions of wheat, or in wheat germ. The variation of rye SF was much smaller than in wheat milling fractions – the highest SF content of rye samples was 7.5-fold compared to the lowest content, whereas in wheat fractions the difference in the SF contents between the highest and the lowest was 25-fold (Figure 3). In all the milling fractions the content of SF correlated positively with the fibre content.

Seitz studied the occurrence of SF in the various bran layers and concluded that SF in wheat could be found in the inner pericarp, but not in the outer pericarp or the endosperm (Seitz, 1989). Further, Hakala et al. (2002) reported only traces of SF in wheat flour with an ash content of 0.6%. In this study all fractions had a significantly higher ash content (ash>1.4%) than the flour in the study of Hakala and co-workers, and in this study some SF was found in all of the milling fractions, likely originating from the bran layers or outer endosperm. Wu and Norton (2001) studied the SF in corn fibre after fine grinding and air-classification to fractions with a narrow particle size distribution, and concluded that a fraction with a particle size <15 µm contained significantly higher content of SF than the original fibre, and that the content of SF in the sieved corn fractions did not correlate with the fibre contents. However, in this study the bran analysed contained the whole bran fraction of the kernel, and the fibre and SF contents were strongly correlated. It is possible that further grinding and sieving the bran into smaller fractions could reveal differences between the different bran layers. The different layers of wheat bran are structurally and compositionally different from each other: inner bran contains more arabinoxylans, whereas the outer bran contains primarily cellulose (Benamrouche et al., 2002). Based on this knowledge and the analyses of Seitz on the SF contents of wheat bran layers, it can be hypothesised that the content of SF in wheat may be more highly correlated with arabinoxylan content than the total fibre.

The richest sources of SF in the literature are rice bran oil and corn fibre oil (extracted mainly from the bran tissue of corn). Also in wheat and rye the highest contents of SF are found in the bran. The total lipid content of wheat and rye bran (4.5% and 4.3%, respectively, www.fineli.fi, Finnish Food Composition Database) is, however, very much lower than that of rice bran (15-20 %) (Orthoefer and Eastman, 2004), and the content of SF in rice bran is

much higher than that of wheat or rye bran (Tables 1, 4 and 5, Figure 3). Therefore, extraction of oil from rye or wheat bran is unlikely to be economically feasible, and thus the content of SF in a product can be elevated by adding rye or wheat bran as such and not by first extracting SF and then adding the extract. The only extracted oil product from these cereals available on the market is wheat germ oil, but as demonstrated in this study wheat germ contains very little SF, and thus it is likely also the SF content of wheat germ oil is low. Further, as demonstrated above, the content of SG varies very little between different tissues, and thus it may be concluded that the exceptionally high content of total sterols in wheat germ (I: Table 2) is mainly in the form of FS and SE.

Sterol composition

Significant differences were observed in the sterol contents of SG, SF and total sterols in wholegrain samples (Figure 4). Sitosterol was the most abundant sterol species in total sterols and SG, but a minor sterol in SF. The predominance of sitosterol in SG is supported by other studies as well (McKillican, 1964; Ruibal-Mendieta et al., 2004). Sterol composition of SG was similar to total sterols with increasing proportion of stanols in the fractions from the outer kernel parts (I: Tables 2 and 4).

As steryl ferulates the saturated sitostanol and campestanol were the most important sterols contributing about 70% of sterols as SF. This is in accordance with the studies of Hakala et al. (2002) and Iwatsuki et al. (2003). In the latter study minor amounts of 24-methylathosteryl ferulate and schottenol (stigmastanol) ferulate were reported in addition to sitosteryl and campesteryl ferulates and their corresponding stanols from wheat and rye bran oils. However, their contribution to total SF in those samples were less than 1 mg/g oil. As the role of these conjugates in plants and possible differences in the functions of different sterols are not known, there is no explanation available to the preference of sitosterol as SG or stanols as SF. However, sterol composition may affect the properties of steryl conjugates, as seen in other sections of this study, and thus it is essential to have information on the sterol composition as well as the content.

6.2 Processing effects on total sterols and steryl conjugates

Effects of processing on total sterols, SF, SG and ASG of rye and wheat bran were studied after various thermal, mechanical and enzymatic processes (II). It was assumed that the processes would not affect the total sterol content *per se*, but that it might have an effect on the *apparent sterol content* i.e. the content of sterols available for analysis. It has been hypothesised in the literature that processing could, for example, break bonds between the

cereal matrix and the minor components in cereals, thus releasing them to be used more effectively (Lane et al., 1997). Further, thermal treatment has been shown to increase the yield of SF from rice bran (Kim et al., 2002). In addition to the total amount of sterols available from the matrix (the apparent sterol content), processing could affect the ease with which the sterols are extracted. This was referred to as the *extractability* of sterols, and was determined using sequential solvent extraction. The method of chemical extraction did not aim to mimic intestinal tract nor to find an ideal solvent for a maximum extraction, but rather aimed at determining the ease of extraction, thus demonstrating how tightly the compounds are bound in the matrix.

Processing effects on apparent sterol contents were moderate (II: Table 1), and significant increases were observed by mechanical treatments, when the particle size of the bran samples was reduced, and by enzymatic treatments when the fibre matrix was partially hydrolysed especially with a combination of the enzymes. The most significant effects were observed as a result of aqueous treatments on the extractability of sterols (Figure 5). The changes in the extractability were particularly significant in rye bran samples, in which the proportion of easily extractable sterols in the heptane extract decreased from 78% to 52% (Figure 5, II: Figure 3). This decrease in extractability was recovered by xylanase and β -glucanase. The overall effects on the apparent sterol content were minor, but more significant when analysed together with the effects on extractability. The amount of easily extractable sterols from rye bran after incubation in distilled water was 1000 $\mu\text{g/g}$, but was increased to 1720 $\mu\text{g/g}$ (42% higher) when the bran was treated with the enzyme mixture (Figure 5). Similar effect was seen with wheat bran, though the increase was only 16%. The difference in the effect between the two cereals can be explained by differences in their fibre composition - rye contains primarily arabinoxylan that has a high hydration capacity, whereas wheat contains more cellulose and less arabinoxylan (Henry, 1985). It is likely that the arabinoxylan in rye bran forms a hydrated structure when incubated in distilled water. This structure may block minor components like sterols inside, which are liberated again as the fibre is partially hydrolysed by enzymes.

Thermal effects on sterols and steryl conjugates were minor under the heating conditions used. Oxidation or degradation would theoretically be possible under these conditions, but sterols have been shown to be relatively stable under e.g. baking conditions (Soupas et al., 2003). Further, studies on rice bran processing have shown that contents of SF are effected less by heat treatment than e.g. tocopherols, and that SF are generally degraded more during an extended period of storage (Shin et al., 1997). Furthermore, as demonstrated also in this study, when SF and/or α -tocopherol were heated in cooking and frying conditions in oil, degradation of SF was much slower than that of α -tocopherol (V). No prior data or literature

is available on thermal stability of SG, but the results indicate good stability under the conditions used in this study.

Aqueous treatments were shown to decrease the apparent content of total sterols in both rye and wheat bran. Some effects were also seen in the contents of SG and rye ASG, but not on SF (II: Table 2). Singh and Paleg (1985) studied the effects of imbibition of wheat aleurone tissue on sterol conjugates, and demonstrated that the SG practically disappeared during the 24 hours treatment. On the other hand, no significant changes were observed in SG contents of aleurone cells in 72 hours germination in another study (Boa et al., 1984). However, the situation in this study is not directly comparable to soaking whole grains, as the signal factors, which are required e.g. for activation of enzyme production in the aleurone layers, originate from the germ, and thus were not present in this study at least in wheat bran. The rye bran from milling also likely contains some/all of the germ, as rye germ cannot be separated in the milling process. However, it is possible that the changes in the SG content of both wheat and rye bran caused by soaking in distilled water are a result of endogenous enzymes in the milling fractions. In addition to incubation in distilled water significant decrease in the content of SG was observed in rye bran by xylanase treatment and in wheat by both xylanase and β -glucanase. Further an increase in the rye ASG was observed after treating with single enzymes, but not their mixture (III: Table 2). Moreau et al. (1994) also reported a xylanase-induced decrease in SG content and an increase in the content of ASG in tobacco cells. On the other hand, data on possible aqueous hydrolysis of SF is limited. Watanabe et al. (2004) showed that the content of SF in rice decreased during germination. However, in this case the difference between the two studies is again the fact that germinating whole grains contain much more enzymatic activity than bran separated from the kernels. Ohtsubo et al. (2005) showed that pregermination decreased the content of SF in brown rice, but not in rice bran, supporting the view that enzymes responsible for the hydrolysis of SF are not present or activated in mere bran. Based on the literature and the data obtained in this study SF contents of rye and wheat bran are unaffected by soaking in distilled water, and thus they can be assumed to keep their bioactive properties also after aqueous processing.

6.3 Enzymatic hydrolysis of sterol conjugates

Steryl ferulates

The mixture of wheat and rye SF was shown to be hydrolysed by porcine and bovine sterol esterases. Of the two enzymes the bovine sterol esterase had somewhat higher activity (III: Table 1), which was in accordance with the one previous study in which the two enzymes were compared (Miller et al., 2004). In that study rice SF (γ -oryzanol) was used as a substrate. Similar results were shown by Huang (2003) that sterol esterase can hydrolyse γ -oryzanol. In

studies by Moreau and co-workers with corn oil steryl ferulates and synthetic sitostanyl ferulate it was demonstrated that SF was hydrolysed by the bovine steryl esterase (Moreau and Hicks, 2004), but not with microbial steryl esterase (Moreau et al., 2003). The inability of the microbial steryl esterase was also reported in the paper by Miller et al. (2004). The data presented in this study, together with the other earlier reports clearly demonstrates that the source of the steryl esterase enzyme has an effect on the hydrolysis of SF.

The substrate composition was shown to affect SF hydrolysis in most cases even more than the origin of the enzyme (III: Table 1). SF mixture from rye and wheat bran was much more effectively hydrolysed than γ -oryzanol (the SF mixture from rice). Further, the hydrolysis of synthetic sitostanyl ferulate was also generally higher than that of γ -oryzanol. The greatest difference between the substrates is the sterol composition: γ -oryzanol is primarily constituted of 4,4'-dimethylsteryl ferulates, whereas sitostanyl ferulate and the SF mixture from rye and wheat contained merely desmethylsteryl ferulates. The most significant structural differences between these two groups are the two extra-methyl groups in C-4 and often also one additional methyl group in C-14 in the sterol ring system of the dimethylsterols. Cholesterol, esters of which are the primary substrates for these enzymes, is a desmethylsterol and thus lacks the methyl groups. Especially the C-4 position is very close to the ester bond, and two additional methyl groups can obstruct the substrate from getting to the active site. Preference of desmethylsteryl ferulates as substrates, or no detected hydrolysis of dimethylsteryl ferulates has also been demonstrated in other studies (Huang, 2003; Miller et al., 2004). Further, it was suggested by Miller et al. that information on the preferences of the enzymes could be used to tailor the sterol composition of γ -oryzanol to increase the proportion of desmethylsteryl ferulates. As rye and wheat contain only desmethylsteryl ferulates they can be thought to have an ideal sterol composition of SF, which compensates for the lower content found in these cereals. In fact, if considered that on average 70% of SF sterols in γ -oryzanol are dimethylsterols and the remaining 30% are desmethylsterols (Table 2), it can be deduced that brown rice contains about 60-210 $\mu\text{g/g}$ desmethylsteryl ferulates. As it is agreed in the literature that SF need to be hydrolysed before they may e.g. act as inhibitors of cholesterol absorption, it could be stated that wheat and rye can be as effective as rice in health promotion by SF constituents.

Enzymes other than steryl esterases have not been conclusively shown to hydrolyse SF. Miller et al. (2004) studied a number of different lipases in addition to steryl esterases. Some hydrolysis of SF (2% hydrolysis) was observed with porcine pancreatic lipase, but it was hypothesised that the hydrolysis was not caused by lipase, but rather a steryl esterase activity present in the lipase preparation. It is also possible that some other enzymes in the gastrointestinal tract could hydrolyse SF. For example ferulic acid esterase activity has been

demonstrated to be found in the mammalian intestinal tract (Andreasen et al., 2001), but there is no data available on the capability of ferulic acid esterase to hydrolyse SF.

Steryl glycosides

Prior studies on enzymatic hydrolysis of SG are scarce, as are studies on the metabolism of SG in mammals. In this study various β -glucosidases were studied for their capability to hydrolyse SG (III). The greatest percent of hydrolysis was gained with a β -glucosidase (Novozym 188) that is marketed as a cellobiase. The same enzyme preparation has been reported to hydrolyse isoflavone glycosides (Richelle et al., 2002), but did not apparently hydrolyse lecithin SG in another study (Both et al., 2006). Recent studies have shown that SG might have a role in cellulose synthesis as a primer, which is cleaved off once the polymer is formed (Peng et al., 2002). This could partially explain the reason why a cellobiase has activity in the hydrolysis of SG. However, the cellobiase from *Aspergillus niger* has been reported to be inhibited by the hydrolysis products (Hong et al., 1981; Grous et al., 1985). This hinders the possibility to utilise these enzymes for the hydrolysis of SG in the analytical procedures, as full hydrolysis cannot be guaranteed. On the other hand, with an indicative knowledge of the amount of SG in the sample, one may adjust the level of enzyme addition to be adequate for total SG hydrolysis. The benefit of enzymatic hydrolysis in the analysis of SG is the fact that it does not destroy the more labile sterols, like acid hydrolysis may do (Kesselmeier et al., 1985). On the other hand, the specificities of the enzymes should also be studied before making a final change from acid hydrolysis to enzymatic hydrolysis, as it cannot be guaranteed that all steryl and carbohydrate moieties have similar affinities for hydrolysis. Nevertheless, the percentages of hydrolysis obtained in this study with cellobiase are a promising start in the search for an enzyme that could be used to replace acid hydrolysis.

In earlier studies β -glucosidases from almonds have been used to hydrolyse SG prior to analysis of sterols as FS (Kesselmeier et al., 1985; Moreau et al., 1994). In this study, however, almond β -glucosidase was ineffective in SG hydrolysis (III). It seems plausible that the enzyme in the earlier studies has been less pure with a number of isoenzymes or side activities, than the one used in this study, which has likely been produced with more modern processes. To further study the capacity of almond β -glucosidase in SG hydrolysis one could perhaps extract the enzymes from almonds, or make more trials with other enzyme preparations as well.

The lack of β -glucosidases from mammalian digestive system available commercially hinders the study of the affinities of digestive enzymes towards SG. It can only be assumed that enzymes with an ability to hydrolyse SG can be found in the gastrointestinal tract. A β -glucosidase with broad substrate specificity has been reported e.g. in the human liver, but its

capability to hydrolyse SG is not known (Daniels et al., 1981). Further, the intestinal microbes, especially in the colon, are known to possess multiple different enzyme activities that may affect the digestion of various nutrients. However, plant sterols are hardly absorbed in the ileum and certainly not in the colon, where the effect of the intestinal enzymes could take place. Therefore, it is highly unlikely that SG metabolism would be significantly affected by intestinal microbiota.

6.4 Antioxidant activity of steryl ferulates

Antioxidant activity of steryl ferulates from rye and wheat bran were evaluated and compared to the activities of compounds with a well established antioxidant activity (ferulic acid, α -tocopherol) at a moderately elevated temperature (+40°C) in bulk MeLo (methyl linoleate) as well as MeLo emulsion (**IV**). Further, synthetic SF were compared to extracted SF, and possible effects of the origin of extraction (rice vs. rye or wheat) were assessed. Antioxidant activity of sitostanyl ferulate was also studied at cooking and frying temperatures (**V**).

As expected, SF demonstrated a capability to donate a hydrogen atom to the stable DPPH-radical (Figure 6), though the activity was less than that of α -tocopherol, which has been shown in other studies as well (Table 11). The SF mixture extracted from rye or wheat bran exhibited higher activity than the synthetic sitosteryl or cholesteryl ferulates. No clear explanation can be given as to why the natural mixture would possess higher activity, but similar phenomenon was seen also in the MeLo bulk lipid-system, in which the rye and wheat SF extracts inhibited hydroperoxide formation more than synthetic SF. Furthermore, SF from rice (primarily dimethylsterols) generally inhibited oxidation less than desmethylsteryl ferulates (sitosteryl and cholesteryl ferulates, and extracts from rye and wheat). Again, no definite trend for the antioxidant activity can be deduced from this data and other literature (Table 10), and to obtain more insight about this, one should analyse the activities of single SF. Due to the minor differences in the structures of these compounds, individual SF are virtually impossible to obtain from mixtures by current chromatographic methods. Other option would be to synthesise the standards. However, not all sterols are available as pure standards, and thus a significant proportion of other sterol species would always be present in the synthesised product. On the other hand, natural mixtures, like SF extracts from rye and wheat or γ -oryzanol, account for the real life situation in foods more fully than any one single synthetic compound.

Effects of antioxidant polarity

According to the polar paradox concept polar antioxidants have higher activities in less polar environments and *vice versa* (Frankel, 1998), and therefore it could be assumed that free ferulic acid without the non-polar sterol would be more active than SF in the non-polar bulk lipid system (IV). However, this was not clearly seen in the results in inhibition of hydroperoxide formation. Free ferulic acid was indeed more or equally active to the synthetic SF and rice SF, but the SF extracts from rye and wheat were better antioxidants than ferulic acid. As described in paper IV the extracts were analysed with three different chromatographic systems and were not shown to contain any other compounds than SF in any of the analyses. Therefore, no simple explanation is available to account for the higher activity of the wheat and rye extracts compared to ferulic acid and other SF samples. In the emulsion system where, according to the polar paradox, SF should have a higher antioxidant activity than ferulic acid, these compounds exhibited equal activity.

Antioxidant activity of steryl ferulates vs. α -tocopherol

α -Tocopherol is used as a reference compound in evaluations of antioxidant activity, as it is known to inhibit oxidation in multiple different systems, and it is readily available commercially at a low price. Therefore α -tocopherol was chosen as a reference compound in this study as well. When antioxidants were added to MeLo and heated at +40°C, SF were added to samples at two different concentrations (0.52mM and 2.58 mM), which corresponded to 100 μ g/g and 500 μ g/g of ferulic acid that is considered to be the active part of the molecule. α -Tocopherol was only analysed at the lower concentration, and its activity was higher than the same amount of any SF. In fact when added at the higher concentration (2.58 mM) the activity of rye and wheat SF extracts were roughly comparable to α -tocopherol (0.52 mM). In the literature the order of antioxidant activity of these two antioxidants, SF and α -tocopherol, has varied between different studies. Higher activity of SF compared to α -tocopherol was reported by Gertz et al. (2000), Xu et al. (2001), Huang et al. (2002) and Kim et al. (2003), whereas α -tocopherol had greater inhibition in the studies of Tajima et al. (1983), Xu and Godber (2001), Kikuzaki et al. (2002) and Juliano et al. (2005). As none of the listed studies used directly comparable methods to those of this study comparisons are not possible. However, it is a very typical phenomenon in antioxidant chemistry that the order of activities of different compounds varies in different systems, and thus it is important to evaluate the compounds in multiple different systems.

The radical scavenging activity of α -tocopherol was significantly higher than that of any SF analysed (Figure 6). Antioxidants that readily donate a hydrogen atom to a hydroperoxyl radical also inhibit the isomerisation of the MeLo-hydroperoxide from *cis,trans*-hydroperoxide to *trans,trans*-hydroperoxide (Mäkinen et al., 2000). Therefore, the proportion

of cis,trans-isomers is higher in systems with more active radical scavengers. The ratio of cis,trans-isomers to trans,trans-isomers in the bulk lipid system was determined (IV), and it was verified that the proportion of cis,trans-isomers was higher in samples with the more active radical scavenger α -tocopherol, when compared to SF samples. Similar effects on isomer distribution have earlier been reported by Xu and Godber (2001).

Differences in the antioxidant activities were less pronounced, when sitostanyl ferulate and α -tocopherol were evaluated at cooking and frying temperatures (V). Significant differences were observed only at the final time points of heating at both temperatures (V: Table 1). Further, SF showed greater heat stability in the mixture samples containing both antioxidants (Figures 7 and 8). Synergism in the activities of these two antioxidants was not demonstrated, though it has been suggested in the literature (Kalitchin et al., 1997; Kochhar, 2000). The results obtained in this study indicate that the activity of SF to inhibit oxidation is not comparable to that of α -tocopherol. However, the natural content of SF in cereals is about 10-fold higher compared to α -tocopherol, which compensates for the inferior activity. Thus naturally occurring SF in cereal products can protect the product from oxidation also at elevated temperatures.

7 CONCLUSIONS

SF and SG are steryl conjugates that are characteristic to cereals and contribute a significant proportion to total plant sterols in both rye and wheat. This study showed that the contents of SF vary significantly within the cereal kernels, and that the content of SF increases in parallel with the ash content in the corresponding samples. Further, there is a strong positive correlation between SF and dietary fibre in both rye and wheat milling fractions. The contents of SG, on the other hand, vary much less between the kernel parts, and either do not correlate or have a negative correlation with the dietary fibre content. The composition of sterol species found as different conjugates varies significantly: sitosterol is preferentially found as glycosides and saturated stanols (campestanol and sitostanol) form the majority of ferulic acid esters.

The apparent content of sterols in rye and wheat bran is only moderately affected by thermal, mechanical and enzymatic processing under the conditions used in this study, and only minor or no changes are observed in the contents of SF, SG and ASG. The extractability of total sterols may be hindered by the aqueous treatments, but they can be overcome with the use of enzymes. The aqueous processes caused some changes especially in the contents of SG, either caused by the endogenous enzymes of the bran, or the exogenous enzymes used in processing. All of the steryl conjugates showed heat stability in the thermal processes.

The enzymatic hydrolysis of SF is significantly affected by the sterol composition of the substrate and the origin of the enzyme. Desmethylsteryl ferulates are preferentially hydrolysed by the mammalian steryl esterases, thus showing a possibly increased nutritional significance of SF from non-rice sources. SG was shown to be hydrolysed by β -glucosidase from *A. niger*, but due to the lack of commercial enzymes, no mammalian digestive enzymes could be evaluated. The results of the study show promising potential for further development and can be used as a basis for the search of enzymes that could be applied in the analytical procedures to replace chemical acid hydrolysis.

Steryl ferulates displayed capacity in radical scavenging and thus prevention of hydroperoxide formation in methyl linoleate bulk and emulsion systems, though their activity was less than what was observed with α -tocopherol. In the antioxidant evaluation systems used in this study, natural SF extracts from rye and wheat seem to be more effective antioxidants than synthetic SF and those originating from rice. Unfortunately, no clear explanation to this phenomenon can be given. At cooking and frying temperatures the capacity of SF to inhibit polymerization was more comparable with the activity of α -tocopherol. Synergism, however, between these two antioxidants was not observed.

Overall, the studies of this thesis illustrate that both rye and wheat are significant sources of steryl ferulates and steryl glycosides. Their contents are only moderately affected by processing, and especially steryl ferulates possess a sterol composition that is favourable to the antioxidant and digestive properties. These steryl conjugates are inarguably components that have advantageous properties to human health despite being minor components in concentration.

Future research perspectives

Both steryl ferulates and steryl glycosides have been studied much less than free sterols or steryl fatty acid esters, and thus they deserve much more attention, particularly as they may have other beneficial effects on human health in addition to modification of blood lipid levels.

The metabolism of both steryl ferulates and steryl glycosides in mammals, as well as their roles in plant metabolism is still poorly understood, and should definitely be studied more. In metabolic studies it would be necessary to evaluate steryl conjugates from a number of sources, since they may differ in their properties, as demonstrated in this study. Well-designed and controlled studies would provide reliable data to many remaining questions and assumptions in biological as well as chemical properties of these steryl conjugates.

In food applications (especially in products like breads, biscuits, pasta and breakfast cereals) the contents of steryl ferulates and steryl glycosides, as well as total sterols, could likely be increased with sophisticated choices of raw materials. And further, their bioavailability should be insured by using for example enzymatic processing. A product with a high content of steryl conjugates and likely also other beneficial bioactive components could then be introduced to nutritional evaluations. However, it is essential that the possible effects of different raw materials and novel processes on the sensory attributes are evaluated in parallel to changes in the composition of the products to ensure consumer acceptability.

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