universität wien

DISSERTATION

Titel der Dissertation

Safety assessment of phytosterol oxidation products

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer.nat.)

Verfasserin / Verfasser:	Karin Koschutnig
Matrikel-Nummer:	0025194
Dissertationsgebiet (lt. Studienblatt):	Ernährungswissenschaften
Betreuerin / Betreuer:	A.o. Univ.-Prof. Dr. Karl-Heinz Wagner

Wien, am 08. März 2009

Acknowledgements

I would like to express my deepest gratitude to my supervisor a.o.Univ.-Prof. Dr. Karl-Heinz Wagner for the possibility to carry out this thesis. I thank you for all the help, support and guidance during all these years as well as for giving me diverse and varying tasks and responsibilities. I also had the possibility to present my results on various conferences. Karl-Heinz, danke für alles!

I would like to thank all members of Prof. Wagner's working group: Mag.[a] Sonja Kanzler, Mag. Oliver Neubauer, Mag.[a] Elisabeth Plasser and Mag.[a] Stefanie Reichhold. Your excellent company allowed me to succeed in completing this study. The many joyful moments we have shared in our workroom, in the lab, on excursions, on congress meetings, during cooking sessions, birthday parties and after-work drinks will never be forgotten.

I would like to extend my thanks to Mag.[a] Christine Mölzer and Mag.[a] Cornelia Fritz-Ton for assisting me in the bacterial assays and in particular for the fun we had.

I would like to thank o.Univ. Prof. Dr. Ibrahim Elmadfa for the possibility to realise this thesis at the Department of Nutritional Sciences.

I further wish to thank all my current and former colleagues at the Department of Nutritional Sciences for all the assistance, discussions and for a friendly working atmosphere.

I am thankful for the opportunity to perform a part of this thesis at the Department of Applied Chemistry and Microbiology, Food Chemistry Division, University of Helsinki, Finland.
I wish to express my warmest gratitude to Professor Dr. Vieno Piironen and Docent Dr. Anna-Maija Lampi. The collaboration made this thesis possible. Kiitos – for adopting me in your group, your valuable advice and ideas for the studies, your guidance and

support – not only during my time at your department - as well as for acting as co-authors on the papers.

My colleagues at the Food Chemistry Division in Viikki deserve special mention for providing a congenial working atmosphere. Thank you for welcoming me into your team. I really enjoyed being one of the 'sterol girls'.
In particular I want to thank Dr. Suvi Kemmo and Susanna Heikkinen for introducing me to the analysis of sterol oxides and their support in the method development. Suvi, thanks for your friendship and all the help during the last three years.

My sincere thanks are due to a.o.Univ.-Prof. Dr. Wolfgang Luf (Institute for Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine Vienna) and a.o.Univ.-Prof. Dr. Michael Murkovic (Institute for Food Chemistry and Technology, Graz University of Technology) for their willingness to review this thesis.

For financial aid, I wish to thank the University of Vienna (F81-B Forschungsstipendium, KWA-Stipendium) and the COST organization (Cost 927 short term scientific mission) for supporting my research and conference attendance.

I am forever thankful to my wonderful friends for sharing many happy moments and for their moral support during difficult times.

This thesis is dedicated to my family, without whom none of this would have even been possible. I am deeply grateful to my parents, my brother, my grandparents and Thomas for always supporting me unconditionally with whatever I have chosen to do and for their absolute confidence in me.

Table of contents

List of Figures

List of Tables

List of Abbreviations

ABC	adenosine triphosphate-binding cassette
ACAT	acyl- CoA cholesterol acyltransferase
AT	adenine-thymine
ATP	adenosine triphosphate
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
BSTFA	*N,O*-bis-(trimethylsilyl)trifluoroacetamide
C	cholesterol
CC	column chromatography
CHD	coronary heart disease
CLA	conjugated linoleic acid
CN	cyano
COP	cholesterol oxidation product
DHA	docosahexaenoic acid
DHE	dihydroethidine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELSD	evaporative light scattering detectors
EFSA	European Food Safety Authority
EtOH	ethanol
FBS	fetal bovine serum
FDA	food and Drug Administration
FID	flame ionization detector
FSC	forward scatter channel
GC	guanine-cytosine
GC-FID	gas chromatography with flame ionization detection
GC-MS	gas chromatography-mass spectrometry
GRAS	generally recognised as safe
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HDL	high density lipoprotein
HE	hydroethidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMDS	hexamethyldisilazane
HPLC	high-performance liquid chromatograph(y)
HPLC-MS	high-performance liquid chromatography-mass spectrometry
ID	inner diameter
ISTD	internal standard
IUPAC-IUB	International Union of Pure and Applied Chemistry and International Union of Biochemistry
KCl	potassium chloride
KH_2PO_4	potassium-dihydrogen-phosphat

LDH	lactate dehydrogenase
LMA	low melting agarose
LOD	limits of detection
log	logarithm
LSD	light scattering detectors
LXR	liver X receptor
MDA	malondialdehyde
MEM	minimal Essential Medium Eagle
MS	mass spectrometry
MTT	3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromid
Na_2EDTA	disodium ethylenediaminetetraacetic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
NMA	normal melting agarose
NOAEL	no-observed-adverse-effect level
NPC1L1	Niemann-Pick C1-like protein
NP-HPLC	normal phase high-performance liquid chromatography
PARNUT	foodstuff for Particular Nutritional Purposes
PBS	phosphate-buffered saline
PDA	photodiode array detection
PE	phycoerythrin
POP	phytosterol oxidation product
PS	phytosterols
r^2	determination coefficient
R*f*	retention factor
RI	refractive index
ROS	reactive oxygen species
RPM	rounds per minute
RT	room temperature
RXR	retinoid X receptor
SCF	European Union Scientific Committee on Food
SCGE	alkaline single cell gel electrophoresis
SD	standard derivation
SPE	solid phase extraction
SSC	side scatter channel
TB	trypan blue exclusion assay
TBA	thiobarbituric acid
tBOOH	tertiary-butyl hydroperoxide
TLC	thin-layer chromatography
TMCS	trimethylchlorosilane
TMSE	trimethylsilyl ethers
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet (detection/detector)
$O_2^{\bullet-}$	superoxide anions
$^1O_2$	singlet oxygen
$^3O_2$	triplet oxygen
7-AAD	7-amino-actinomycin D

1. Introduction

Over the past few years phytosterols (plant sterols) have become a focus of interest in science as well as in public due to their serum cholesterol lowering effect. According to literature a daily phytosterol uptake of 1.5-3 g can lower serum LDL cholesterol levels by 10-15 % (Katan et al., 2003), thus a protection against cardiovascular disease is assumed (Hendriks et al., 1999). Consequently, an increasing number of products fortified with plant sterols has been introduced to the international market during the last decade. However, besides their positive aspects concerns in terms of health are emerging.

Due to their unsaturated ring structure, oxidation is the reaction of main concern. Similar to the structurally related cholesterol, phytosterols are susceptible to oxidation when exposed to heat, air, light or radiation (Dutta and Savage, 2002). Intense research has been performed on possible health implications induced by cholesterol oxidation products (COPs). Their mutagenic, carcinogenic, angiotoxic, cytotoxic and atherogenic properties can be considered as generally acknowledged (Guardiola et al., 1996; Osada, 2002).

In contrast, at present only limited information is available on the biological effects of phytosterol oxidation products (POPs) (Hovenkamp et al., 2008). However, in first studies on cytotoxicity similar patterns for phytosterol and cholesterol oxides were observed, although in general higher concentrations of POPs were needed for the same effect (Roussi et al., 2005; Roussi et al., 2007; Ryan et al., 2005).

Due to the fact that standards of phytosterol oxidation products are not commercially available so far, in many investigations blends rather than individual oxides were used. Although based on former publications (Maguire et al., 2003; O'Sullivan A et al., 2005) it might be assumed that mixtures of sterol oxides and single compounds act in a different way.

Since POPs have been detected in human plasma (Grandgirard et al., 2004b), more investigations to better understand their biological significance are necessary.

Therefore the overall aims of the present thesis were:

1. **To scale up a method for the isolation of single phytosterol oxidation products**, as for the subsequent toxicity assessment gram scale amounts of toxicologically

relevant oxides were needed. This part of the work was carried out in co-operation with the Department of Applied Chemistry and Microbiology, University of Helsinki under the supervision of Prof. Dr. Vieno Piironen.

2. **To investigate the biochemical potential of various isolated phytosterol oxidation products** derived from β-sitosterol, namely 7-ketositosterol, 7- β -OH-sitosterol, 7- α -OH-sitosterol, a mixture of 6-β-OH-3-keto-sitosterol/6-α-OH-3-keto-sitosterol (ratio 4:3) and a mixture of polar β-sitosterol oxidation products.
   Therefore the following *in vitro* tests were conducted:
   - Salmonella microsome assay (mutagenic/oxidative properties were analyzed using Salmonella thyphimurium strains TA98, TA100 and TA102 in different setups)
   - Cell culture assays using HepG2-cells
     - Viability assays (Trypan blue exclusion assay, MTT- assay)
     - Flow cytometric measurements (apoptosis versus necrosis, superoxide anion production ($O_2^{\bullet-}$))
     - Measurement of lipid oxidation (MDA)
     - Comet assay (detection of DNA-strand breaks)
     - Quantification of the phytosterol oxide uptake in HepG2-cells, which was done at the Department of Applied Chemistry and Microbiology, University of Helsinki as well.

2. Review of Literature

2.1 Phytosterols

2.1.1 Chemical structure

Phytosterols (plant sterols) as secondary plant metabolites are structural and biological counterparts of cholesterol, the main sterol in mammalian cells. Plant sterols are responsible for permeability and fluidity of cell membranes. Further they act as precursors of brassinosteroids, thus regulating storage and transport processes, and of numerous other metabolites such as glycoalkaloids and saponins (Piironen et al., 2000).

To date over 250 different phytosterols and related compounds have been identified in plant and marine materials (Salo et al., 2003). The main important plant sterols are β-sitosterol (24α-ethylcholest-5en-3β-ol), campesterol (24α-methyl-5-cholesten-3β-ol) and stigmasterol (5,22-cholestadien-24 α –ethyl-3β-ol), which represent about 45-95 %, 30 % and 25 % of total sterols present in plants, respectively (Lutjohann, 2004).

In general sterols are derived from squalen and consist of a tetracyclic cyclopenta[α]phenanthrene structure with a hydroxyl group at C-3 and a flexible side chain with 8-10 carbons at C-17 (Piironen et al., 2000). Plant sterols and cholesterol are structurally nearly similar, differing mainly in their side chain by an additional methyl or ethyl group at C-24 (figure 2.1).

On structural and biosynthetic basis plant sterols can be divided into 4-desmethyl sterols, 4α-monomethyl sterols and 4,4-dimethyl sterols according to the number of methyl groups at C-4. 4α-monomethyl sterols and 4,4-dimethyl sterols are both precursors of the 4-desmethyl sterols and only found in trace amounts in plants. 4-desmethyl sterols, with no methyl group at C-4, represent the major group of phytosterols. Depending on the position and number of double bonds in the B ring they can further be classified as Δ5-, Δ7- or Δ5,22-sterols, the former being the most common one (Moreau et al., 2002).

Phytostanols are saturated analogies of 4-desmethyl sterols with no double bond in their structure and occur in certain cereals (corn, wheat, rye, and rice), fruits and vegetables (figure 2.1). Concentrations are far lower than that of unsaturated plant sterols.

In plants, sterols occur not only as free alcohols, but also as conjugated forms as steryl fatty acid esters, hydroxycinnamate steryl esters, steryl glycosides and esterified steryl glycosides (Moreau et al., 2002).

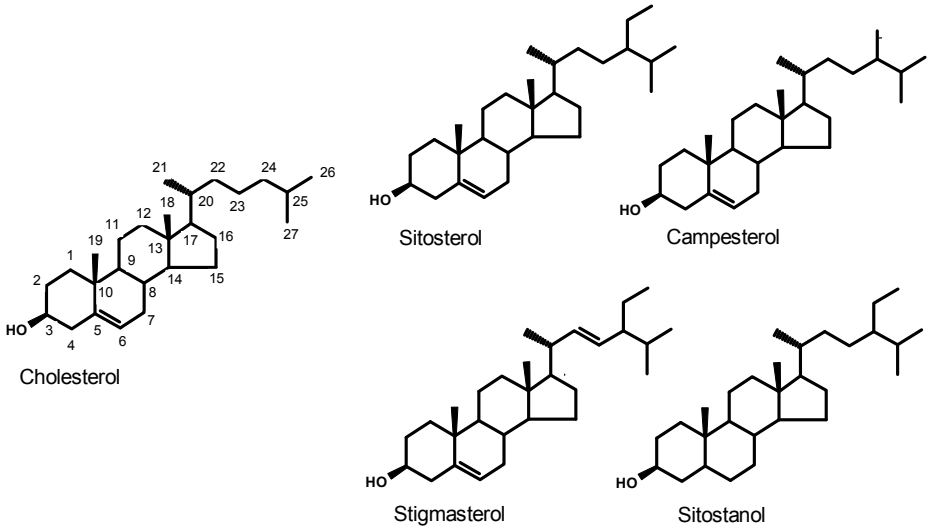


Fig 2.1 Structures of main sterols. Numbering is according to the International Union of Pure and Applied Chemistry and International Union of Biochemistry (IUPAC-IUB) recommendations (Moss, 1989).

2.1.2 Dietary sources of plant sterols

Phytosterols are not synthesized by animals or humans. In a natural form they only occur in plants. Cereals, margarine, vegetables and vegetable oils contribute as main sources in the human diet (Piironen et al., 2004). Total levels of phytosterols in vegetable oils range between 1-5 g/kg, with corn oil, rice bran oil and sesame oil being the richest source. Through the refining process plant sterol amounts get reduced. Depending on the applied conditions losses of 10-70 % were observed (Piironen et al., 2000).

For cereals total levels of 350-1200 mg/kg fresh weight were found, with rye (955 mg/kg) and barley (761 mg/kg) being the richest sources (Piironen et al., 2004). Although levels are lower than those of vegetable oils cereals are the main contributors of plant sterol intakes as in general higher amounts of cereals than oils are consumed (Piironen et al., 2000).

Also vegetables, fruits and berries are essential sources, accounting for 20-25 % of plant sterol consumption (Valsta et al., 2004). Among vegetables broccoli, Brussels sprout, cauliflower, green and black olives represent the richest sources with amounts over 300 mg/kg fresh weight. Plant sterol levels in fruits were found to be between 116–228 mg/kg. In berries contents ranging from 60–279 mg/kg were detected, whereas remarkable higher concentrations were measured in wild than in cultivated berries (Piironen et al., 2003).

2.1.3 Estimated daily intakes

The average daily intake of plant sterols from natural sources is estimated to range between 150-450 mg (Ostlund, 2002; Ellegard et al., 2000), depending on the respective country and the type of diet. For vegetarians intake levels of 1 g/day and more have been found (Piironen et al., 2000). Recent studies have shown intakes of 305 mg/d for men and 237 mg/d for women in Finland and 307.3 mg/d for men and 262.9 mg/d for women in the Netherlands (Valsta et al., 2004; Normen et al., 2001). Density of plant sterols in the diet (mg/1000kJ) was higher for women (34.9) than men (32.3) (Valsta et al., 2004). When analysing the amount of plant sterols in different US diets, a rise in phytosterol intake levels with increasing consumption of polyunsaturated fat has been observed, whereas the intake of saturated fat was inversely related to plant sterol contents in the diet (Phillips et al., 1999). In general plant sterol intakes are steadily increasing, reflecting the change from animal fat to vegetable oils, but also the increasing number of phytosterol enriched products on the market (Morton et al., 1995; Valsta et al., 2004).

2.1.4 Phytosterol metabolism

While cholesterol absorption in humans varies from 30-60 % (Bosner et al., 1999), absorption of plant sterols is rather low. Observed levels for campesterol (9.4-14.8 %) are approximately 3 times higher than for sitosterol (3.1-4.5 %) and stigmasterol (~4 %). Absorption of campestanol and sitostanol was shown to be even lower (0.1-2 %) (Miettinen et al., 2000; Piironen et al., 2000; Sanders et al., 2000).

Differences in the absorption efficiency of sterols depend strongly on their molecular structure. The presence of a double bond between C5 and C6 has been shown to increase the absorption rate, whereas it decreased by increasing length of the side chain (Ostlund et al., 2002).

Absorption of plant sterols occurs under the same conditions as that of cholesterol and other lipids (figure 2.2). They have to be emulsified and incorporated into mixed micelles to be absorbed from the lumen. For a long period a passive transfer into the enterocyte has been assumed. Recent studies suggest an involvement of a specific protein the Niemann-Pick C1-like protein (NPC1L1) in the brush border membrane in the uptake of sterols by the enterocyte (Jessup et al., 2008).

The rather poor absorption of plant sterols compared to cholesterol could be due to the low affinity of acyl- CoA cholesterol acyltransferase (ACAT) for plant sterols (De Jong et al., 2004). After being taken up by the enterocytes both cholesterol and phytosterols have to be esterfied for transportation in chylomicrones by ACAT. Esterfication rate of plant sterols is however 60 times lower than that of cholesterol. Hence, only a small part of the absorbed phytosterols is esterfied in enterocytes and incorporated into chylomicrons. Therefore the absorption of phytosterols is mostly prevented. Adenosine triphosphate (ATP) – binding cassette (ABC) - half transporters ABCG5 and ABCG8 are suggested to be responsible for the transportation of unesterfied sterols back in the intestinal lumen. Mutations in these two transporters lead to sitosterolemia, a plant sterol storage disease, which is characterised by increased serum levels of plant sterols (7-16% of total cholesterol concentration in plasma) and causes mainly premature atherosclerosis, coronary heart disease, tendon and tuberous xanthomas (Salen et al., 1992). Yet the full mechanism is not completely understood.

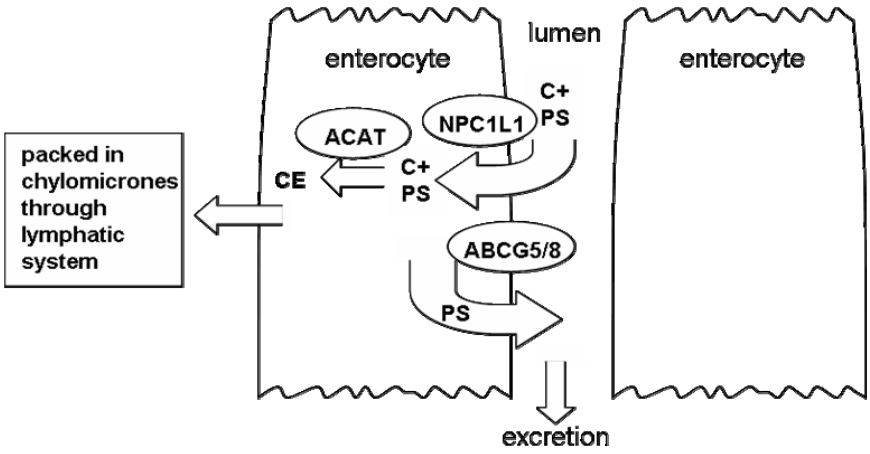


Fig 2.2 Absorption of cholesterol (C) and phytosterols (PS) in the intestine (adopted from Chen et al. (2008)).

Those parts of plant sterols taken up by the liver are incorporated into very low density lipoproteins (VLDL) or secreted via the biliary route (Plat and Mensink, 2005). Plant sterols have been shown to be stored in the cytoplasma or incorporated into the cell membrane. Labelled phytosterols were located in adrenal glands, ovary and testis of animals, which suggests similar metabolic pathways to cholesterol (Trautwein et al., 2003).

To date it is assumed that plant sterols are not metabolized into normal C24-bile acids but excreted as polar compounds, presumably di- and trihydroxylated C21-derivates (Boberg et al., 1990). Biliary excretion of phytosterols appears to be faster than that of cholesterol. This, together with the lower absorption rate, leads to serum levels (0.3-1.7 mg/dl) far lower than that of cholesterol (140-320 mg/dl) (Piironen et al., 2000).

	Cholesterol	Plant sterols	Plant stanols
Dietary intake	300-500 mg/d	150-450 mg/d	10-60 mg/d
Dietary scources	meat, egg yolk, dairy products	vegetable oils, cereals, fruits, vegetables, berries	wheat, rye, corn
Endogenous synthesis	800-1200 mg/d	not synthesized	not synthesized
Absorption	30-60 %	5-15 %	0.1-2 %
Plasma concentration	140-320 mg/dL	0.3-1.7 mg/dL	0.01 mg/dL
Excretion	40-60 %	85-95 %	>98 %

Tab 2.1 Summary of sterol metabolism (modified according to Salo et al. (2003)).

2.1.5 Cholesterol lowering action of plant sterols

2.1.5.1 Historical perspective

As early as in 1951 Peterson (1951) for the first time observed the cholesterol lowering property of plant sterols when feeding soysterols to chicken on a high cholesterol diet. Shortly after, this effect has also been shown in humans after administration of 5-10 g sitosterol a day (Pollak, 1953). These findings were followed by a broad range of investigations. Knowledge on the poor absorption of plant sterols in their crystalline form led to the use of rather high doses (up to 20-30 g/d).

In the late 1970s first studies with plant stanols were conducted and their better efficiency compared to sterols in reducing cholesterol levels was suggested (Sugano et al., 1976; Heinemann et al., 1986). Already small doses (1.5 g/day) resulted in a measurable decrease in cholesterol levels (Piironen et al., 2000). However, inconsistent data focused the research on the importance of their physical state (Katan et al., 2003).

Finally esterfication of first plant stanols and later also sterols with fatty acids of vegetable oils was developed. Hereby their solubility was improved. Hence their application in a wide variety of food was facilitated and through their better dispersion in the intestine their efficiency in cholesterol reduction was enhanced as well (Katan et al., 2003). Soon, in the year 1995, the first plant stanol ester enriched commercial

application, Benecol® (Raisio Plc., Raisio, Finland) margarine was launched in Finland (Moreau, 2004). In 1999 Unilever's Take Control® (also marketed under the brands Becel, Rama or Flora Pro active^{TM}), a phytosterol enriched margarine, was introduced to the US-market. Because of their simpler and less expensive production, Unilever had a distinctive marketing advantage especially since the effectiveness of both sterol and stanol esters was gradually accepted as equipollent (Moreau et al., 2002).

To date two main sources for phytosterols exist: tall oil, a by-product of the pulp industry and deodorizer distillate of vegetable oils. Although phytosterols could in fact be isolated of most vegetable oils (for example corn, rape, peanut, corn fibre and rice bran oil) soybean oil is the most commonly used source. Composition of the obtained phytosterols differs between tall oil and soy oil. Whereas the former contains mainly sitosterol (92 %, 8 % campesterol) in the latter the ratio of sitosterol and campesterol is 68:32 (w/w). However, it seems that statistically there is no difference in cholesterol-lowering efficiency (Salo et al., 2003). For the production of stanols as an additional step catalytic hydrogenation for saturation of the ring structure is necessary.

In recent years further strategies for the incorporation of plant sterols and stanols in food matrixes have been developed. In 2001 Christiansen et al. (2001b) investigated the effect of microcrystalline free phytosterols and found reduced total- and LDL - cholesterol levels after administration of only 1.5 g/day. This process is being commercialized under the name Diminicol® by the Finnish company Teriaka (Helsinki, Finland).

Emulsification of phytosterols and stanols with lecithin or diacylglycerol reduces the required amount of sterols and enhances their solubility. Both methods seem to be promising approaches to a simpler and more effective cholesterol lowering effect (Salo et al., 2003; Ostlund et al., 1999). After having been marketed in Japan for several years a diacylglycerol rich oil fortified with phytosterols is now also approved in the European Union (EU) (see table2.2).

Further Multibene, a product that combines phytosterols with various minerals as calcium, magnesium, and potassium or phytosterols esterfied with conjugated linoleic acid (CLA) and docosahexaenoic acid (DHA) has been developed. A further improvement of the health effects through these combinations is expected.

2.1.5.2 Current spectrum of plant sterol/-stanol enriched food

After the start in Finland and the USA plant stanols and sterols enriched food is now being sold in over 20 countries all over the world (Moreau, 2004). The success of the first fortified applications have given rise to a number of other products, meanwhile varying form fruit juices to ice cream and snack bars. However, in the EU growth has been slow-going as the regulatory system is quite complicated (for detailed information see table 2.2).

While Benecol spread was already approved in Finland as Foodstuff for Particular Nutritional Purposes (PARNUT) before the country became a member of the EU, Unilever's Take Control had to pass a long approval process, since in the EU phytosterol and -stanol enriched products have to be applied as novel food or novel food ingredient according to the EC Regulation No. 258/97. However, the complicated regulatory application process can be simplified if a newly launched product is substantially equivalent to an existing food application (http://ec.europa.eu/food/food/biotechnology/novelfood).

In the USA products enriched with plant sterol esters, plant stanol esters, plant sterols, plant stanols and microcrystalline phytosterols received GRAS (generally recognised as safe) status by the Food and Drug Administration (FDA). In the year 2000 FDA has authorised a health claim for reducing the risk of coronary heart disease for food products containing plant stanol and sterol esters. Stanol ester enriched spreads, salad dressings, snack bars and dietary supplement as well as spreads and salad dressings fortified with sterol ester were included in this regulation. Total fat has to be restricted to 13 g per serving or 50 g in the whole product. Spreads and salad dressings, however, were excluded from this regulation (Moreau, 2004).

While in Japan, Australia and New Zealand plant sterol and stanol enriched food is also marketed, Canada withdrew the approval of Becel Pro-Active after only a few months in 2001, as health risks for certain groups are possible (Moreau, 2004).

In Austria, currently only phytosterol enriched milk products are available. Unilever markets 3 Becel Pro-Active products: a low fat spread containing 7.5 g plant sterols / 100 g, a low-fat yoghurt drink containing 2 g plant sterols / 100 g and a low fat milk containing 0.3 g plant sterols / 100 mL. Recently also Danone launched a low fat

phytosterol enriched yoghurt drink (1.6 g / 100 g) using the brand Danacol, which is also marketed in several but not all EU-countries.

In general, spreads were the first phytosterol enriched commercial product available on the markets and still they are the most widespread application. Nearly all studies on the cholesterol lowering effect have been done with spreads (Berger et al., 2004). Their fatty food matrix provides optimal solubility of the added compounds. Furthermore spreads can be easily incorporated into the daily diet.
Currently dairy food represents the steeply rising area of sterol enriched applications. Similar to spreads plant sterols can be incorporated into the fat-phase and low fat products are in general considered as part of a healthy diet (Salo et al., 2003). Especially yoghurt and milk based drinks can be used as convenient single servings.

Food type	Manufacturer	Active components	Status
yellow fat spread	Unilever	phytosterol esters	EU Commission Decision (2000/500/EC)
	Archer Daniels Midland Company	phytosterols/phytostanols	EU Commission Decision (2004/333/EC)
	Pharmaconsult Oy Ltd.	phytosterols/phytostanols	EU Commission Decision (2004/334/EC)
	Teriakia Ltd.	phytosterols/phytostanols	EU Commission Decision (2004/336/EC)
cheese type products	Archer Daniels Midland Company	phytosterols/phytostanols	EU Commission Decision (2004/333/EC)
	Teriakia Ltd.	phytosterols/phytostanols	EU Commission Decision (2004/336/EC)
fermented milk type products	Archer Daniels Midland Company	phytosterols/phytostanols	EU Commission Decision (2004/333/EC)
milk based beverages	Novartis Consumer Health	phytosterols/phytostanols	EU Commission Decision (2004/845/EC)
milk based fruit drink	Teriakia Ltd.	phytosterols/phytostanols	EU Commission Decision (2004/336/EC)
milk type products	Archer Daniels Midland Company	phytosterols/phytostanols	EU Commission Decision (2004/333/EC)
	Pharmaconsult Oy Ltd.	phytosterols/phytostanols	EU Commission Decision (2004/334/EC)
	Unilever Bestfood Europe	phytosterol esters	EU Commission Decision (2004/335/EC)
yoghurt type products	Pharmaconsult Oy Ltd.	phytosterols/phytostanols	EU Commission Decision (2004/334/EC)
	Teriakia Ltd.	phytosterols/phytostanols	EU Commission Decision (2004/336/EC)
salad dressings	Archer Daniels Midland Company	phytosterols/phytostanols	EU Commission Decision (2004/333/EC)
spicy sauces	Pharmaconsult Oy Ltd.	phytosterols/phytostanols	EU Commission Decision (2004/334/EC)
soya drinks	Archer Daniels Midland Company	phytosterols/phytostanols	EU Commission Decision (2004/333/EC)
rye bread	Pharmaconsult Oy Ltd.	phytosterols/phytostanols	EU Commission Decision (2006/58/EC)
	Oy Karl Fazer	phytosterols/phytostanols	EU Commission Decision (2006/59/EC)
oil (containing diacylglycerol rich fat components and free phytosterol esters)	Enzymotech Ltd.	phytosterols/phytostanols	EU Commission Decision (2007/343/EC)
rice drink	Teriaka Ltd.	phytosterols	EU Commission Decision (2008/36/EC)
frankfurter sausages	Pouttu Ltd.	phytosterols	waiting for approval since March 2000
juices and nectars	Coca-Cola	phytosterols	waiting for approval since October 2004

Tab 2.2 Plant sterol/-stanol enriched products approved/ waiting for approval on the EU-market in the end of 2008.

2.1.5.3 Factors affecting the influence of plant sterol and stanol enrichment on cholesterol reduction

Effective dose

Numerous studies investigating the cholesterol lowering effect of plant sterols and stanols in a wide variety of subjects including normo- and hypercholesterolemic adults and children as well as patients with familiar hypercholesterolemia, type II diabetics on statin therapy or type II diabetic hypercholesterolemic subjects have been conducted (Berger et al., 2004). Administered amounts ranged from 0.8 to 3.8 g/day (Nguyen, 1999).

A meta-analysis of 41 randomized trails comparing the effects of plant sterol and stanol ester enriched products found that an average daily intake of 2 g of plant sterols and stanols induces a reduction of LDL-cholesterol levels by approximately 10 % (Katan et al., 2003). Hallikainen et al. (2000b) compared the effects of increasing concentrations of plant stanol esters (0, 0.8, 1.6, 2.3, 3.2 g/d) and found a dose dependent effect. The reduction at 2.3 and 3.2 g/d, however, didn't really differ from that reached by an intake of 1.6 g/d.

In general a reduction of 5-15 % in total cholesterol levels and 10-20 % in LDL-cholesterol levels is achieved by an intake of 1.5-3 g plant sterols or stanols (Normen et al., 2004; Nguyen, 1999). Levels below 1.5 g/day were shown to exhibit no significant cholesterol lowering actions (Nguyen, 1999; Hallikainen et al., 2000b). Doses higher than 3 g/d should be avoided as - if at all - only a small improvement has been observed and negative side effects of large intakes can't be absolutely excluded (Katan et al., 2003). The cholesterol-lowering effect was already noted after two weeks of treatment (Wester, 2000). A stop in phytosterol or -stanol intake, however, leads to a return to cholesterol baseline levels within two weeks (Jones et al., 1997).

Intake frequency

From the very beginning it has been suggested that for achieving an optimal cholesterol lowering effect plant sterols or stanols have to be consumed together with meals. This theory was based on the assumption that plant sterols/stanols have to be present in the lumen for interference with cholesterol uptake (Katan et al., 2003). Plat et al. (2000)

compared the effect of one single daily dose to 3 divided doses of stanol-ester enriched margarine and found similar efficiency in LDL-cholesterol reduction. This finding was confirmed by a second investigation (Matvienko et al., 2002). Hence, it seems that the number of phytosterol/-stanol doses per day is not important (Rozner and Garti, 2006) and that plant sterols and stanols exert a longer-lasting effect on cholesterol metabolism. Yet it seems that the time of intake affects the cholesterol lowering effect as this is more pronounced when the single dose is consumed with lunch or the principal meal than with or before breakfast (Doornbos et al., 2006).

Plant sterols versus plant stanols

Several intervention trials have shown that plant sterols and stanols consumption efficiently lower LDL cholesterol levels. Yet it was always assumed that phytostanol intake was the more effective option, although reliable comparison was not possible as conducted studies varied a lot regarding the administered dose, intervention periods and analysing methods (Moreau et al., 2002). With the performance of the first side-by-side comparisons (Weststrate and Meijer, 1998; Hallikainen et al., 2000a; Jones et al., 2000; Normen et al., 2000; Noakes et al., 2002) no significant difference in the plasma cholesterol reducing properties of both sterols and stanols was found.

However, besides equal effectiveness of sterols and stanols in cholesterol reduction in the aforementioned short term studies, their effects in the quite limited number of long term studies (>1 year) doesn't seem to be that certain. While consumption of a stanol enriched margarine led to significantly different serum cholesterol levels compared to the control group even after one year of treatment (Miettinen et al., 1995), spreads fortified with sterols showed only little long term effects (Hendriks et al., 2003). As it has been observed that cholesterol reduction has to last for at least one year before positive effects on the patient's clinical manifestations of atheromatous arterial disease could be noted (Law et al., 1994), research on long term intervention studies has to be encouraged (Katan et al., 2003).

Low-fat versus high-fat formulations

As incorporation of plant sterols and stanols in high-fat products like margarine is contradictory to dietary recommendations for a healthier life style, the development of

low-fat and also not-fat alternatives was encouraged. In various studies (Mensink et al., 2002; Volpe et al., 2001; Nestel et al., 2001; Korpela et al., 2006) reduction of LDL-cholesterol (7-14 %) by low-fat applications was found to be comparable to that observed in investigations using high-fat spreads. Yet it has to be mentioned that the enriched low-fat products were consumed with meals. Already Doornbos et al. (2006) found cholesterol reduction by two yoghurt drinks (2.2 and 3.3 % fat) to be irrespective of the fat content, but a higher cholesterol lowering effect was found when the drink was consumed as part of a meal.

On the other hand Jones et al. (2003) couldn't find reductions in total and LDL-cholesterol using low-fat and non-fat beverages enriched with unesterfied phytosterols. The authors concluded that solubility of the added compound is important for optimal efficacy.

Similar Clifton et al. (Clifton et al., 2004) testing four types of phytosterol ester-enriched low-fat food (bread, breakfast cereal, milk and yoghurt) found that they were all able to reduce cholesterol levels, low-fat milk, however, was almost three times more effective than bread or cereals. These findings may be due to phytosterol incorporation into the milk globule membrane, thus being easier available for the transport into micelles.

It seems that for achieving the best possible action in cholesterol metabolism an accurate solubilisation in the food vehicle (independent of the fat content) is crucial.

Impact of background diet

The cholesterol lowering effect of plant sterols and stanols was found to be independent of the various background diets. As they reduce both bilary and dietary cholesterol absorption, this finding doesn't really seem surprising (Berger et al., 2004). However, also lack of efficiency when combined with a low-cholesterol diet was reported (van Heyningen, 1999). Yet observed reduced activity may be due to solubility problems as sitosterol was administered in its unesterified, crystalline form.

Since plant sterol and stanol enrichment lower cholesterol levels also in people not eating healthy diet, a certain risk of neglecting other recommendations for the prevention of CHD such as enhanced intake of dietary fiber, essential fatty acids, fruits and vegetables does exist.

2.1.5.4 Mechanism of action

Dietary intake of plant sterols has shown to lower serum total cholesterol levels and LDL-cholesterol, without altering high density lipoprotein (HDL) cholesterol or triglycerides (Katan et al., 2003). Absorption of both dietary and endogenously synthesised cholesterol is decreased. Reduced absorption of cholesterol leads to suppressed feedback-regulation of enterohepatic cholesterol circulation, hence increasing the endogenous cholesterol synthesis. The net result is, however, a decreased serum cholesterol concentration. The exact mechanism is not yet completely understood, but several theories are being discussed.

Co-crystallization of cholesterol and plant sterols

Through co-crystallization of cholesterol and plant sterols in the gastrointestinal tract poorly absorbable mixed crystals are formed, leading to a reduced intestinal uptake of cholesterol. A limited number of studies analysing this co-precipitation have confirmed this theory (Christiansen et al., 2001a), information on crystal formation in triacylglycerol oil or under *in vivo* conditions is, however, lacking (Trautwein et al., 2003).

Further for plant stanols, which proved to be as efficient as phytosterols in lowering cholesterol levels, no crystal formation was observed. Hence, reduced absorption may not solely depend on the formation of poorly soluble precipitations.

Competition for incorporation into mixed micelles

Cholesterol as virtually water insoluble compound has to be incorporated in mixed micelles for its transfer through the brush border membranes into the enterocyte. Also phytosterols require the micelle mediated transport for their absorption and as more hydrophobic compounds they even have a higher affinity to the micells (Ling and Jones, 1995). Therefore it is believed that increased uptake of plant sterols leads to a reduced capacity to incorporate cholesterol.

The fact that the cholesterol lowering effect showed to be the same regardless whether the whole dose of phytosterols was consumed at once or split into 3 doses over the day supports the assumption that several mechanism are involved in the hypocholesterolemic process.

Competition between cholesterol and plant sterols for esterase activity

Before being absorbed cholesterol esters have to be hydrolysed by the pancreatic cholesterol-esterase. Due to their structural similarity also plant sterol esters could serve as substrates. This may lead to a reduction of cholesterol in its absorbable form or in case that the esterase activity is not sufficient enough remaining cholesterol esters could be transferred to more distal parts of the intestine, where absorption is not as efficient (Trautwein et al., 2003).

Competition between cholesterol and plant sterols for cholesterol transporters

It is assumed that uptake of sterols is facilitated by transporters as the Niemann Pick like 1 protein in the intestinal brush border membrane. Due to their similar structure plant sterols instead of cholesterol could be transported into the enterocyte, reducing the uptake of cholesterol.

Plant sterols have been shown to upregulate ABCA1 gene expression, thus increasing the efflux of unesterified cholesterol back into the lumen. ABCA1 expression has been shown to be controlled by receptors like the liver X receptor (LXR) or the retinoid X receptor (RXR), to which plant sterols may act as ligands (Trautwein et al., 2003).

However, further research on cholesterol transporters is necessary before the influence of plant sterols can be elucidated.

Inhibition of the ACAT activity by plant sterols

Once absorbed in the mucosal cell cholesterol is re-esterified by the intestinal ACAT. It has been shown that mainly esterified cholesterol is incorporated into chylomicrones, therefore the esterification step seems to be essential for cholesterol absorption (Salo et al., 2003). Esterification of plant sterols has been shown to occur to a lesser extent, causing suppressed activity of ACAT. As a possible consequence the uptake of cholesterol is reduced due to the higher concentration of intracellular free cholesterol. On the other hand in rabbits an enhanced induction of ACAT gene expression stimulated through the increasing concentration of free cholesterol has been observed (Trautwein et al., 2003).

Competition for the incorporation into chylomicrones

As cholesterol and plant sterols have to be incorporated into chylomicrones to be transported from the enterocytes into the lymph a competition between both sterols might take place. It seems that the incorporation depends mainly on the esterification of the sterols as 70-80% of the transported cholesterol is present in its esterified form (Ikeda et al., 1988). As discussed above plant sterols are esterified to a far lesser extent than cholesterol. Therefore their ratio in chylomicrones is low. Up to now no studies exist suggesting that plant sterols in the cytoplasm could alter the uptake of cholesterolesters in chylomicrones. Also in patients with sitosterolemia plant sterol concentration in the lymph is lower than that of cholesterol (Salen et al., 1992). It seems that a decreased rate of cholesterol in chylomicrones could be only due to a lower absorption of cholesterol from the lumen.

2.1.6 Safety

Plant sterols and stanols have been in use as functional food components for many years. To date no distinct adverse health effects have been observed (Katan et al., 2003; Ling and Jones, 1995). Long term experience, however, is lacking.

Hepburn and co-workers have performed a comprehensive safety evaluation of plant sterol and stanol esters. Although some earlier studies suggested their estrogenic effect, plant sterols were not found to bind to estrogen receptors *in vitro* using a recombinant yeast strain. In addition no indication for estrogenicity was observed by oral administration of up to 500 mg/kg body weight/day to immature female rats (Baker et al., 1999). In a two-generation reproduction study an intake of up to 8.1 % plant sterols esters in the diet (w/w) showed no effect on the development of pups, on reproduction and on sexual maturation. Also in a further 90 days feeding study on subchronic toxicity with rats using same plant sterol ester doses no treatment related changes were noticed. Therefore the highest applied dose of 8.1 % plant sterols esters in the diet (w/w) corresponding to 6 g/kg body weight/day was determined as no-observed-adverse-effect level (NOAEL). In a later study of Kim et al. (2002) an oral dose of 9 g/kg body weight/day of plant sterol esters led to decreased body weight gain in both sexes and

increased incidence of cardiomyopathy in male rats and was therefore considered as the absolute toxic dose.

Plant sterols and their esters showed no genotoxic activity in various *in vitro* assays (bacterial mutation assay, chromosome aberration assay in human peripheral blood lymphocytes and mammalian cell gene mutation assay in L5178Y mouse lymphoma cells) and *in vivo* assays (rat bone marrow micronucleus assay and unscheduled DNA synthesis in rat liver) (Wolfreys and Hepburn, 2002).

Similar results were obtained for stanol esters. No evidence for genotoxicity, estrogenic activity or adverse effects on reproduction were found in several toxicity studies (Turnbull et al., 1999b; Turnbull et al., 1999a; Whittaker et al., 1999). Subchronic ingestion of stanol esters at levels up to 5 %, however, resulted in a decrease in liver weight, plasma levels of vitamin K1, E and D and hepatic levels of vitamin E and D in Wistar rats (Turnbull et al., 1999c).

High amounts of phytosterol ester intake (8.6 g/day) were also investigated in humans. No effects on gut microflora and serum sex hormone levels in females were found (Ayesh et al., 1999). The significant reduction of progesterone levels in female subjects was considered to be of no importance (De Jong et al., 2004).
So far only a few studies exist investigating the effects of high intake levels of phytosterols in humans. Usually no endpoints other than the reduction of cholesterol and fat soluble vitamins were included. Therefore to date detailed clinical information in humans on a daily consumption exceeding 8.6 g is lacking (Brufau et al., 2008).
Based on the currently available data the European Food Safety Authority (EFSA) concluded that an intake of more than 3 g per day of added phytosterols should be avoided (EFSA, 2008).

2.1.6.1 Possible negative side effects

Several studies with animals, cell models and humans did not show any adverse effects of plant sterols and stanols. However, there exist some aspects that may require further attention.

2.1.6.1.1 Effect on plasma levels of fat soluble vitamins

Besides their cholesterol lowering effect, the uptake of plant sterols and stanols also lead to a reduction in plasma concentrations of lipophilic hydrocarbon carotenoids. In a metaanalysis of 18 trails testing the impact of 1.5 g/d or more of plant sterols and stanols on the plasma levels of fat soluble vitamins a reduction of α-carotene by 9 %, β-carotene by 28 % and lycopene by 7 % was found. As LDLs act as carrier molecules for carotenes, adjustment for their decrease after plant sterol or stanol consumption has to be done. After correction a statistically significant reduction in the plasma concentration was found for β-carotene (-12.1 %) only (Katan et al., 2003).

As low levels of β-carotenoids have been associated with health risks such as cardiovascular disease and cancer, questions arose concerning a consequently higher potential risk of chronic diseases due to the lower amount of circulating lipid soluble antioxidants (Katan et al., 2003).
However, observed β-carotene reduction remained within the seasonal changes noted in individuals (Ntanios and Duchateau, 2002).
In 2000 the Scientific Committee on Food (SCF) concluded that no β-carotenoid fortification of phytosterol enriched products is necessary, but recommended the use of natural β-carotenoids sources to compensate the undesirable effects. For people with a higher requirement for vitamin A, as for example pregnant or lactating women and young children, β-carotene reduction should be considered with more caution (SCF, 2000).

2.1.6.1.2 Effects on membrane properties

Plant sterols and stanols are easily incorporated into membranes, thus increasing membrane rigidity (De Jong et al., 2003). In stroke-prone spontaneously hypertensive rats high levels of plant sterols were shown to shorten life span, presumably due to the replacement of cholesterol in membranes (Ratnayake et al., 2000). Similar results were found for plant stanols, although different mechanisms seem to be responsible (Ratnayake et al., 2003). Whether these findings are relevant in humans as well has to be further explored. However, in patients with sitosterolemia episodes of hemolysis have been reported (Moghadasian, 2000). Conversely, De Jong et al. (2006) found no

modification of the osmotic fragility of erythrocytes after 16 weeks of phytosterol and -stanol treatment in patients receiving statin therapy.

2.1.6.1.3 Increasing serum levels of plant sterols

Numerous intervention trails in humans have shown an increase in serum levels of plant sterols by 34 to 73 % after an intake of 1.5-3 g plant sterols per day, while plant stanols reduced both cholesterol and plant sterol serum levels but increased serum concentrations of stanols (Hallikainen et al., 2000a; Weststrate and Meijer, 1998; Fransen et al., 2007). The effects of this increase have not been elucidated yet.

However, the rare disease sitosterolemia (less than 100 have been reported in literature worldwide (Lee et al., 2001)), which induces high serum levels of plant sterols mostly in absence of hypercholesterolemia and leads to premature atherosclerosis, suggests plant sterols to be an additional risk factor for coronary heart disease (Katan et al., 2003). The fact that also patients with a positive family history for coronary heart disease were found to have increased serum levels of plant sterols supports this hypothesis (Jessup et al., 2008). Recently Weingärtner et al. (2008) found a significant correlation of plasma sitosterol and campesterol levels to their concentrations in tissue and aortic valve cusps in humans. Further an increase in plant sterol concentrations was also reported for people on statin medication.

Numerous studies on a potential atherosclerotic effect of plant sterols have also been done in animal models. Very recently Weingärtner et al. (2008) found that an increase in serum plant sterol concentration worsens arterial function in normal and apolipoprotein-E-deficient mice. Yet animal studies demonstrating a reduction of atherosclerotic plaque formation have been published as well (Brufau et al., 2008). However, due to differences in the sterol metabolism of rodents and humans, these animal species may not present optimal models for the evaluation of possible negative effects of increased serum phytosterol levels (Jessup et al., 2008).

Thus, the role of plant sterols in the progress of atherosclerosis is not known. More treatment studies including cardiovascular end points are necessary, especially since a combined intake of statins and enriched food products is suggested to improve cholesterol reduction.

2.1.7 Impact of increasing levels of plant sterols and stanols in nutrition, long term efficiency

After the introduction of their first phytosterol ester enriched product, Unilever started a post –launch monitoring programme, in order to elucidate among others if the product is used as recommended. There the authors concluded that the intake levels were even less than expected (Lea and Hepburn, 2006). However, although evidence existed that besides the phytosterol ester enriched margarine also stanol spreads and other enriched food were consumed only intake data of the Unilever product were included in the calculation.

As the spectrum of plant sterol and stanol enriched food is further increasing, concern about the extent of their eventual intake is emerging. The recommended daily dose of 1.5-3 g alone leads to a consumption of 8-12 times the amount obtained of natural sources (SCF, 2000). Two investigations of De Jong et al. (2004) and Raulio et al. (2001) estimated the extent of plant sterols and stanols intake in case more than one enriched product is consumed simultaneously. Both found similar results: replacement of 3-4 conventional products by their sterol enriched forms easily led to intake levels of more than 4 g/day in men and women. For men intakes of up to 9 g/d were noted as well.

Further, although plant sterols and stanols seem equally effective in reducing cholesterol levels in short-term studies, only plant stanols have been shown to maintain their efficiency over longer periods (O'Neill et al., 2005).
After a five year consumption of a phytosterol and -stanol enriched margarine total cholesterol levels had not significantly changed from baseline values (Wolfs et al., 2006). Intake of plant stanol fortified margarine for one year yielded to an average reduction of 14.4 % in LDL-cholesterol (Miettinen et al., 1995), whereas Hendriks et al. (2003) only found a slight reduction of 6 % in LDL levels after one year of sterol ester spread consumption. In women, however, long-term treatment with plant sterols was reported to be ineffective (Miettinen and Gylling, 2004).
As shown in a meta-analysis of Law et al. (1994) long term reduction (up to one year or longer) of cholesterol levels (10 %) is necessary in order to reduce the risk of ischaemic

heart disease. Based on the limited available data plant stanols seem to be the better option, as in addition to maintaining their cholesterol lowering ability they also reduce plant sterol levels. However, more long term investigations and head to head comparisons of plant sterols and stanols are of importance.
Further one should bear in mind that the link between lower cholesterol levels and a reduction in clinical manifestations of heart disease is based on the assumption that persons with a lower cholesterol level are in general people with a healthier life style. The use of plant sterol or stanol enriched food will neither change fat or energy content of the diet nor increase vegetable, fruit, fibre intake and daily exercise levels. Additional targeted education at least on the fortified products would be necessary (Thurnham, 1999).

2.1.8 Formation of phytosterol oxidation products

The increasing availability of products fortified with phytosterols rose questions concerning their stability and reactivity. Hereby their susceptibility towards oxidation is of main concern (Piironen et al., 2000). This is attributed to the presence of an unsaturated bond between C-5 and C-6 in the ring structure.

The most common oxygen species involved in the oxidation of sterols is triplet oxygen ($^3O_2$), which is in general rather unreactive due to its small electrostatic energy (Frankel, 1998). However, during processing and storage conditions sterol oxidation may be initiated by activating factors such as heat, light, air, metal ions and photosensitizers.

Extensive research has been done on the oxidation mechanism of cholesterol. Useful information therefore was gained from earlier investigations conducted on fatty acid oxidation. Although studies on the oxidation mechanism of plant sterols are scarce, due to their structural similarity, it is assumed that oxidation of other Δ^5-sterols than cholesterol follows the same reaction pathways (Piironen et al., 2000; Lercker and Rodriguez-Estrada, 2002).

Basically, sterols can be oxidized via the enzymatic and the non enzymatic pathway (Dutta, 2004), whereas the nonenzymatic pathway can further be divided into the free radical mechanism (i.e. autoxidation) and the nonradical mechanism (i.e. photo-oxidation) (Lercker and Rodriguez-Estrada, 2002). This chapter will mainly focus on the autoxidation pathway, as the most common oxidation mechanism for sterols.

Within the oxidation products two groups can be distinguished based on the polarity of the generated products (Dutta and Savage, 2002). The present work was done with oxidation products more polar than unoxidized sterols, therefore called polar oxidation products. The systematic and trivial names of the main polar secondary oxidation products of β-sitosterol are listed in table 2.3. Non-polar oxides, compounds less polar than unoxidized sterols, result from dehydration or dehydrogenation reactions occurring at high heating conditions.

Abbreviation	Trivial name	Systematic name
sitosterol	**sitosterol**	**(24R)-ethylcholest-5-en-3β-ol**
6α-OH-3-ketositosterol	6α-hydroxy-3-ketositosterol	(24R)-ethylcholest-4-en-3-one-6α-ol
6β-OH-3-ketositosterol	6β-hydroxy-3-ketositosterol	(24R)-ethylcholest-4-en-3-one-6β-ol
5α,6α-epoxysitosterol	sitosterol-5α,6α-epoxide	(24R)-5α,6α-epoxy-24-ethylcholestan-3β-ol
5β,6β-epoxysitosterol	sitosterol-5β,6β-epoxide	(24R)-5β,6β-epoxy-24-ethylcholestan-3β-ol
7-ketositosterol	7-ketositosterol	(24R)-ethylcholest-5-en-3β-ol-7-one
6β-OH-sitosterol	6β-hydroxysitosterol	(24R)-ethylcholest-5-en-3β,6β-diol
7α-OH-sitosterol	7α-hydroxysitosterol	(24R)-ethylcholest-5-en-3β,7α-diol
7β-OH-sitosterol	7β-hydroxysitosterol	(24R)-ethylcholest-5-en-3β,7β-diol
sitostanetriol	sitostanetriol	(24R)-ethylcholestan-3β, 5α,6β-triol

Tab 2.3 Trivial and systemaic names and commonly used abbreviations of the main secondary polar oxidation products of β-sitosterol.

2.1.8.1 Autoxidation

Autoxidation, a free radical chain reaction, is the most common oxidation mechanism for sterols. It has been reported to follow the same pathway as monounsaturated fatty acids. The first free radical is formed by the abstraction of an allylic hydrogen form the double bond between C-5 and C-6 in the ring structure. Basically both C-4 and C-7 seem to be equally susceptible to oxidative attack, allowing the reaction of molecular oxygen at the position 4, 5, 6 or 7. Yet abstraction occurs predominantly at C-7, since C-4 is stabilized by the hydroxyl group at C-3 and the tertiary C-5 (Smith, 1981).

The free radical formed then reacts with molecular triplet oxygen, which leads to the formation of 7-peroxyl radicals. These radicals are stabilized by hydrogen abstraction,

forming the more stable 7-hydroperoxides (7-OOH) as primary oxidation products (Dutta, 2004; Tai et al., 1999; Lercker and Rodriguez-Estrada, 2002). 7α-OOH and 7β-OOH further decompose to the more stable 7-hydroxysterols (7α-OH and 7β-OH). 7-ketosterol, the most abundant oxidation product, is formed either through dehydration of 7-OOH or dehydrogenation of 7-OH compounds.

Another major oxidation route is the formation of epoxy-compounds, 5α,6α- and 5β,6β-sterols, by a bimolecular reaction between hydroperoxides and intact sterol molecules (Lercker and Rodriguez-Estrada, 2002). It has been noted that the generation of the 5β,6β-epimer is more favoured than 5α,6α-sterol. This may be explained by the steric hindrance of the hydroxyl-group at C-3 (Lercker and Rodriguez-Estrada, 2002). The hydration of both epimers of 5,6-epoxysterol in acidic environment leads to the formation of the same compound, 3β, 5α, 6β-triol.

Also side chain oxidation products can be formed by oxygen attack of the tertiary carbons in the lateral chains. Degradation of the primary formed hydroperoxides results in the formation of the more stable hydroxides. In heating studies with plant sterols 24-OH- and 25-OH-sterols were detected (Johnsson and Dutta, 2003; Johnsson et al., 2003). In the case of cholesterol also 20-OH- and 26-OH-compounds were found (Lercker and Rodriguez-Estrada, 2002). For cholesterol, oxidation of the side chain was reported to occur only in solid state or crystalline form, not in solution or dispersion. Further it seems to be far less pronounced than oxidation at C-7. However, research of plant sterol side chain oxidation products is rather limited (Dutta, 2004; Tai et al., 1999).
The formation pathways of some β-sitosterol oxides are summarised in figure 2.3.

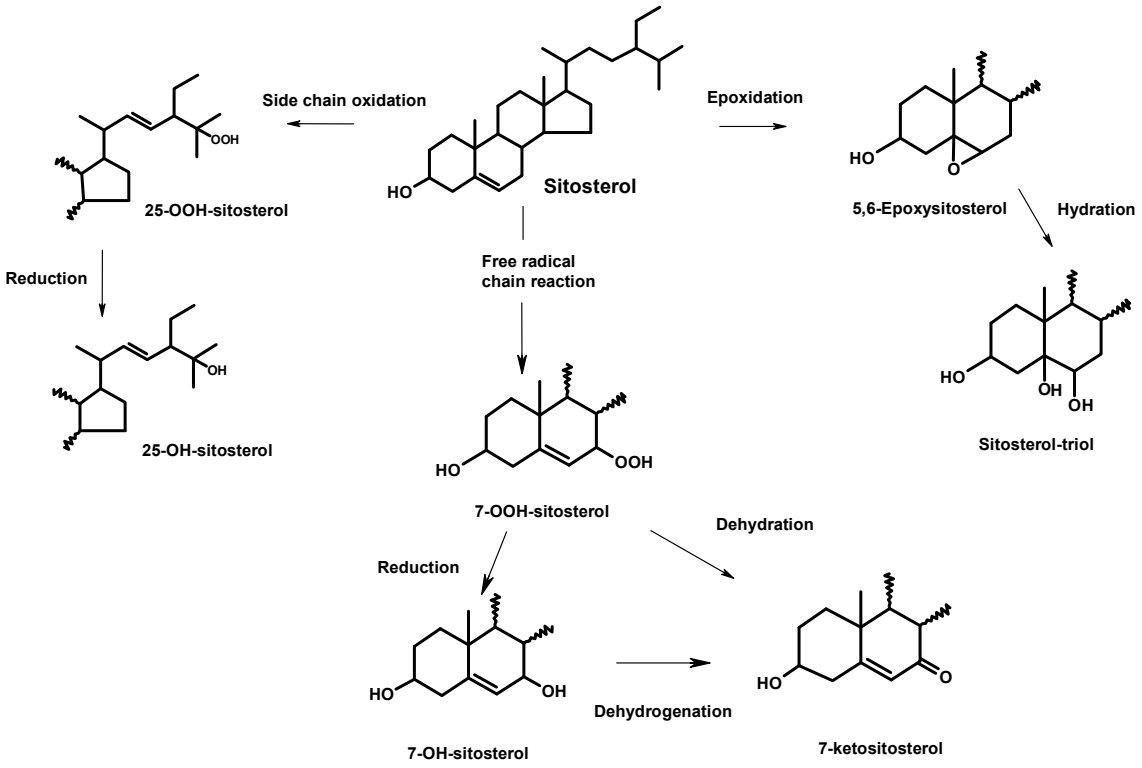


Fig 2.3 Autoxidation pathways of some β-sitosterol oxidation products (adapted from Tai et al. (1999)).

2.1.8.2 Photo-oxidation

In the photo-oxidation pathway singlet oxygen ($^1O_2$) instead of triplet oxygen ($^3O_2$) initiates the oxidation process. Photosensitizers (e.g. chlorophyll) absorb energy as radiation and transfer it to triplet oxygen to form singlet oxygen (Dutta, 2004; Min and Boff, 2002).

Information on photo-oxidation of plant sterol oxides is rather scarce. However, it was already shown that the oxidation behaviour of plant sterols is similar to that of cholesterol (Bortolomeazzi et al., 1999; Säynäjoki et al., 2003).

Singlet oxygen is highly reactive and can rapidly attack the double bond in ring B of sterols. This leads to the generation of mainly 5α-OOH-sterol with lower quantities of 6α-OOH- and 6β-OOH-sterols. In non polar solvents 5α-OOH-sterol can be rearranged

to 7α-OOH-sterol which can further epimerize to 7α-OOH-sterol. The fact that the oxidation products formed by photo-oxidation differ qualitatively and quantitatively from those generated by autoxidation facilitates the identification of the occurring process (Lercker and Rodriguez-Estrada, 2002; Tai et al., 1999).

2.1.8.3 Oxidation behaviour of sterols

In general, sterols are rather stable molecules when heated in pure state. Osada et al. (1993) investigated the stability of cholesterol during heating and nearly no oxidation products could be detected after 24 hours at a temperature of 100 °C. This behaviour changed at heating conditions of 120 °C and above. When cholesterol was heated at 200 °C total degradation was observed already after 6 hours of heating.

Research on the oxidation behaviour of plant sterols has not been as extensive as that of cholesterol. However, the results obtained from the limited studies conducted on phytosterols support the assumption that observed reaction mechanisms could be valid for both cholesterol and plant sterols.

Recently Zhang et al. (2005b) found similar results as Osada et al. (1993) for β-sitosterol. After 30 min of heating at 100 °C no oxidation products could be found, whereas at temperatures of 150 and 200 °C all common secondary oxides, as 7α-hydroxy, 7β-hydroxy, 5α,6α-epoxy, 5β,6β-epoxy, and 7-ketositosterol, were formed.

Johnsson and Dutta (2003; 2005) also detected side chain oxidation products, 24-hydroxy- and 25-hydroxy- compounds, triols as well as 6α-OH-3-keto- and 6β-OH-3-ketosterol, oxides with medium polarity, after heat treatment of stigmasterol and a mixture of campesterol/sitosterol for 72 hours at 120 °C. Further oxidation products found include 6-hydroxy derivates, 6-keto derivates and 4-hydroxy-derivates (Grandgirard et al., 2004c). Formation of the various oxidation products were highly influenced by the oxidation conditions applied. Although always the same kind of oxidation products were formed, irrespective of the different heating temperatures, the amount of the single products differed (Kemmo et al., 2005).

In contrast to oxidation studies carried out on pure sterols, in food numerous other factors such as the presence of lipids and water could affect the oxidation mechanism (Chien et al., 1998).

Lampi et al. (2002) investigated the oxidative stability of phytosterols in rapeseed oil or tripalmitin at various temperatures. While at heat treatments of 80 °C sterols showed to be rather stable, significant losses could be observed at 120 °C and above. When phytosterols were heated in different oils a greater variety of oxidation products was found at lower temperatures (100 °C and 150 °C) than after heating at 180 °C. At temperatures of 150 °C and 180 °C degradation of sterols occurred (Oehrl et al., 2001).
Conflicting results were reported on the influence of the lipid matrix. On the one hand plant sterols seemed to be more stable in rapeseed oil than in tripalmitin when heated at 180 °C (Lampi et al., 2002). On the other hand a greater amount of sterol losses was observed for sterols heated in canola and soybean oil instead of coconut and peanut oil, thus a higher ratio of polyunsaturated fatty acids could be linked to higher levels of oxidation (Oehrl et al., 2001). Recently Soupas et al. (2004a) found plant sterols to be more stable in unsaturated lipid matrices when heated at high temperatures, whereas at temperatures of 140 °C and beneath the situation seemed to be reverse.

2.1.9 Content of POPs in food

At the moment only little information is available on the content of plant sterol oxides in various food products. The lack of commercial standards, the great number of compounds with very similar structures and the fact that sterol oxides in food usually represent only trace components in a huge complex of interfering matrix compounds account for the complexity of the analysis of POPs (Guardiola et al., 2004). In contrast to food of animal origin containing only cholesterol oxides the number of different oxidation products found in plant based food is at least 3 times higher (Dutta, 2002), thus analysis is even more challenging.
To date small amounts of plant sterol oxides have been detected in various food, including wheat flour (Nourooz-Zadeh and Appelqvist, 1992), potato chips, potato crisps and French fries fried in different oils as well as commercial samples (Dutta and Appelqvist, 1997; Dutta, 1997; Tabee et al., 2008a; Tabee et al., 2008b), a range of plant oils (Dutta, 1997; Bortolomeazzi et al., 2003; Zhang et al., 2005b; Johnsson and Dutta, 2006) plant sterol enriched and non-enriched spreads (Grandgirard et al., 2004c; Conchillo et al., 2005; Johnsson and Dutta, 2006), enriched milk and milk powder

(Soupas et al., 2006) and liquid infant food (García-Llatas et al., 2008) (for detailed information see table 2.4) .

The main oxides detected were 7-OH and 7-keto derivatives, followed by 5,6-epoxy and triol compounds. In general detected plant sterol oxides resemble those commonly found in the analysis of COPs. Oxidation products of β-sitosterol dominated, but those of campesterol and brassicasterol were found as well.

Total amounts of the quantified plant oxides seem to be low and not all the tested food may represent an important source of oxidation products. The increase in fortified products could, however, change this situation. Grandgirard et al. (2004c) found 68 µg plant sterol oxides /g enriched spread. In order to achieve the recommended daily dose of 1.5-3 g of plant sterols, an intake of 19-37 g of spread would be necessary, containing at the same time 1.3-2.6 mg of oxidation products. That is an amount quite close to the reported daily consumption of 2-4 mg of cholesterol oxides per day (Grandgirard, 2002; Dutta, 1999).

Food source	treatment	total amount of plant sterol oxides	Reference	quantified POPs	
				parent sterol	oxides
wheat flour	2 months old	35 µg/g the lipids	Nourooz-Zadeh and	β-sitosterol	5,6-epoxy epimeres,
	storage (36 months)	328 µg/g lipids	Appelqvist, 1992		7-OH epimeres
potatoe chips fried in palm oil	fresh	5 µg/g in lipids	Dutta and	β-sitosterol,	5,6-epoxy epimeres,
	storage (10 weeks)	6 µg/g in lipids	Appelqvist, 1997	campesterol	7-OH epimeres,
	storage (25 weeks)	9 µg/g in lipids			triols,
potatoe chips fried in sunflower oil	fresh	6 µg/g in lipids			7-keto compounds
	storage (10 weeks)	50 µg/g in lipids			
	storage (25 weeks)	47 µg/g in lipids			
potatoe chips fried in high-oleic sunflower oil	fresh	35 µg/g in lipids			
	storage (10 weeks)	55 µg/g in lipids			
	storage (25 weeks)	59 µg/g in lipids			
rapeseed oil/palm oil blend	untreated	41 µg/g oil	Dutta, 1997	β-sitosterol,	5,6-epoxy epimeres,
		60 µg/g oil		campesterol,	7-OH epimeres,
sunflower oil	untreated	40 µg/g oil		stigmasterol	triols,
	frying, 2 days	57 µg/g oil			7-keto compounds
high-oleic sunflower oil	untreated	46 µg/g oil			
	frying, 2 days	56 µg/g oil			
french fries fried in rapeseed oil/palm oil blend	200°C, 15 min	2.4 µg/g sample (32 µg/g lipid)			
french fries fried in sunflower oil	200°C, 15 min	2.8 µg/g sample (37 µg/g lipid)			
french fries fried in high-oleic sunflower oil	200°C, 15 min	4.0 µg/g sample (54 µg/g lipid)			
sunflower oil		4.5 - 67.5 µg/g oil	Bortolomeazzi	β-sitosterol,	7-OH epimeres,
maize oil		4.1 - 60.1 µg/g oil	et al., 2003	campesterol,	7-keto sitosterol
peanut oils		2.7 and 9.6 µg/g oil		stigmasterol	

Food source	treatment	total amount of plant sterol oxides	Reference	quantified POPs	
				parent sterol	oxides
palm nut oil		5.5 µg/g oil			
lampante olive oils		1.5 - 2.5 µg/g oil			
enriched spread (8% plant sterols)		68µg/g spread	Grandgirard et al., 2004	β-sitosterol, campesterol, stigmasterol	5,6-epoxy epimeres, 7-OH epimeres, triols 7-keto + 6-keto compounds, 6β-OH + 4β-OH derivatives
enriched spread (6% plant sterols)		46 µg/g sample	Conchillo et al., 2005	β-sitosterol, campesterol, stigmasterol	7-OH epimeres, 7-keto compounds, 5,6-α-epoxid
nonenriched spread (0.3% plant sterols)		13 µg/g sample			
sunflower oil	no treatment	50.5 µg/g oil	Zhang et al., 2005	β-sitosterol,	5,6-epoxy epimeres, 7-OH epimeres, triols, 7-keto compounds
	150°C, 60 min	241 µg/g oil			
	200°C, 60 min	815 µg/g oil			
oilve oil	no treatment	-			
	150°C, 60 min	37 µg/g oil			
	200°C, 60 min	365 µg/g oil			
olive oil	no treatment	7.7 µg/g oil	Johnsson and Dutta, 2006	β-sitosterol, campesterol, stigmasterol	5,6-epoxy epimeres, 7-OH epimeres, triols, 24-OH, 7-keto compounds
	180°C, 60 min	10.2 µg/g oil			
	180°C, 120 min	17.6 µg/g oil			
peanut oil	no treatment	7.1 µg/g oil			
	180°C, 60 min	5.4 µg/g oil			
	180°C, 120 min	6.8 µg/g oil			
maize oil	no treatment	4.3 µg/g oil			
	180°C, 60 min	12.4 µg/g oil			

Food source	treatment	total amount of plant sterol oxides	Reference	quantified POPs	
				parent sterol	oxides
enriched spread	180°C, 120 min	12.2 µg/g oil 12 µg/g spread			
milk powder (enriched with 7% plant sterols)	no treatment	14 µg/g sample	Soupas et al., 2006	β-sitosterol	7-OH epimeres
	storage (12 months, 22°C)	19 µg/g sample			
	storage (12 months, 38°C)	34 µg/g sample			
milk (enriched with 0.4% sitosterol)	127°C, 2s	2 µg/g sample			
milk (enriched with 0.4% sitosterol)	127°C, 2s	2 µg/g sample			
milk (enriched with 0.4% sitosterol)	127°C, 2s	0.2 µg/g sample			
potatoe chrisps (16 commercial samples)		0.5 - 6.8 µg/g sample	Tabee et al., 2008	β-sitosterol, campesterol, stigmasterol	5,6-epoxy epimeres, 7-OH epimeres, 7-keto compounds
French Fries (5 different commercial prefried		0.1 - 2.1 µg/g sample	Tabee et al., 2008	β-sitosterol, campesterol,	7-OH epimeres, triols,
French Fries (4 samples of fast food restaurants)		1.5 - 8.1 µg/g sample		stigmasterol	7-keto compounds
liquid infant food with fruits	fresh	0.7 µg/g sample	García-Llatas et al., 2008	β-sitosterol, campesterol, stigmasterol	5,6-epoxy epimeres, 7-OH epimeres, triols, 7-keto compounds
	storage (9 months)	0.6 µg/g sample			
liquid infant food with honey	fresh	0.64 µg/g sample			
	storage (9 months)	0.57 µg/g sample			

Tab 2.4 Overview of published studies on the content of plant sterol oxides in food.

2.1.10 Absorption and metabolism of phytosterol oxidation products

Information on the absorption mechanism and plasma levels of plant sterol oxidation products is still quite limited.

Grandgirard et al. (1999b) investigated the lymphatic absorption of β-sitosterol and campesterol oxides (7-keto and 5, 6-epoxid derivates) in male Wistar rats. Absorption of 7-ketositosterol (1.5 %) and -campesterol (2.9 %) was quite low, although absorption rates of 7-ketositosterol were not significantly different from those of its parent sterol. For 5, 6-epoxy compounds a higher absorption rate (4.7 %) was found, whereas 5β,6β-epoxysterols were more efficiently absorbed than 5α,6α-epoxysterols. In addition also stigmasteroltriol was detected in the lymph, although it hadn't been administered to the animals, which indicates a possible in-vivo transformation of epoxides to triols.

Corresponding results were found when the lymphatic absorption of 7α-OH, 7β-OH, 5α,6α -epoxy, 5β,6β -epoxy, triols and 7-keto compounds of β-sitosterol or campesterol was analysed in Sprague-Dawley rats. Highest recovery was observed for 7-hydroxy derivates. Absorption rates of β-sitosterol oxides were lower than those of corresponding campesterol oxides. However, recovery of all oxidation products was higher than that of their parent sterols (Tomoyori et al., 2004).

A dose dependency between the amount of oxysterols in the diet and their recovery in plasma, aorta, liver, kidneys and heart was found in Golden Syrian hamsters receiving a diet containing different levels (0.1, 0.5 or 0.25 % of the diet) of a mixture of β-sitosterol and campesterol oxides. 7β-OH, 5β,6β -epoxy, triols and 7-keto compounds were found in noticeable amounts in all tissues after feeding with 0.25 % of oxidation products. 7α-hydroxysitosterol and 5α,6α-epoxysitosterol, however, were only detected in the plasma, which could be due to a better metabolism of these two oxides. The transformation of 5α,6α-epoxysitosterols to triols is also possible. In animals fed a diet containing 0.1 % of an oxidation mixture only sitostanetriol was found. Equally to previous studies cited above the amount of campesterol oxides recovered in the plasma was higher than provided in the diet, whereas amounts of β-sitosterol oxides were approximately the same. As already observed for non-oxidized phytosterols the length of the side chain could be responsible for different absorption degrees (Grandgirard et al., 2004a).

Information on *in vivo* metabolism of phytosterol oxides is missing. However, some investigations on their *in vitro* formation have been conducted. Conversion of β-sitosterol to oxidized derivates (mainly 7α-OH, 7β-OH, 5,6-epoxide and triols) has been accomplished by the use of rat liver preparations (Aringer and Eneroth, 1973). Also the enzymatic oxidation of the side chain, probably induced by hydroxylases, has been determined. 26-OH and 29-OH products of β-sitosterol, sitostanol and campesterol were detected, whereas the hydroxylation of C-24 and C-25 seemed to be prevented by the additional ethyl or methyl group at C-24 (Hovenkamp et al., 2008).

Excretion of plant sterol oxidation products does most likely proceed via the bile, whereas different excretion rates according to different oxides structures are assumed. For example, sitostanetriols were found to be slowly eliminated from the organism (Grandgirard et al., 2004d). Besides comparable absorption rates, 7α-OH-phytosterols seemed to be faster metabolized than 7β-OH-sitosterol. Their transformation in bile acids, similar to what was observed for 7α-OH-cholesterols, is possible (Tomoyori et al., 2004; Hovenkamp et al., 2008).

Some studies on the plasma concentration of phytosterol oxides were conducted with humans. Grandgirard et al. (1999a) detected 7-ketositosterol, 5β,6β –epoxysitosterol and sitostanetriol in human plasma samples with a total concentration of 0.3 µg/mL.
High amounts of phytosterol oxides were found in plasma of a patient with phytosterolaemia. Approximately 1.4 % of the amount of β-sitosterol in plasma was found in its oxidized form (7-keto, 7β-OH, 5α,6α –epoxid and 5β,6β –epoxid). In the same study no oxidized plant sterols were detected in a pooled plasma sample of healthy volunteers, presumably due to the relatively high detection limit of the method applied (Plat et al., 2001).
On the contrary, oxyphytosterols in the range from 4.8 to 57.2 ng/mL were quantified in the plasma of 13 healthy volunteers (Grandgirard et al., 2004b). Oxides of β-sitosterol (7-keto, 5α,6α –epoxid, 5β,6β –epoxid and triol) dominated. However, compared to plasma levels of the main oxycholesterols (3-154 ng/mL) detected levels were comparable or lower (Hovenkamp et al., 2008).

2.1.11 Safety

In recent years extensive research has been done on cholesterol oxidation products. Numerous in vitro studies have proven their mutagenic, carcinogenic, angiotoxic, cytotoxic and atherogenic potential. Further, COPs may play part in the onset and development of atherosclerosis (Guardiola et al., 1996; Osada, 2002).
By contrast, data on phytosterol oxides are limited. Moreover a large part of the conducted studies was done with mixtures instead of single oxides, although different effects of mixtures and purified compounds have already been shown for COPs. However, due to their structural similarity with COPs, analogous functions and biological effects are expected.

Meyer et al. (1998) investigated the influence of different β-sitosterol, stigmasterol and cholesterol oxides on the mortality of meal worms (Tenebrio molitor). The effects of phytosterol oxides were comparable to those of cholesterol oxides, especially triols showed toxic effects. However, tested phytosterol oxidation products were by a factor of five less active than oxycholesterols.

As part of a comprehensive safety evaluation of plant sterols Lea et al. (2004) investigated a mixture of phytosterol oxides in a series of *in vitro* genotoxicity assays (bacterial mutation, chromosome aberration and micronucleus). No mutagenic effect on Salmonella typhimurium strains (TA98, TA100, TA102, TA1535 and TA1537) and no significant increase in chromosome aberrations or frequency of micronuclei were found. In addition, in a 90 days subchronic feeding study in Wistar rats (0.44-0.45% POPs in the diet) no evidence of a genotoxic potential was found.

Likewise no genotoxic effects were found when a mixture of β-sitosterol oxidation products were investigated in the single-cell gel electrophoresis - and the sister chromatid exchange assay using U937 cells (Maguire et al., 2003).

Very recently, an *in vivo* evaluation of the genotoxic potential of triol and epoxid compounds derived from β-sitosterol or campesterol using the micronucleus assay has

been conducted. In line with the previous mentioned investigations no significant effects were observed (Abramsson-Zetterberg et al., 2007).

For the evaluation of cytotoxicity different cell lines were used. The activity of oxide mixtures of β-sitosterol (Maguire et al., 2003) and β-sitosterol/campesterol (Adcox et al., 2001) was evaluated using a human monocytic blood cell line (U937) and a cultured-derived macrophage cell line (C57BL/6), respectively. In the former study reduced cell viability, incidence of apoptotic cell death and decreased glutathione levels were observed, in the latter study cell viability, mitochondria dehydrogenase activity, lactate dehydrogenase (LDH) leakage and protein content were determined. In both investigations similar cell damage to that caused by COPs was found, although in general higher concentrations of POPs were needed. Non oxidized sterols showed no cytotoxic effects. Further Magurie et al. (2003) assumed that isolated phytosterol oxidation products might behave differently to individual compounds.

Isolated oxidation products of β-sitosterol (7β-OH, 7-keto, sitostanetriol and a mixture of 5α, 6α-epoxide/5β, 6β-epoxide (6:1)) and their corresponding COPs were tested in a human monocytic cell line (U937), a colonic adenocarcinoma cell line (CaCo-2) and a hepatoma liver cell line (HepG2). While oxides caused apoptotic cell death in U937 cells, necrosis was detected in CaCo-2 and HepG2-cells. 7-keto- and 7β-OH-sitosterol were found to be the most cytotoxic compounds, when cell viability, apoptitic cell death and DNA fragmentation was assayed, while 5α,6α-epoxysitosterol showed no toxicity at all. 7β-OH-sitosterol was the only β-sitosterol oxide that caused glutathion depletion (Ryan et al., 2005). As already observed for oxide mixtures, toxicity caused by POPs is similar but less severe compared to the corresponding COPs.

Roussi et al. (2005) compared the antiproliferative effects of 7β-OH-sitosterol and 7β-OH-cholesterol. In contrast to Ryan et al. (2005) for both compounds apoptotic cell death was observed in CaCo-2 cells. However, different apoptotic mechanisms may be involved as 7β-OH-sitosterol enhanced caspase-3 and -9 activities and DNA fragmentation, whereas for 7β-OH-cholesterol no activation of caspase-3 and a delay in the activation of caspase-9 and DNA fragmentation was observed. In a further study

different modulators of apoptosis were assessed. Both 7β-OH-sitosterol and 7β-OH-cholesterol caused apoptosis by mitochondrial membrane permeabilization, independently of Bcl-2 or Bax alterations. Endonuclease G expression was enhanced after exposure to both sterol oxides, although for 7β-OH-cholesterol a certain delay was observed. Enhanced endonuclease G expression and enhanced production of reactive oxygen species were detected in 7β-OH-cholesterol treated cells only. 7β-OH-sitosterol was more potent in increasing lysosomal membrane integrity (Roussi et al., 2007).

2.1.12 Analysis of phytosterol oxidation products

In the past research on the oxidation of sterols concentrated on cholesterol while phytosterol oxides remained rather disregarded. Consequently, to date analysis of POPs is mostly based on methods developed for oxycholesterols, which mainly consist of extraction of total lipids of the sample material, saponification of lipids (preferably at room temperature), purification and enrichment of oxidation products and chromatographic analysis. In general, high temperatures, exposure to oxygen and light should be avoided as this would lead to artefact formation and further reactions of the sterol oxides (Piironen et al., 2000).

However, compared to the research of COPs, methods for the analysis of phytosterol oxides require higher selectivity and higher sensitivity, as the number of plant sterol oxides found in plants are 3 to 4 times higher than those of cholesterol oxidation products in animal based sources and therefore single oxides may be present in relatively low amounts (Dutta, 2002).

Another challenge in the POP-analysis is the lack of pure phytosterol standards. To date only Δ5-stigmasterol (purity: 95%) and β-sitosterol (purity: ≥ 97% or ≥ 95%) are commercially available, the latter one in very small amounts and at an excessively high price (Zhang et al., 2005a). Hence, POPs standards are also not available and have to be laboratory-prepared.

2.1.12.1 Formation of oxidation products

Applied methods are manifold and depend on the respective oxides to be obtained. Basically at the moment no validated, internationally accepted methods for the

preparation of oxidation products exist. Distinction may be drawn between formation of oxides by irradiation (Bortolomeazzi et al., 1999; Säynäjoki et al., 2003), by chemical synthesis (Zhang et al., 2005a; Geoffroy et al., 2008; Julien-David et al., 2008) and by thermo-oxidation, whereas in this case preparation can further be divided in thermo-oxidation in solid state (Daly et al., 1983; Lampi et al., 2002) or in aqueous dispersion (Dutta and Appelqvist, 1997; Conchillo et al., 2005).

2.1.12.2 Purification and Enrichment of oxidation products

Because of the small amount of oxides compared to the excess of native sterols and the complexity of the gained blend, cleanup and enrichment is essential before separation techniques to isolate single oxidation products are applied (Dutta, 2002). Thus co-elutions of oxidized and non oxidized material may be prevented.

In the sample clean up solid phase extraction (SPE) cartridges and preparative TLC are commonly employed (Guardiola et al., 2004; Piironen et al., 2000). For the purification of bigger sample amounts, column chromatography (CC) on alumina or silica is generally used (Dinan et al., 2001). Compared to SPE-columns self prepared glass columns offer a higher loading capacity. In both cases separation is achieved by a stepwise elution with increasing solvent polarity (Piironen et al., 2000). A mixture of n-hexane/diethylether has proven to be the most effective combination, whereas for the elution of the oxidation products containing fraction acetone is commonly employed (Ulberth and Buchgraber, 2002).

In TLC analysis polar eluent mixtures, n-heptane/ethyl ether and diethyl/cyclohexane, are generally used. Compared to SPE and CC, TLC offers the possibility to isolate less polar oxidation products as their own fraction (Piironen et al., 2000).

2.1.12.3 Separation, Identification and Quantification of oxidation products

2.1.12.3.1 Thin layer chromatography (TLC)

Besides HPLC and GC, TLC is frequently used in the analysis of sterol oxides, especially when a rapid procedure is favoured. For compounds with a higher polarity than non-oxidized sterols good resolution was observed. However, its application is

usually limited to the qualitative determination as quantitative results are not as reliable as other techniques (Lebovics, 2002). Identification is achieved by comparing the position of the sample spots with those of COP standard reference substances. Visualisation of the generated spots can easily be done by spraying with sulphuric acid and following heat treatment. Noteworthy to say that 7-ketosterols do not give a colour reaction, but can be detected using UV light (Lebovics, 2002).

Nevertheless, in the preparative analysis TLC is rather appropriate for the processing of small sample amounts. In addition to its low loading capacity it also allows long exposure of the sample to air, which facilitates the possibility of artefact formation. Further it involves laborious scraping steps (Dutta and Appelqvist, 1997; Csallany et al., 1989).

2.1.12.3.2 High pressure liquid chromatography

In the analysis of sterol oxides HPLC has become an alternative to GC methods. Due to its non destructive characteristic and its possibility to operate at room temperature HPLC is particularly suitable for the investigation of thermolabile compounds. Both normal- and reverse phase chromatography has already been successfully used for the analyses of cholesterol (Caboni et al., 1997; Chien et al., 1998; Mazalli et al., 2006) and plant sterol (Kemmo et al., 2007; Kemmo et al., 2005) oxidation products. In general normal phase chromatography is considered to be the more effective option. However, no full resolution over the entire polarity range of the oxidation products is possible under isocratic conditions (Maerker et al., 1988). In normal phase applications silica or cyano (CN) columns are most frequently used. As mobile phases binary systems consisting of usually heptane or hexane with varying percentages of polar modifiers, in most cases 2-propanol, are employed (Abidi, 2001).

Further HPLC can be performed either as preparative or analytical technique and can be coupled to a wide range of detectors. Among all detection systems available UV detection is still the most frequently applied for sterol analyses. In general single sterol oxidation products have their maximum UV absorption at different wavelengths (Caboni et al., 1997; Osada et al., 1999). However, wavelengths between 205-210 nm are those commonly employed for oxysterol analysis (Rodriguez-Estrada and Caboni, 2002). Compared to measuring the absorbance at one single wavelength photodiode

array detection (PDA) has the advantage to acquire a series of spectra over different UV wavelengths (Rodriguez-Estrada and Caboni, 2002). However, both UV and PDA detection possess limitations since products without double bonds as epoxy- epimers and triols do not respond to UV detection and are therefore not seen with this kind of detectors (Caboni et al., 1997; Moreau, 2005).

By the use of refractive index (RI) detectors (Chen and Chen, 1994), light scattering detectors (LSD)(Caboni et al., 1997) or evaporative light scattering detectors (ELSD) (Lakritz and Jones, 1997) also those compounds which are transparent to UV detection can be included in the measurement. RI detection is approximately 1000 times less sensitive than UV detection (Chen and Chen, 1994). Further it is not suitable for gradient elution systems (Rodriguez-Estrada and Caboni, 2002). Regarding the sensitivity LSD is similar to UV detection, except in the case of 7-ketocholesterol, where far higher detection limits were observed (Caboni et al., 1997). In recent years ELSD has become more popular in the lipid analysis. For detection the analyte has to be more volatile than the mobile phase, the detector response is based on mass. ELSD works with isocratic and gradient elution systems. It has been shown to be more sensitive than RI, but less sensitive than UV (Rodriguez-Estrada and Caboni, 2002; Abidi, 2001). In addition it shows a limited range of linear response (Lakritz and Jones, 1997).

Recently also HPLC-MS has become more common in the analysis of sterol oxides (Kemmo et al., 2007; Mazalli et al., 2006; Razzazi-Fazeli et al., 2000).

In HPLC analysis quantification with both internal and external standards is possible. Again COP reference solutions are commonly used in the POP analysis. In the more common external standard method the preparation of calibration curves for each oxide is necessary (Rodriguez-Estrada and Caboni, 2002). Similar to GC analysis 19-hydroxycholesterol can be used as internal standard (Caboni et al., 1997).

2.1.12.3.3 Gas chromatography

To date GC is still the most common method used for the determination of sterol oxides (Guardiola et al., 2002). It is normally coupled to a flame ionization detector (FID) but also to mass spectrometry (MS) (Abidi, 2001).

Due to the high boiling points of sterols and their oxides, it is necessary to apply elevated temperatures in GC methods. In order to avoid degradation and artefact formation oxysterols are usually converted to trimethylsilyl ethers (TMSE). For derivatisation combinations of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) (Johnsson and Dutta, 2006; Conchillo et al., 2005) or *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and TMCS (Lampi et al., 2002; Soupas et al., 2004b) are quite commonly used. Usually silulation is performed in pyridine at room temperature or accelerated at elevated temperatures. As water would compete with hydroxyl groups of sterol oxides an anhydrous condition is essential (Guardiola et al., 2004).

Compared to FID the use of MS for the detection of sterol oxides also allows the quantification and identification of overlapping peaks. Further its sensitivity was shown to be similar to or better than that of FID (Guardiola et al., 2004). Quantification of sterol oxidation products is generally done by the internal standard (ISTD) method. The use of 19-hydroxycholesterol has been proven to be the most effective option. As commercial standards of plant sterol oxidation products are not available, also in the analysis of phytosterol oxides oxycholesterols are used as standard compounds (Plat et al., 2001).

3. Material and Methods

3.1 Production and isolation of phytosterol oxidation products

3.1.1 Chemicals and Reagences

Substance	Supplier	Product number
24β-Ethylcholest-5-en-3β-ol	Fluka	85451
5-Cholesten-3β-ol	Sigma	S957623
Cholest-5-en-3β-ol-7-one	Sigma	C2394
Cholestan-5α,6α-epoxy-3β-ol	Sigma	C2773
Cholest-5-en-3β,7β-diol	Steraloids	C6430-000
5-Cholesten-3β,19-ol	Steraloids	C6470-000
Acetone	Rathburn Chemicals	
Bis(trimethylsilyl) trifluoroacetamide	Merck	110255
Diethyl ether	Merck	100921
Ethyl acetate	Merck	100868
n -Heptane	Rathburn Chemicals	
Methanol	Rathburn Chemicals	
2-Propanol	Rathburn Chemicals	
Pyridine	Fluka	82703
Sulphuric acid	Merck	112080
Trimethylchlorsilan	Fluka	92360

Tab 3.1 Chemicals and Reagences used for plant sterol oxide analysis

3.1.2 Equipment

Equipment	Supplier	Product number
Acrodisc Syringe Filters with GHP Membrane, 13 mm, 0.45 µm	PALL	4563
Silica gel 60 (0.2-0.5mm)	Merck	107733
TLC silica gel 60 aluminium sheets (0.5mm layer thickness)	Merck	105553

Tab 3.2 Equipment used for plant sterol oxide analysis

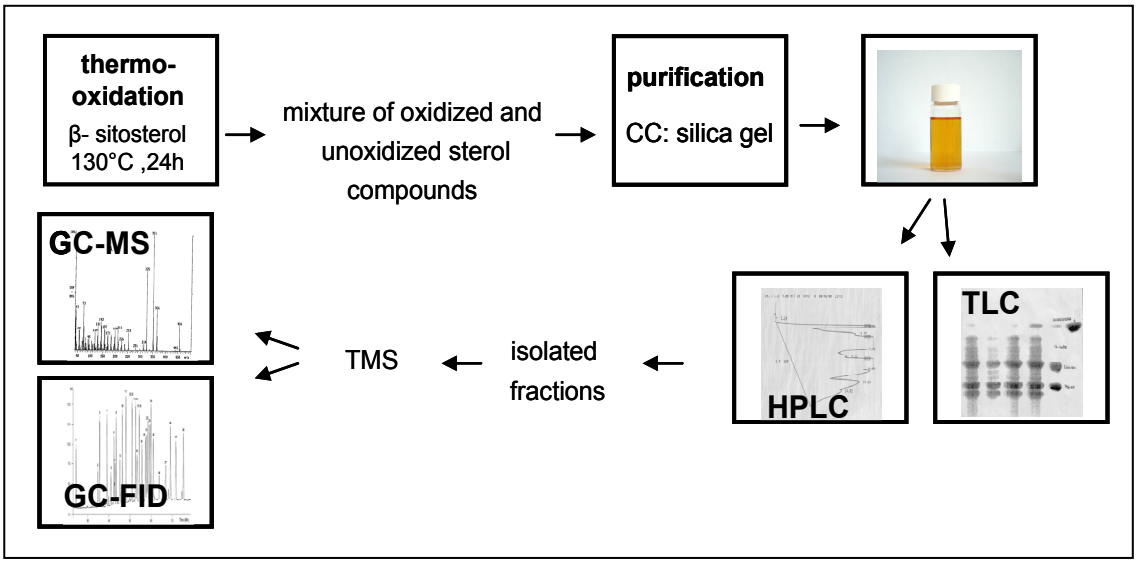


Fig 3.1 Schematic overview of β–sitosterol oxides production

3.1.3 Preparation of oxides by thermo-oxidation

Commercial available β–sitosterol was used for the generation of phytosterol oxidation products. First its composition was determined by GC-FID and GC-MS.

purity	%
β-sitosterol	76
sitostanol	13
campesterol	9.5
campestanol	1.5
rest	1

Tab 3.3 Composition of employed β–sitosterol

POPs were formed by thermo-oxidation. Commercial available β–sitosterol was heated in open glass vials (300 mg, 25 mm, I.D.) in a ventilated oven. After the heating period the samples were cooled down in a dessicator, dissolved in 15 mL of n-heptane/diethyl-ether (90/10, v/v), solubilised using a sonicator and finally stored in a freezer (- 20 °C).

To determine the optimal conditions different temperatures and heating periods (130 °C/24 h, 120 °C/48 h and 130 °C/48 h) were tested. Resulting oxidation mixtures were analysed using GC-FID and GC-MS.

3.1.4 Purification and enrichment of phytosterol oxidation products by column chromatography (CC)

The purification method used was based on former investigations by Lampi et al. (2002) and Apprich and Ulberth (2004).

72 g silica gel was dry packed into a glass column and pre-wetted with 150 mL n-heptane. All oxides obtained of 300 mg heated β–sitosterol and diluted in 15 mL n-heptane/diethyl-ether (90:10, v/v) were applied to the column. First nonoxidized sterols and apolar components were eluted with 150 mL n-heptane/diethyl-ether (90/10, v/v) followed by 450 mL n-heptane/diethyl-ether (50/50, v/v). Thereafter POPs were extracted with 150 mL acetone. The acetone fraction was evaporated to dryness. To get rid of the water ethanol was added during the evaporation step. Finally the residue was dissolved in 550 µL n-heptane/2-propanol (93/7, v/v).

The yield of oxidation products was increased when the silica gel was loaded with 10 % distilled water.

3.1.5 Confirmation of the purification by thin layer chromatography (TLC)

100 µL of the purified sample were applied to silica gel G 60 TLC plates. As eluent n-heptane/ethyl-acetat (50/50, v/v) was used. Components were visualized by spraying with 10 % sulphuric acid in methanol and a following heat treatment of a few minutes at 100 °C. The identification of the single oxidation products was conducted using a cholesterol oxides standard solution (5α,6α-epoxy-, 7-hydroxy- and 7-ketocholesterol).

3.1.6 Separation of single oxidation products by a NP-HPLC-UV system

For the collection of single oxidation products a preparative normal–phase HPLC-method was used. The method was based on papers published by Kemmo et al. (2005; Säynäjoki et al., 2003) with some modifications.

In pre-testes several mobile phase systems (90/10, 92/8, 93/7, 94/6, 95/5 and 97/3 n-heptane/2-propanol, v/v) and flow rates (5, 7, 9.9, 15 and 17 mL/min) were examined. For the first tests a semi-preparative silica column (25 cm x 10 mm, 5µm, Supelcosil) was used. Good seperation was achieved with a mobile phase of n-heptane/2-propanol (92/8, v/v) and a flow rate of 9.9 mL/min, but obtained yields of oxidation products were rather low. Therefore a preparative silica column (25 cm x 21.1 mm, 12µm, Supelcosil) was used for the following HPLC runs.

HPLC system	
Waters Delta Prep 3000, preparative HPLC - instrument (Walters, Milford, USA)	
Supelcosil silica column (25 cm x 21.1mm, 12µm) (Supelco, Bellefonte, PA, USA)	
Waters 484 UV detector (Walters, Milford, USA)	
HPLC conditions	
mobile phase	*n* -heptane/2-propanol (93/7, v/v)
flow rate	17 mL/min
injection volume	1.8 mL
detection	UV-detection, 206 nm

Tab 3.4 HPLC conditions used for the separation of POPs

The performance of the separation was checked daily using a cholesterol oxide standard solution (7-ketocholesterol, 7β-hydroxycholesterol and 7α-hydroxycholesterol) by monitoring the retention times.

3.1.7 Identification and quantification

For identification and quantification of the collected fractions GC-mass spectrometry (GC-MS) and GC-flame ionization detection (GC-FID) were used, respectively. Both methods have been developed earlier by co-workers (Lampi et al., 2002; Soupas et al., 2004b), are routinely used for analysing POPs and were implemented under supervision of the respective person responsible.

Prior to the GC analysis the samples were converted to TMS-ether derivatives. Therefore 100 µL aliquots of each fraction and 1 mL of internal standard solution (19-

OH-cholesterol, 18.55 µg/mL) were evaporated to dryness under nitrogen, dissolved in 100 µL of pyridine and subjected to silylation by 100 µL BSTFA/TMCS (100 µL, 99/1, v/v) over night at room temperature. The reagent mixture was then evaporated and the residue was dissolved in 200 µL n-heptane before GC analysis.

3.1.7.1 GC–MS analysis

For identification of the collected oxidation products and verification of the purity of the fractions GC–MS analysis was done as described in Soupas et al. (2004b).

GC-MS system	
Hewlett Packard 6890 Series gas chromatograph (Wilmington, PA, USA)	
Rtx-5MS w/ Integra fused-silica capillary column (60m x 0.25mm i.d., crossbond 5% diphenyl – 95% dimethyl polysiloxane, 0.1µm film with 10m Integra-Guard column; Restek, Bellefonte, PA, USA)	
Agilent 5973 mass spectrometer (Palo Alto, CA, USA)	
GC-MS conditions	
carrier gas	helium (>99.996%)
flow	1.2 mL/min (constant flow)
temperature programm	70°C (1 min)
	40°C/min to 280°C (kept for 35 min)
interface temperature	280°C
ion source	230°C
ionization	electron impact 70 eV
scan modus	full scan mode, m/z 100-600

Tab 3.5 GC-MS conditions used for the identification of POPs

3.1.7.2 GC–FID analysis

Quantification of the collected oxides was done by GC-FID as described by Lampi et al. (2002).

GC-FID system	
Hewlett Packard 6890 Series II gas chromatograph (Hewlett-Packard, Karlsruhe, Germany)	
HP-7673 autosampler (Hewlett-Packard, Karlsruhe, Germany)	
flame ionization detector (Hewlett-Packard, Karlsruhe, Germany)	
RTX-5w/ Integra fused-silica capillary column (crossbond 5% diphenyl - 95% dimethyl polysiloxane; with film thickness 0.10 µm, 60 m × 0.32 mm i.d.) (Restek, Bellefonte, PA, USA)	
GC-FID conditions	
carrier gas	helium (>99.996%)
flow	1.4 mL/min (constant flow)
temperature programm	70°C (kept for 1 min)
	60°C/min to 245°C (kept for 1 min)
	3°C/min to 275°C (kept for 41 min)
detector temperature	300°C

Tab 3.6 GC-FID conditions used for the quantification of POPs

3.2 Salmonella microsome assay

The Salmonella microsome assay (Salmonella test; Ames test) is a short-term bacterial reverse mutation assay and a well established marker for the detection of a wide range of chemical substances that can produce genetic damage thus leading to gene mutations. The principle behind this assay is that the used Salmonella typhimurium strains need the amino acid histidine as a growth factor, which is a consequence of a pre-existing mutation. By adding a mutagenic substance this pre-existing mutation can be reverted (Göggelmann, 1993). The intensity of the increase in bacterial growth is an indicator for the mutagenic potential of a tested substance (Mortelmans and Zeiger, 2000).

In this study the Salmonella strains TA98, TA100 and TA102 were used. TA98 gives an indication of frameshift mutations and TA100 gives information on base-pair substitutions. The strain TA102 was developed as a strain that, in contrast to the others, contain its mutant site at AT instead of GC base pairs. It is able to detect cross- linking agents and can be reverted by mutagens that cause oxidative damage (table 3.7). In

order to detect mutagens acting via different kind of mechanisms experiments including different tester strains are necessary (Mortelsmann & Zeiger, 2000).

Allele	Strains	DNA target	Reversion event
HisG46	TA 100	-G-G-G-	Base-pair substitution
HisD3052	TA 98	-C-G-C-G-C-G-C-G-	Frameshifts
HisG428	TA 102	-T-A-A-	Transitions/transversions

Tab 3.7 Genotypes of Salmonella tester strains used in the Ames test

3.2.1 Chemicals and Reagences

Substance	Supplier	Product number
Agar nr. 1	Oxoid/ Bertoni	LP011P
2-Aminofluorene	Sigma	A9031
Ampicillin trihydrat	Sigma	A6140
Citric acid monohydrate	Sigma	C1909
Crystal violet	Sigma	C3886-25G
D-Biotin	Sigma	B4639
Dimethylsulfoxid	Sigma	D5879
Dulbecco´s phosphate buffered saline	PAA Laboratories	H15002
D-(+)-Glucose	Sigma	G8270
D-Glucose 6-phosphate	Sigma	G7250
Hydrochloric acid	Riedel-de Haën	30723
Hydrogen peroxide, 30%	Riedel-de Haën	31642
L-Histidine.HCl	Sigma	H8125
Magnesium chloride	Sigma	M9272
Magnesium sulfate	Sigma	434183
Nicotinamide adenine dinucleotide	Sigma	N0505
Nutrient broth nr. 2	Oxoid/ Bertoni	CM 067B
Potassium chloride	Sigma	P5405
Potassium phosphate dibasic	Sigma	P3786
Rat liver homogenate	Biomedica/ ICN	50412
Salmonella thyphimurium strains TA98, TA100 and TA102	Trinova Biochem GmbH (Giessen, Germany)	
Sodium ammonium phosphate	Sigma	S9506
Sodiumazide	Sigma	S8032
Sodium chloride	Sigma	S5886
Sodium hydroxide	Sigma	O6203
Tert-butylhydroperoxide	Sigma	B2633
Tetracycline hydrochloride	Sigma	T3383
2,4,7-trinitro-9-fluoren	unknown	unknown

Tab 3.8 Chemicals and Reagences used in the Salmonella microsome assay

3.2.2 Equipment

Equipment	Supplier	Product number
Petri dishes	Bertoni	101VR20
Diluting loops	Semadeni	3224
Autoclavable bags	Semadeni	2054
Autoclave band	Semadeni	4343
Cryogenic tubes, 2 mL	Semadeni	4190
Helipur disinfectant	VWR-Merck	148F4124
Incubation tubes (100 x 16-mm)	Dr. F. Bertoni	2775/14
Analytical balance (LC 4801P Sartorius)		
Incubator (Memmert Modell 500)		
Water bath (GFL Müller und Scherr)		
Vortexer (Heidolph Reax 2000)		
Laminar flow (Holten LaminAir HB 2472)		
Autoclaves (Melag Autoklav 23, Varioklav 500)		

Tab 3.9 Equipment used in the Salmonella microsome assay

3.2.3 Investigated samples

Three individual oxidation products, 7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol, a mixture of 6β-OH-3-keto-sitosterol/6α-OH-3-keto-sitosterol (ratio 4:3) and a mixture of the polar oxidation products of β-sitosterol were investigated.

3.2.4 Solubility experiments

Solubility of the plant sterol oxidation products was determined in different solvents (H_2O, DMSO, acetone (95 %, 100 %), hexane, EtOH (95 %) and 2-propanol). Unfortunately phytosterol oxides are not water-soluble therefore unpolar solvents had to be tested as well. These unpolar solvents are at the same time considered to be potentially toxic, thus maximal added concentrations had to be identified in pre-tests. However, either the oxides were not dissolved in the solvents, or the solvents were too toxic for the used bacteria. Therefore for dissolving the samples the emulsifier Tween80 had to be used.

3.2.5 Preparation of reaction mixtures

In order to obtain appropriate dilutions, respective amounts of oxidation products (dissolved in 2-propanol) were evaporated with nitrogen and pre-dissolved in a mixture of acetone/Tween80 (3/1, v/v). Afterwards two parts of sterile, distilled water was added to keep the concentration of acetone as low as possible and to avoid potential toxic effects on the bacterial strains. This procedure was tested to be safe for the strains in pre-experiments.

The concentrations range used was broad, from very low to non physiologically high concentrations, but all below the solubility range, which is recommended for this test procedure (Ames et al., 1973; Mortelmans and Zeiger, 2000). Considering the results of pre-tests, four concentrations (0.04, 0.2, 1.0 and 5.0 mg per plate (≈ %)) of each compound were prepared.

Due to precipitation problems in the highest concentration of 7α-OH-sitosterol, only 3 doses of this compound could be used (0.04 – 1 %). There were similar but minor solubility problems with the mixture of 6β-OH-3-keto-sitosterol/6-α-OH-3-keto-sitosterol, therefore as highest concentration 2.5 mg/plate was tested. On the other hand the mixture of all oxidation products showed very good solubility, so a 10 mg/plate dilution could also be included in the experiments (table 3.10).

	10%	5%	2.5%	1%	0.2%	0.04%
7-ketositosterol		x		x	x	x
7β-OH-sitosterol		x		x	x	x
7α-OH-sitosterol				x	x	x
6α-OH-3-keto-/ 6β-OH-3-keto-sitosterol			x	x	x	x
mixture	x	x		x	x	x

Tab 3.10 Overview of the used concentrations of the reaction mixtures

3.2.6 Metabolic activation

Some carcinogenic chemicals such as aromatic amines or polycyclic aromatic hydrocarbons are biologically inactive unless they are metabolized to active forms. On the other hand some mutagens are active but are inactivated during metabolization. Bacteria do not have a cytochrome-based P450 metabolic oxidation system. In order to simulate in vivo conditions the oxidation products were treated with a rat liver enzyme mixture (S9, which mainly consists of phase I enzymes) for metabolic activation (Mortelmans and Zeiger, 2000). The S9 mix was prepared according to the recipes of Maron and Ames (1984). It was stored on ice throughout the whole experiment and discarded after 50 min.

3.2.7 Experimental design

The Salmonella microsome assay was performed according to Maron and Ames (1984). In order to allow a closer contact of the test compounds and the indicator strain as well as to detect short term reacting mutagens the preincubation assay with an incubation period of 25 min (37 °C) was chosen, as successfully applied prviously (Wagner et al., 2007).

Because of the highly sensitive nature of plant sterol oxides, samples had to be prepared freshly for every test run.

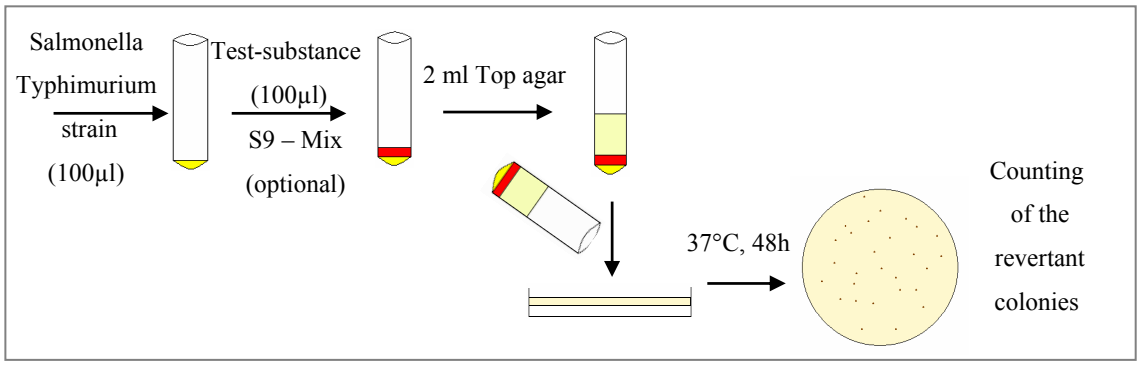


Fig 3.2 Scheme of the Salmonella microsome assay

Briefly, 500 µL of PBS or S9 mix, 200 µL of reaction mixture and 100 µL of overnight bacterial culture were added to test tubes. The tubes were shortly vortexed and then

placed in an incubator on a rotary shaker and incubated for 25 min at 37 °C. Thereafter 2 mL of molten top agar were added to each tube. The mixture was vortexed and poured on minimum glucose plates. As soon as the agar had solidified the plates were inverted and stored in an incubator for 48 h at 37 °C. Thereafter his^+- revertants were counted manually (figure 3.2).

To further investigate the anti-/pro-oxidative effects the pro-oxidant tertiary-butyl hydroperoxide (tBOOH, 0.7 mM) was used for challenge tests. The concentration of the oxidant was chosen in order to obtain suitable numbers of revertants on the plates. Therefore 720 µL of the test sample and 720 µL of tBOOH were first mixed and then 400 µL of this mixture was added to 500 µL of PBS or S9 mix and 100 µL of overnight culture. Besides, these challenge tests were performed in the same way as without pro-oxidative stressing.
All test procedures were conducted with and without metabolic activation.

For each compound and each concentration three plates were prepared and every test was repeated under the same conditions on another day. So, altogether 6 replicates of each test sample were produced.
In addition, each test includes a positive control to confirm the reversion properties and specificity of each tester strain as well as a negative control, the sample solvent, for the assessment of the spontaneous revertants (Mortelmans and Zeiger, 2000).
For the strains TA98 and TA102 2,4,7-Trinitro-9-fluorenone and for TA100 Sodiumazide was used as positive controls for tests without metabolic activation, while for tests with metabolic activation 2-Aminofluorene was used for all strains.
Detailed description of the test performance and solvent recipes can be found in other Master and PhD Theses that have been done at the Department of Nutritional Sciences, University of Vienna: e.g. 'Wirkungen von Phytosterin-Oxidationsprodukten im Ames Test', Cornelia Fritz-Ton, 2007.

3.2.8 Statistical analysis and evaluation of the mutagenic experiments

All data are expressed as mean ± SD (standard derivation). Obtained data (n = 6 for each concentration used) were analysed by one-way analysis of variance (ANOVA) and

the Student's t-test since they were all normally distributed, using SPSS 15.0 for Windows. Statistical differences were considered significant at a value of $p < 0.05$.

In addition to the statistical, a nonstatistical evaluation was carried out. According to Mortelmans and Zeiger (2000) a compound is considered as 'mutagenic' if the total number of his^+- revertants per plate was at least twice as high as the negative control (200 %). Moreover a dose related increase of the number of his^+- revertants has to be shown.

3.3 Cell culture assays

3.3.1 Solutions and Reagents

Substance	Supplier	Product number
Amino acids non essential	PAA Laboratories	M11-003
AnnexinV-PE detection kit I	BD Biosciences	559763
Camptothecin	Sigma	C9911
Dimethylsulfoxid	Sigma	D5879
3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide	Sigma	M5655
Dulbecco´s phosphate buffered saline	PAA Laboratories	H15002
Ethanol	Riedel-de Haën	32221
Ethylenediaminetetraacetic acid	Sigma	E6758-500G
Ethylenediaminetetraacetic acid disodium salt dihydrate	VWR	443882G
Ethidium bromide aqueous solution	Sigma	E1510
Foetal Bovine Serum "GOLD"	PAA Laboratories	A15-151
Dihydroethidine	Sigma	37291
Hydrogen peroxide, 30%	Riedel-de Haën	31642
Hydrochloric acid	Riedel-de Haën	30723
Low melting Agarose	Invitrogen	15517014
Methanol	Merck	106007
Minimal essential medium (MEM) with Earle's Salta with L-Glutamine	PAA Laboratories	E15-825
Sodiumhydroxide	Riedel-de Haën	6203
Normal melting Agarose	Invitrogen	15510019
Phosphoric acid, 85%	Riedel-de Haën	30417
Potassium chloride	Sigma	P5405
Potassium dihydrogen phosphate	Riedel-de Haën	30407
Sodium chloride	Sigma	S5886
Sodium hydroxide	Sigma	S5881
Sodium pyruvate	Sigma	P2256
1,1,3,3-Tetraethoxypropane	Sigma	T9889
2-Thiobarbituric acid	Sigma	T5500
Tris	Sigma	T1503
Triton X-100	Serva	T8787
Trypsin/EDTA	PAA Laboratories	L11-659
Trypan blue solution	Sigma	T8154

Tab 3.11 Chemicals and Reagences used in the cell tests

3.3.2 Equipment

Equipment	Supplier	Product number
Centrifuge tube, 15 mL	Dr. F. Bertoni	15PPR
Centrifuge tube, 50 mL	Dr. F. Bertoni	36050NPG
Coverslips (24x50 mm)	Dr. F. Bertoni	990
Disposable syringe, 10 mL	Dr. F. Bertoni	309110
Disposable syringe, 20 mL	Dr. F. Bertoni	309296
Electrophoresis chamber, horizontal	VWR	730-1796
Frosted slices	VWR	6311304
Gloves (nitril)	VWR	112-2220
Syringe filters (22µm)	Dr. F. Bertoni	2052-025
Injection cannula Terumo Neolus Nr 18, 26G x23, 0,45x 23mm	pharmacy	Lot 0606023
Round-Bottom Tubes, 5mL	VWR	734-0443
Tissue culture flasks, 25cm^2	Dr. F. Bertoni	3103-025
Tissue culture flasks, 75cm^2	Dr. F. Bertoni	3123-075
6 well plates	Dr. F. Bertoni	3810-006
24 well plates	Dr. F. Bertoni	3820-024
96 well plates	Dr. F. Bertoni	3860-096
Microscope (Axioskop, Zeiss; Wilovert, Hund Wetzlar)		
Incubator (Heraeus Instruments Function Line Typ BB16)		
Water bath (GFL Müller und Scherr)		
Analytical balance (LC 4801P Sartorius)		
FACSCalibur flow cytometer (multicolor system, BD Biosciences)		
BD CellQuest Pro Software (BD Biosciences)		
Fluostar Optima microplate reader (BMG labtechnologies)		
Fluorescence microscope (Axioskop 20, Zeiss)		
Komet 5.5, image analysis system (Kineting Imaging)		
POWER Supply (Peqlab)		
Rotary Shaker (ELMI Ltd. laboratory equipment)		

Tab 3.12 Equipment used in the cell tests

3.3.3 Preparation of general solutions and reagents for cell tests

Culture media (500 mL):

445 mL Minimal Essential Medium Eagle (MEM)

50 mL fetal bovine serum (FBS)

5 mL non essential amino acids

1 mL Sodium pyruvate (500 mM)

NaCl –solution (0.9 %):

0.9 g NaCl

100 mL Aqua bidest.

Sodium pyruvate (500 mM):

1.1 g Sodium pyruvate

20 mL NaCl- solution (0.9 %)

Positive control:

0.00375 % H_2O_2 in MEM (incubation time: 10 min)

3.3.4 HepG2-cells

For the following cell culture experiments human hepatoma cells (HepG2) were used. The cells were isolated from the liver tissue of a 15 year-old Caucasian male from Argentina in 1975. HepG2-cells are perpetual adherent, epithelial in morphology and routinely used for toxicology studies (Aden et al., 1979).

3.3.5 Cell maintenance

HepG2 were cultured as monolayers in MEM supplemented with 10 % (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 % (v/v) non-essential amino acids in a humidified atmosphere at 37 °C in 5 % CO_2 in the absence of antibiotics. Cells were passaged weekly and the culture medium was changed every 3-4 days. Cultures were allowed to reach 80 % confluence before experiments were performed.

3.3.6 Passage

Subconfluent cells were washed with Dulbecco´s Phosphate-buffered saline (PBS), harvested with trypsin-EDTA solution (1.5 mL for T25-flasks, incubation time: 3 min) and gently centrifuged (5 min, 800 RPM). Thereafter the cell pellet was resuspended in 10 mL pre-warmed culture medium and a single-cell suspension was prepared by

pressing the cell suspension through an injection cannula. 1/15 of the cells were seeded in a new flask.

3.3.7 Investigated samples

Three individual oxidation products, 7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol, a mixture of 6β-OH-3-keto-sitosterol/6α-OH-3-keto-sitosterol (ratio 4:3) and a mixture of the polar oxidation products of β-sitosterol were investigated.

3.3.8 Sample preparation

For delivery to the cells all compounds were dissolved in ethanol and added to reduced serum media (2.5 % (v/v) FBS). Within all experiments 3 different concentrations (30 µM, 60 µM, 120 µM) and an incubation period of 24 hours were considered in order to guarantee uniformity and comparability. The final concentration of ethanol in cultures did not exceed 0.4 % (v/v) and did not affect cell proliferation. Equivalent quantities of ethanol were added to control cells.

3.3.9 Cell treatment

For treatment cells were cultured in different multiwell dishes depending on the respective experiment at a density of either $2x10^4$ cells/well in 250 µL (96-well plates), $2x10^5$ cells/ well in 2 mL (24-well plates) or $1x\ 10^6$ cells/ well in 3 mL (6-well plates) of complete medium. After 24 hours medium was removed and cells were incubated with the different β-sitosterol oxide samples. At the end of the incubation period, both floating and attached cells were collected for analysis.

3.3.10 Trypan blue exclusion assay

Trypan blue is an acid di-azo group dye. Its anions can bind to cell proteins, but as long as the cell membranes are intact it is not able to interact with the cells. Therefore, viable cells exclude the dye, whereas dead cells will be stained blue, thus can be easily counted using a light microscope.

3.3.10.1 Assay procedure

At the end of the incubation period culture media was collected in order to include floating cells in the analysis. Cells were washed once with 1 mL pre-warmed PBS, harvested with trypsin/EDTA solution (100 µL, 4 min incubation), resuspended in 1 mL of complete media and mixed with the collected media containing the floating cells. With the help of an injection cannula a single cell suspension was prepared. 100 µL of this cell suspension were mixed with 100 µL of trypan blue solution and incubated for 3 minutes. Thereafter 20 µL of this mixture were filled into the haemocytometer and the number of viable (bright cells) and non-viable cells (stained blue) were counted using a light-optical microscope.

Results were presented as number of viable cells expressed as percentage (%) of negative control values. Additionally percentage of cell death was calculated according to the percentage of dead cells of the total cell population.

Experiments were performed in triplicates.

3.3.11 MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromid) assay

In metabolically active cells the yellow, water soluble tetrazolium salt MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromid) is converted to purple, water insoluble formazan by dehydrogenase enzymes of the mitochondria (figure 3.3). The resulting intracellular formazan is directly proportional to the number of metabolically active cells. This colorimetric reaction can be measured spectrophotometrically (Mosmann, 1983).

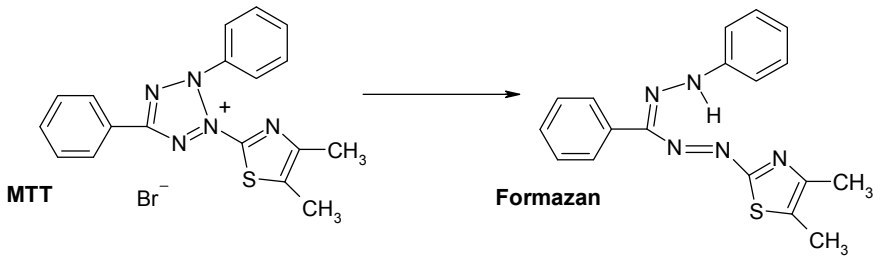


Fig 3.3 Reduction of yellow MTT to purple formazan.

3.3.11.1 Preparation of solutions and reagents

MTT-solution (5 g/L):

50 mg MTT

10ml PBS

MTT-solution was protected from light and stored at 4°C. For delivery to cells 20 µL MTT-solution was added to 180 µL media (final concentration 0.5 mg/mL).

3.3.11.2 Assay procedure

At the end of the incubation period culture medium was discarded and cells were washed with 200 µL of pre-warmed PBS. 200 µL of MTT in culture medium was added and incubated for 1 hour at 37°C. Thereafter the MTT containing media was carefully removed and cells were washed with 200 µL of pre-warmed PBS. 100 µL of DMSO was added and formazan crystals were dissolved under gentle shaking (30 min). Absorbance was read at 540 nm with a Fluostar Optima microplate reader

Results were expressed as the number of viable cells as percentage (%) of control cells. Measurements were made in triplicates.

3.3.12 Flow cytometric measurments

With the help of flow cytometry it is possible to simultaneously examine multiple characteristics of single cells. Based on light scatter properties (forward scatter channel (FSC), side scatter channel (SSC)) it is possible to determine cell size and granularity. At the same time specific cell parameters can be analysed by labelling definite targets with special fluorescence markers. In most cases these markers are antibodies which are also bound to fluorescence groups.

3.3.12.1 Detection of apoptosis

During the early phase of apoptosis phosphatidylserine, which is normally located on the inner surface of the cell membrane, is translocated from the inner to the outer surface (Koopman et al., 1994). The expression of phosphatidylserine on the external surface is a universal event during apoptosis occurring before the loss of membrane

integrity arise, independent of the respective cell type. This loss of plasma membrane asymmetry is precedent for the recognition and removal of damaged cells by macrophages (van Engeland et al., 1998).

AnnexinV, a phosphlipid binding protein, specifically binds to phosphatidylserine in a Ca^{2+} dependent reaction. Thus staining with fluorescent labelled AnnexinV-PE allows identifying cells in the early stage of apoptosis (figure 3.4).

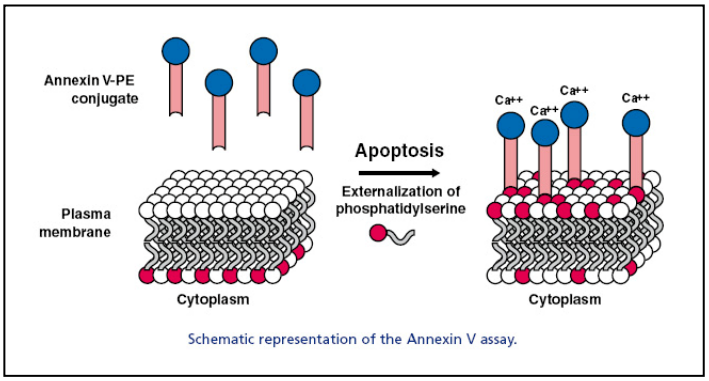


Fig 3.4 Schematic representation of the Annexin V assay (www.bdbiosciences.com).

Annexin V is not an absolute marker of apoptosis, as phosphatidylserine translocations can also occur during the process of necrosis. Therefore in addition to Annexin V the dye 7-amino-actinomycin D (7-AAD) was used to mark necrotic cells. 7-AAD binds to nucleic acids inside the cell but is not able to diffuse intact cell membranes. Hence only those cells with damaged membranes, a condition commonly occurring in the later stages of apoptosis or in necrosis, are marked with 7-AAD (Vermes et al., 1995).

AnnexinV positive/7-AAD negative cells were defined as early apoptotic, while AnnexinV positive/7-AAD positive cells were classified as late apoptotic or necrotic.

3.3.12.1.1 Preparation of solutions and reagents

AnnexinV-PE detection kit I:

AnnexinV-PE

7-AAD

Binding buffer (0.1 M HEPES/NaOH (pH 7.4) 1.4 M NaCl, 25 mM $CaCl_2$)

Positive control:

Camptothecin

Stock solution (1 mM)

6.967 mg Camptothecin

20 mL DMSO

For delivery to cells 5 µL Camptothecin-solution was added to 12 mL media (24 h incubation).

3.3.12.2 Assay procedure

For detection of apoptosis an AnnexinV-PE detection kit (BD Pharmingen) was used and analysis was done following the manufacturer's instructions.

Briefly, at the end of the incubation period culture media was collected in order to include floating cells in the following analysis. Cells were washed with 1 mL pre-warmed PBS and harvested with trypsin/EDTA solution (300 µL, 4 min incubation). After the incubation period 700 µL of complete media was added, mixed with the collected media of step 1 and centrifuged (5 min, 800 RPM).

Resulting cell pellets were washed twice with cold PBS. Then cells were resuspended in binding buffer at a concentration of approximately 1 x 10^6 cells/mL. 100 µL of this suspension was transferred into 5 mL culture tubes and 5 µL of Annexin V-PE and 7-ADD were added. Cells were incubated for 15 min at RT in the dark. Then 400 µL of binding buffer was added to each tube and samples were analysed by flow cytometry within one hour.

For each sample 10.000 cells were acquired and data management was done with CellQuest Pro Software (FACScan, BD Biosciences, USA). Experiments were performed in triplicates.

3.3.12.3 Intracellular Superoxide anion ($O_2^{\bullet-}$) generation

Hydroethidine (HE) was used for the detection of $O2^{\bullet-}$ in living cells. It is a non-fluorescent compound, which diffuses easily through the cell membrane. Under the action of $O2^{\bullet-}$ HE is dehydrogenated to ethidium bromide and intercalates DNA. The

red fluorescence of ethidium can be measured by flow cytometry (absorption/emission: 518/605 nm) (Rothe and Valet, 1990).

3.3.12.3.1 Preparation of solutions and reagents

Stock solution (1 mM):

5 mg Hydroethidine

16 mL DMSO

3.3.12.4 Assay procedure

At the end of the incubation period culture media was collected in order to include floating cells in the analysis. Cells were washed with 1 mL pre-warmed PBS, harvested with trypsin/EDTA solution (300 µL, 4 min incubation) and then 700 µL of complete media was added. Cells were mixed with the collected media of step 1, centrifuged (5 min, 800 RPM) and cell pellets were resuspended in PBS at a concentration of approximately 1x 10^6 cells/mL. 5 µL of HE-solution in DMSO was added to 1 mL of cell suspension. Then cells were incubated for 10 min at 37 °C in the dark, then stored on ice and analysed as quickly as possible by flow cytometry.

For each sample 10.000 cells were analysed and data management was done with CellQuest Pro Software (FACScan, BD Biosciences, USA). Experiments were performed in triplicates

3.3.13 Measurement of Lipid Peroxidation

To include a second maker for oxidative stress, malondialdehyde (MDA) was measured. MDA constitutes an end product of lipid peroxidation and was determined as described earlier (Ramel et al., 2004), with some modifications.

HPLC system	
LaChrom Merck Hitachi chromatography system	
Hitachi L-7100 pump	
column: LichroCART 250-4 Lichrosher 100 RP-18.10 µm	
pre-column: LichroCART 125-4 Lichrosher 100 RP-18.5 µm	
Hitachi F-1050 flourescence detector	
Hitachi D-7500 integrator	
HPLC conditions	
mobile phase	phosphate buffer/ methanol (60/40, v/v)
flow rate	1 mL/min
injection volume	20 µL
detection	fluorescence (emission/excitation: 563/532 nm) sensitivity: 20

Tab 3.13 HPLC conditions used for MDA-determination

3.3.13.1 Preparation of solutions and reagents

Phosphoric acid (44 mM):

3 mL orto-phosphoric acid (85%)

ad 100 mL aqua bidest.

Thiobarbituric acid (TBA):

0.6 g TBA

100 mL aqua bidest.

Methanol/ NaOH (90/10, v/v):

5 mL NaOH (1N)

45 mL methanol (chromasolv)

NaOH (1N)

4 g NaOH

100 mL aqua bidest.

KCl (75 mM):

5.592g KCl/1000 mL aqua bidest.

Phosphate buffer (pH 6.8):

6.8 g potassium dihydrogen phosphate

ad 1L aqua bidest.

standard solutions	
stock-solution 1 (4.06 mM)	**stock-solution 2** (8.12 µM)
50 µL 2-Thiobarbituric acid ad 50 mL EtOH/ Aqua bidest. (40/60, v/v)	100 µL stock 1 ad 50 mL EtOH/ Aqua bidest. (40/60, v/v)

standard 1 (81.2 µM):	100 µL stock1 + 4.9 mL aqua bidest.
standard 2 (40.6 µM):	50 µL stock1 + 4.95 mL aqua bidest.
standard 3 (20.3 µM):	1 mL standard 2 + 1 mL aqua bidest.
standard 4 (8.12 µM):	= stock 2
standard 5 (4.06 µM):	1 mL stock 2 + 1 mL aqua bidest.
standard 6 (0.81 µM):	100 µL stock 2 + 900 µL aqua bidest.

Tab 3.14 Composition of standard solutions used for MDA-determination

3.3.13.2 Assay procedure

At the end of the incubation period culture media was collected (to include floating cells in the analysis). Cells were washed with 1 mL pre-warmed PBS, harvested with trypsin/EDTA solution (300 µL, 4 min incubation), media including floating cells were added and centrifuged (5 min, 800 RPM). Cell pellets were resuspended in 2 mL of media. A single cell suspension was prepared using an injection cannula and 100 µL thereof were used for the determination of cell numbers. The remaining cell suspensions were centrifuged (5 min, 800 RPM) again. 150 µL KCl, 400 µL aqua bidest., 700 µL phosphoric acid and 260 µL TBA was added to the cell pellets. At the same time 500 µL aqua bidest., 700 µL phosphoric acid and 260 µL TBA were added to 50 µL of the standard solutions. Standards and cell samples were incubated in boiling water for 60 minutes and then cooled on ice. 100 µL of the cell samples or standards were mixed with 100 µL of methanol/NaOH and centrifuged (3 min, 3000 RPM). Then 20 µL were injected into the HPLC and MDA was measured by fluorescence detector.

Data was expressed as nanomoles per 10^9 cells. Experiments were performed in triplicates.

3.3.14 Alkaline single cell gel electrophoresis (SCGE) assay

The single cell gel electrophoresis assay (Comet assay) represents a rapid technique for the quantification of DNA damage in individual mammalian cells. The assay was designed by Ostling and Johanson (Ostling and Johanson, 1984) for the detection of double-strand breaks. Due to further developments (Singh et al., 1988; Olive, 1989) today various forms of DNA damage (e.g., single- and double-strand breaks, alkali labile sites, oxidative DNA base damage and DNA cross-linking with DNA or protein) can be confirmed.

The principle of the assay is that cells embedded in agarose gel on microscope slides are lysed to remove all cellular proteins. Thereafter DNA is allowed to unwind under alkaline conditions and later electrophoresed. In the electric field broken DNA fragments (damaged DNA) migrates faster than undamaged DNA, as it remains mainly in the nucleus. The result looks like a comet with a 'head' of intact DNA and a 'tail' full of DNA fragments. The extent of DNA drifted away from the head of the comet was directly proportional to the DNA damage.

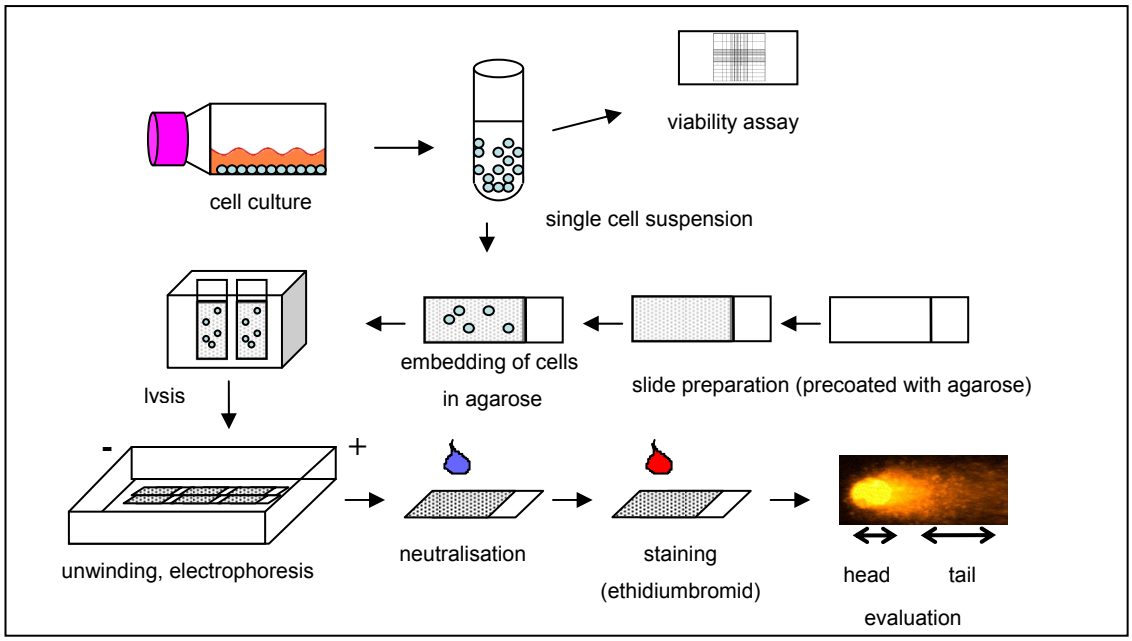


Fig 3.5 Scheme of the alkaline single cell gel electrophoresis assay

3.3.14.1 Preparation of solutions and reagents

Lysis solution

2.5 M NaCl (146.1 g)

100 mM Na_2EDTA (37.2 g)

10 mM Tris (1.2 g)

in 1 L aqua bidest.

PH was set to 10 by addition of NaOH (~ 8 g).

Prior to use 1% Triton X 100 and 10% DMSO were added and the lysis solution was refrigerated for at least one hour.

Electrophoresis buffer

stock solutions:

10 N NaOH (200 g/500 mL aqua bidest.)

200 mM Na_2EDTA (14.89 g/200 mL aqua bidest.)

For 2200 mL of electrophoresis buffer (pH 13.6-13.7) 2130 mL aqua bidest., 59 mL NaOH and 11 mL Na_2EDTA was mixed. Electrophoresis buffer should be cooled before use.

Neutralising buffer

0.4 M Tris (48.5 g/1000 mL aqua bidest.)

pH was set to 7.5 with HCl solution (3.5 %).

Low melting agarose (LMA)

125 g LMA

25 mL PBS

For experiments LMA had to be heated in the microwave and stored at 37 °C in the waterbath.

Normal melting agarose (NMA)

1.5 g NMA

100 mL PBS

NMA was heated in the microwave until near boiling. In order to pre-coate the slides they were dipped in hot agarose. On the underside the agarose was wipped off, slides were air dried and stored at room temperature until needed.

Ethidium bromide (20 µg/mL)

10 µL stock solution (10 mg/mL)

5 mL aqua bidest.

3.3.14.2 Assay procedure

The SCGE assay (comet assay) was carried out according to the guidelines developed by Tice et al. (1990; 2000) and Singh et al. (1988).

At the end of the incubation period culture media was collected to include also floating cells in the analysis. Cells were washed with 1 mL pre-warmed PBS, harvested with trypsin/EDTA solution (300 µL, 4 min incubation), mixed with floating cells and centrifuged (5 min, 800 RPM). Cell pellets were resuspended in 500 µL of media and a single cell suspension was prepared using an injection cannula. 100 µL of the cell suspension were mixed with 100 µL of trypan blue solution (incubated 3 min) and cell numbers were determined. Aliquotes of $1x10^5$ cells were mixed with 80 µL of LMA and transferred to NMA-coated slides. Slides were covered with cover slips and placed on ice packs until the agarose has solidified. Then cover slips were removed, slides were placed in lysis solution and stored at 4 °C for 24 hours.

After lysis slides were incubated in alkaline electrophoresis buffer (40 min) for DNA unwinding. Then electrophoresis was performed using a horizontal gel electrophoresis (25 V, 50 W, 300 mA, 20 min). Thereafter slides were rinsed 2 times with neutralization buffer and once with cold aqua bidest. Dried slides were stained with 5 µL ethidium bromide solution and analysed using a fluorescence microscope coupled with a computerized image analysis system (Komet 5.5, Kineting Imaging, Liverpool, UK).

For each sample, three replicate gels were analysed and from each replicate slide 50 cells were randomly selected. As parameter of DNA damage percentage of DNA in the tail (% DNA in tail) was determined. Each experiment was done twice.

3.3.15 Statistical analysis of cell assays

All data are expressed as mean ± SD (standard derivation). Obtained data were analysed by the Student's t-test since they were all normally distributed, using SPSS 15.0 for Windows. Statistical differences were considered significant at a value of $p < 0.05$ and are reported as $p < 0.05$, $p < 0.01$ and $p < 0.001$.

3.3.16 Measurement of the uptake of oxidation products in HepG2-cells

3.3.16.1.1 Extraction of the oxidation products

At the end of the incubation period culture media was removed and collected separately. Cells were harvested using trypsin/EDTA solution (1.5 mL, 4 min), centrifuged (800 RPM, 5 min), washed twice with PBS and resuspended in 5 mL of KCl (incubation for 20 min). Then the lipid fraction was extracted from cell and media samples. Therefore 5 mL of diethyl ether was added and after centrifugation (1200 RPM, 10 min) the ether phase was collected. This extraction procedure was repeated 3 times.

The ether phases of all samples were evaporated and dissolved in ethanol. Then the samples were sent to the Department of Applied Chemistry and Microbiology and there stored at -20 °C until measurement.

3.3.16.1.2 Sample preparation for HPLC-analysis

Extracted samples were evaporated again, dissolved in 1 mL of n-heptane/2-propanol (95/5, v/v) and filtered (0.45 µm GHP membrane filters) and stored at -20 °C until analysis.

3.3.16.1.3 Quantification of the extracted oxidation products by a HPLC-UV-ELSD system

For the separation of the extracted oxides a mobile phase system of n-heptane/2-propanol (97/3, v/v) and a flow rate of 0.6 mL/min were used based on methods published by Kemmo et al. (2005; 2004).

In order to ascertain co-elutions of oxysterols and lipid extracts of the cell membrane standard solutions consisting of 7-ketocholesterol, 7β-OH-cholesterol, 7α-OH-cholesterol, the laboratory prepared mixture of 6-β-OH-3-keto-sitosterol/6-α-OH-3-keto-sitosterol (also used for incubation experiments) and cell lipid residues (derived as result of the extraction) were used.

In several pre-testes ELSD conditions were examined with the help of cholesterol oxide standard solutions (7-ketocholesterol, 7α-OH-cholesterol and 7β-OH-cholesterol). The effect of various temperatures (50 °C/42 °C, 55 °C/42 °C, 60 °C/42 °C and 45 °C/36 °C for drift tube/nebulising temperature), nebuliser gas pressures (20, 30, 40 and 50 PSI) and injection volumes (5 and 10, 20 µL) were tested.

HPLC system	
Waters 717 plus Autosampler (Walters, Milford, USA)	
Supelcosil silica column (250 mm x 2.1 mm i.d., 5 µm) (Supelco, Bellefonte, PA, USA)	
Waters pump (model 515) (Walters, Milford, USA)	
Waters 996 Photodiode Array Detector (Walters, Milford, USA)	
Waters 2420 ELS-detector (Walters, Milford, USA)	
HPLC conditions	
mobile phase	*n* -heptane/2-propanol (97/3, v/v)
flow rate	0.6 mL/min
injection volume	20 µL
detection	UV-detection, 206 nm ELSD-detection: nebulization temperature 42 ˚C drift tube temperature 50 ˚C pressurised air 20 PSI

Tab 3.15 HPLC conditions used for the detection of POPs

3.3.16.1.4 Assessment of detection limits and linearity of UV and ELSD responses

Detection limits and linearity were determined using standard solutions containing 7-ketocholesterol, 7β-OH-cholesterol and 7α-OH-cholesterol.

standard solutions		
	µg/mL	cholesterol oxides
solution 1	63.5	7-keto
	79.9	7β-OH
	61.9	7α-OH
solution 2	328.6	7-keto
	395.6	7β-OH
	305.6	7α-OH

Tab 3.16 Cholesterol oxide standard solutions

Detection Limits (LOD) were calculated on the basis of a signal to noise ratio of 3. Standard solution 1 was diluted down to one hundred times and multiple injections on different days were performed.
For assessment of linearity triplicate injections of 6 different volumes (5, 10, 20 and 30 µL of solution 1; 10 and 15 µL of solution 2) of the standard solutions were processed.

3.3.16.1.5 Quantification of sterol oxides

Quantification was performed by PDA - and ELS - detection. The concentrations of the oxidation products were estimated by cholesterol standards of the corresponding cholesterol oxidation products, whereas 7-ketocholesterol was also used for the quantification of 6α-OH-3-keto-sitosterol and 6β-OH-3-keto-sitosterol. Concentration and purity of the standards was confirmed by GC–FID as described above. Standard curves were analysed at the beginning and at the end of each compound, concentration of the oxides was calculated using the mean standard curve.

3.3.16.1.6 Statistical analysis

The data was recorded as means ± standard deviations. Analysis was carried out by simple regression testing using Statgraphics 4.0 software (STCC Inc., Rockville, ML).

4. Results and Discussion

4.1 Production and isolation of phytosterol oxidation products

As already mentioned POPs are commercially not available. For the assessment of their potentially harmful effects in bacterial and cell culture assays individual oxides had to be laboratory-prepared. Various methods for the preparation of sterol oxidation products exist. However, most of them are designed for the collection of only small amounts of oxides.

Therefore the initial target of this thesis was the development of a fast, simple and effective method for the isolation of common β-sitosterol oxides.

4.1.1 Preparation of oxides by thermo-oxidation

For the preparation of β-sitosterol oxidation products thermo-oxidation in solid state was conducted. Different heating conditions (130 °C/ 24 h, 130 °C/ 48 h and 120 °C/ 48 h) were tested and generated amounts of oxysterols were estimated by GC-FID. As can be seen in figure 4.1 after heat treatment for 24 h at 130 °C highest amounts of the single oxides were formed. Longer heat exposure, however, decreased the total amount of oxidation products detected. This may be due to conversion reactions to other secondary oxidation products or further reactions such as polymerisation leading to non polar compounds, dimers and polymers (Soupas et al., 2005).

Similar to our findings Kemmo et al. (2005) reported that heat treatments at different temperatures induced the formation of the same kind of oxidation products, yet the amount of the single oxides varied. Already Caboni et al. (1997) noticed that the ratios of the formed products were influenced by the oxidation conditions applied.

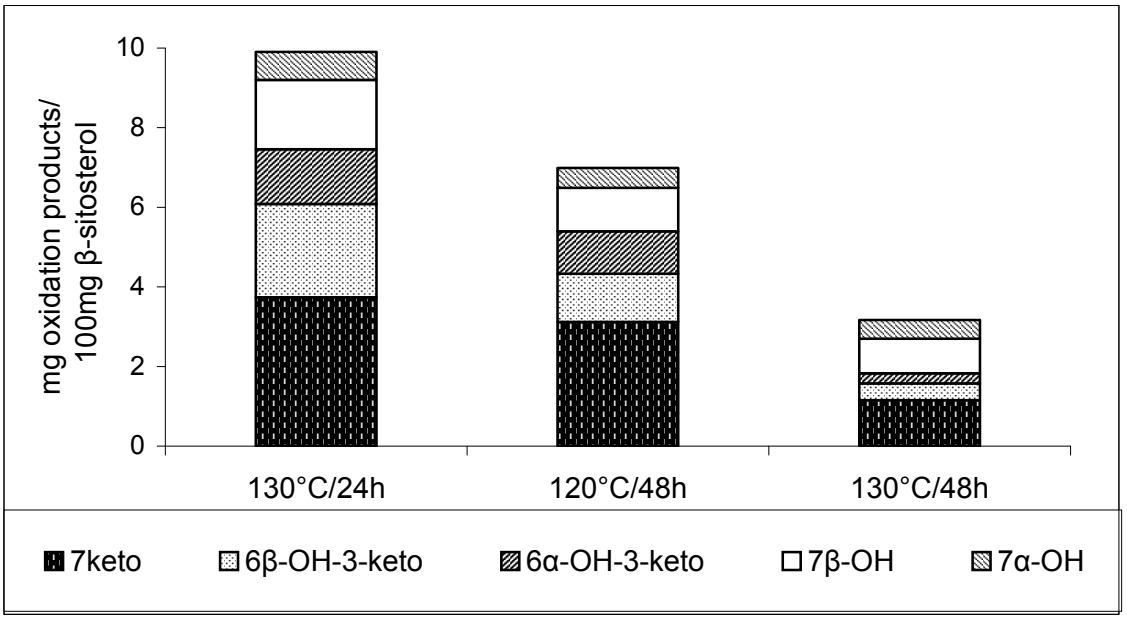


Fig 4.1 Proportion of collected β-sitosterol oxidation products (mg) after different oxidation conditions (130 °C/24 h, 120 °C/48 h and 130 °C/48 h) measured by GC-FID.

The oxidation products observed in these experiments, 7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol, 5,6β-epoxy-sitosterol, 5,6α-epoxy-sitosterol, 6β-OH-3-keto-sitosterol, 6α-OH-3-keto-sitosterol, 6β-OH-sitosterol and 6keto-sitosterol, were analogous to those commonly found in literature.

Grandgirard et al. (2004c) generated 7α-OH-, 7β-OH-, 5,6α-epoxy, 5,6β-epoxy- 7keto- and triol-compounds of various plant sterols after heat-treatment at 135 °C for 24 h.

Johnsson and Dutta (2003) heated plant sterols for 72 h at 120 °C and found 7α-OH-, 7β-OH-, 5,6α-epoxy, 5,6β-epoxy- and 7keto- compounds. Additionally 24-OH-, 25-OH- as well as 6-OH-3-keto- compounds were detected.

Same as (Daly et al., 1983; Apprich and Ulberth, 2004; Conchillo et al., 2005) no triols were observed in our experiments, presumably due to the lack of water (Zhang et al., 2005b).

The high amount of 7-ketositosterol and the generation of 7β-OH-sitosterol rather than 7α-OH-sitosterol was in accordance with previous studies (Chien et al., 1998; Kemmo et al., 2005).

4.1.2 Purification and enrichment of phytosterol oxidation products

After heat treatment of sterols a mixture of oxidation products, non oxidized material and several unknown by-products is formed. Because of the complexity of the gained blend usually a combination of different separation techniques is applied (Daly et al., 1983).

To exclude the main part of unknown and non oxidized compounds, column chromatography using silica gel was conducted. In recent years column chromatography and TLC have been gradually replaced by the use of disposable ready-to-use SPE cartridges (Guardiola et al., 2004). However, considering the great amount of samples to be purified, in our case CC with its higher loading capacity allowed a more efficient working procedure.

Since our interest was focused on POPs with a higher polarity than non oxidized β-sitosterol a stepwise elution order to remove apolar components up to free β-sitosterol was applied. Solvent mixtures of n-heptane/diethyl-ether with increasing polarity in combination with silica gel and a final elution of the oxidized components with acetone were already successfully employed by others (Apprich and Ulberth, 2004; Lampi et al., 2002; Piironen et al., 2002).

The activity of polar sorbents like silica gel depends strongly on the hydration of the silanol groups (Guardiola et al., 2004). Therefore, as parts of the oxidation products were obviously retained in the column, 10 % of water was added to the silica gel.

Analytical TLC was used to confirm the separation process performed by CC (figure 4.2). Products with a lower polarity than β-sitosterol were successfully removed. Although non oxidized β-sitosterol was still present in the purified samples a relevant reduction could be noted.

Identification of the oxidation products was not only done by comparison of the respective Rf-values with those of the corresponding cholesterol oxides, but also by colour development after spraying with sulphuric acid and following heat treatment. In accordance with earlier publications (Daly et al., 1983; Bortolomeazzi et al., 1999) non oxidized sterols, β-sitosterol and cholesterol, turned reddish brown, epoxide epimeres yellow and 7α-OH- and 7β-OH-compounds blue. Under the conditions applied separation of 7-ketosterols and epoxy derivatives was not achieved. However, 7-

ketosterols don't show colour development but are detectable under UV light (Lebovics, 2002; Daly et al., 1983).

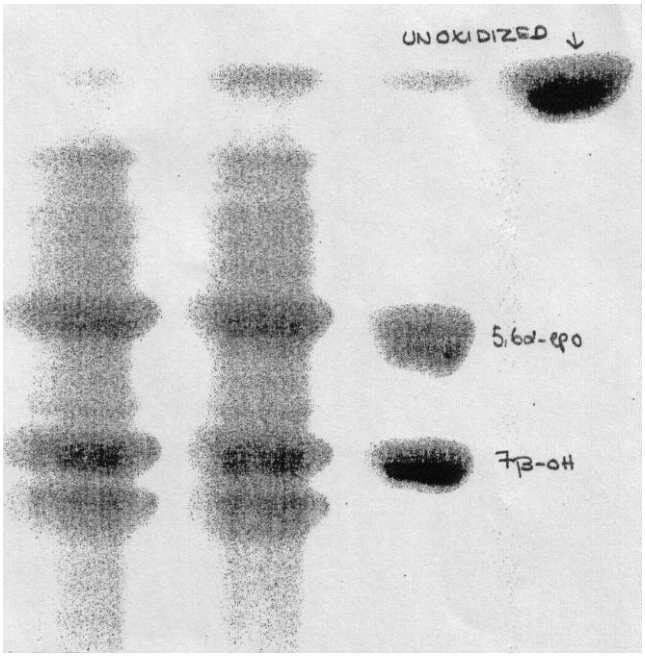


Fig 4.2 Oxidation products of β-sitosterol observed on silica TLC plates developed after spraying with 50% sulfuric acid and following heat treatment.

4.1.3 Separation of single oxidation products by a NP-HPLC-UV system

Due to the high sample amounts to be separated and in accordance with earlier publications (Daly et al., 1983; Ansari and Smith, 1979) the application of an HPLC method was more effective for the isolation of single oxidation products than more generally used TCL methods. Further in TLC analysis artefact formation due to the long exposure of the samples to air is possible (Rose-Sallin et al., 1995).

In general both normal (Kemmo et al., 2007; Kemmo et al., 2008; Csallany et al., 1989; Chien et al., 1998) and reverse phase chromatography (Osada et al., 1999; Razzazi-Fazeli et al., 2000; Manini et al., 1998) has already been successfully used for the analysis of cholesterol- and plant sterol oxidation products. Yet normal phase chromatography is considered to be the more effective option (Saldanha et al., 2006).

Correspondingly the final separation of the oxidation products was achieved by a normal phase HPLC-UV system with a silica column. Based on investigations of Kemmo et al. (2007; 2005) different mobile phase systems with changing percentages

of n-heptane and 2-propanol were tested. Rapid separation within 20 min could be achieved in an isocratic system with a mobile phase of n-heptane/2-propanol (93:7, v/v).

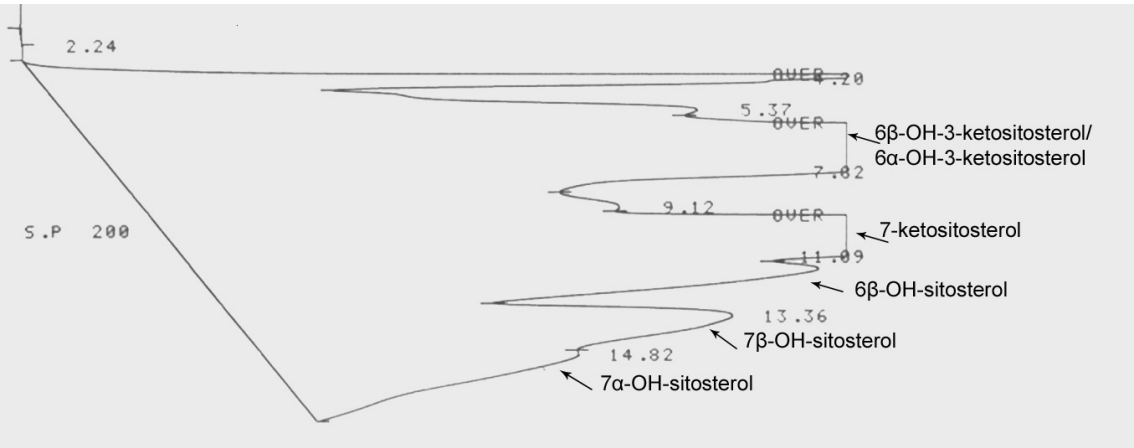


Fig 4.3 Separation of β-sitosterol oxidation products by NP-HPLC.

Figure 4.3 shows the elution order in this system: 6β-OH-3-ketositosterol/6α-OH-3-ketositosterol, 7-ketositosterol, 6β-OH-sitosterol, 7β-OH-sitosterol and 7α-OH-sitosterol.

Due to the high injection volume of purified samples 6β-OH-3-ketositosterol and 6α-OH-3-ketositosterol co-eluted. However, our interest was focussed on the isolation of large amounts of the major oxidation products 7-ketositosterol, 7β-OH-sitosterol and 7α-OH-sitosterol and good separation was achieved for them. Therefore the applied method was retained and 6-OH-3-keto - epimeres were collected in one fraction.

Injection of 1.8 mL oxide sample (containing oxidation products derived from approximately 1000 mg β-sitosterol) led to yields in the range of several milligrams for the single fractions in one HPLC run (table 4.1).

compound	retention time (min)	mg/ injection	overall collected amounts (mg)
6β-OH-3-keto-sitosterol	7.82	5.7	430.7
6α-OH-3-keto-sitosterol		4.34	315.7
7keto-sitosterol	11.09	16.9	1753.2
7β-OH-sitosterol	13.36	11.03	1044.4
7α-OH-sitosterol	14.82	6.12	592.6

Tab 4.1 Retention times and yields of single oxidation products (mg) within one HPLC-run or in total.

Separation of the sterol oxides was monitored using UV detection at a wavelength of 206 nm, which is the common wavelength employed for oxysterols (Csallany et al., 1989). Therefore epoxy-compounds, although being formed during the oxidation process, couldn't be isolated with this method, as in general products without double bonds do not absorb well at UV wavelengths (Moreau, 2005).

4.1.4 Purity of the collected oxidation products

For identification and quantification of the collected fractions GC-MS and GC-FID were used, respectively (Soupas et al., 2004b; Lampi et al., 2002).

The applied commercial β-sitosterol was almost pure, but included approximately 10 % of campesterol. Therefore during the heating process campesterol oxides were formed too. In accordance with earlier investigations (Dutta and Appelqvist, 1997) it was not possible to separate campesterol oxides from their sitosterol counterparts during the HPLC run. On average 10 % campesterol oxidation products were present in the corresponding β-sitosterol oxides. However - considering the velocity of our method - we decided to put up with these impurities.

Moreover, high proportions of 7-ketositosterol were found in the 6β-OH-sitosterol-sample. Therefore 6β-OH-sitosterol was excluded from subsequent toxicology testing. Table 4.2 gives detailed information on the purity of the respective compounds.

fraction	purity (%)	impurities (%)	
		campesterol counterpart	others
6β-OH-3-keto-sitosterol/ 6α-OH-3-keto-sitosterol	88	12	
7keto-sitosterol	82	10	8
7β-OH-sitosterol	90	10	
7α-OH-sitosterol	70	10	20

Tab 4.2 Purity of the obtained oxidation products as measured by GC-FID.

4.2 Salmonella microsome assay

For the evaluation of the anti-/mutagenic and anti-/oxidative potential of the collected oxidation products, 7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol, a mixture of 6β-OH-3-ketositosterol/6α-OH-3-ketositosterol (ratio 4:3) and a mixture containing polar β-sitosterol oxides, were tested in Salmonella typhimurium indicator strains TA98, TA100 and TA102 in the Ames test. The preincubation assay was applied for all test runs. According to the literature this is the most sensitive test form, since it allows a closer contact between test compounds and indicator strain, which is an advantage for detecting short- living mutagens (Mortelmans and Zeiger, 2000).

To our knowledge single oxides of β-sitosterol have never been tested before on their effects towards Salmonella typhimurium strains.

4.2.1 Mutagenicity testing

Mutagenicity assays were performed with all three indicator strains (TA98, TA100, TA102) with and without metabolic activation. Results are shown in table 4.3 and 4.4, respectively.

compound	concentration (mg/plate)	TA 98 -S9 revertants/ plate	TA 98 +S9 revertants/ plate	TA 100 -S9 revertants/ plate	TA 100 +S9 revertants/ plate
negative control		22 ±5[b)]	50 ±11[b)]	205 ±65[b)]	199 ±10[b)]
7keto-sitosterol	5	22 ±5[b)]	46 ±4[b)]	236 ±62[b)]	192 ±12[b)]
	1	27 ±5[b)]	52 ±13[b)]	**276 ±40[a)b)]**	191 ±22[b)]
	0.2	25 ±3[b)]	46 ±7[b)]	237 ±72[b)]	187 ±14[b)]
	0.04	22 ±5[b)]	49 ±7[b)]	210 ±60[b)]	**175 ±20[a)b)]**
7β-OH-sitosterol	5	21 ±4[b)]	53 ±6[b)]	202 ±31[b)]	212 ±10[b)]
	1	23 ±3[b)]	48 ±4[b)]	197 ±43[b)]	183 ±18[b)]
	0.2	24 ±8[b)]	46 ±5[b)]	195 ±51[b)]	**175 ±3[a)b)]**
	0.04	24 ±3[b)]	47 ±7[b)]	195 ±36[b)]	182 ±16[b)]
7α-OH-sitosterol	1	**17 ±3[a)b)]**	56 ±3[b)]	220 ±17[b)]	187 ±48[b)]
	0.2	19 ±3[b)]	50 ±9[b)]	209 ±28[b)]	**163 ±6[a)b)]**
	0.04	16 ±8[b)]	46 ±12[b)]	202 ±45[b)]	**176 ±8[a)b)]**
6α-OH-3-keto/ 6β-OH-3-keto -sitosterol	2.5	25 ±3[b)]	46 ±3[b)]	204 ±38[b)]	**179 ±9[a)b)]**
	1	24 ±4[b)]	54 ±6[b)]	212 ±55[b)]	180 ±33[b)]
	0.2	23 ±9[b)]	54 ±9[b)]	217 ±66[b)]	**175 ±5[a)b)]**
	0.04	21 ±4[b)]	49 ±9[b)]	240 ±57[b)]	142 ±26[b)]
mixture	10	22 ±5[b)]	37 ±5[b)]	200 ±56[b)]	**174 ±4[a)b)]**
	5	20 ±2[b)]	44 ±3[b)]	202 ±44[b)]	182 ±13[b)]
	1	22 ±8[b)]	56 ±3[b)]	216 ±59[b)]	**168 ±11[a)b)]**
	0.2	22 ±4[b)]	48 ±5[b)]	204 ±71[b)]	**173 ±9[a)b)]**
	0.04	22 ±6[b)]	46 ±4[b)]	213 ±63[b)]	**173 ±3[a)b)]**
positive control		211 ±15	965 ±74	2553 ±473	468 ±61

Tab 4.3 Overview on the data obtained in the preincubation assay with the strains TA98 and TA100. Numbers of his^+ - revertants are listed as means ± SD ([a)] $p< 0.05$ to negative control, [b)] $p< 0.05$ to positive control). In addition values significantly different to the negative control are coloured in orange.

For all three strains the spontaneous mutation frequency was in accordance with the control levels published (Mortelmans and Zeiger, 2000) and the number of revertants of each sample was significantly lower than the positive control ($p< 0.05$).

In the preincubation assay using strain TA98 the number of his^+- revertants of each sample was in the range of the negative control. Further no concentration dependency was observed. Therefore no mutagenic activity could be observed.

In TA100 number of his^+- revertants which significantly differed to that of the negative control were obtained for individual samples. In assays including metabolic activation, contrary to a rather expected mutagenic indication, a certain reduction of revertant numbers was observed. However, neither a twofold reduction nor a significant dose related decrease was induced. Thus no evidence for a mutagenic or protective effect of phytosterol oxides was seen.
In accordance with Lea et al. (2004), who tested a mixture of oxidized and unoxidized products of β-sitosterol using different Salmonella strains (TA98, TA100, TA102, TA1535 and TA1537), in the strains TA98 and TA100 β-sitosterol oxidation products were mutagenically not active. However, in the mentioned publication only an unspecific mixture containing 30% of phytosterol oxides was used. Differing results for individual oxides and mixtures have already been repeatedly documented (O'Sullivan A et al., 2005; Leonarduzzi et al., 2001).

Also in TA102 none of the phytosterol oxidation products were able to increase the number of his^+ - revertants beyond the doubled negative control, which was set as threshold for mutagenic activity. Yet for 7-ketositosterol, the mixture of 6β-OH-3-ketositosterol/6α-OH-3-ketositosterol and the mixture of polar oxides a significant increase in revertant numbers was observed. Results for the two mixtures, however, were rather inconclusive as no dose relation was found.
In the case of 7-ketositosterol also a concentration-dependent tendency was seen for the lower concentrations but not for the 5%- fraction, mainly due to its higher standard deviation. Moreover, in the test with metabolic activation the increase of 7-ketositosterol (5%) was even more pronounced (figure 4.4).
On the other hand 7β-OH-sitosterol was rather inactive in the normal preincubation assay. In the test approach with metabolic activation a significant reduction of the

number of revertants was detected for the highest concentration of 7β-OH-sitosterol (5%).

Besides its capability of detecting cross-linking agents the indicator strain TA102 additionally informs on oxidative stress (Grey and Adlercreutz, 2003; Mortelmans and Zeiger, 2000). Based on the latter, the increase in revertants induced by 7-ketositosterol (5%) could be an indication of a pro-oxidant tendency. On the other hand the decline in revertant numbers of 7β-OH-sitosterol (5%) could be interpreted as a marginal sign of antioxidative effects. However, it could also be the result of increased cytotoxicity (Mortelmans and Zeiger, 2000).

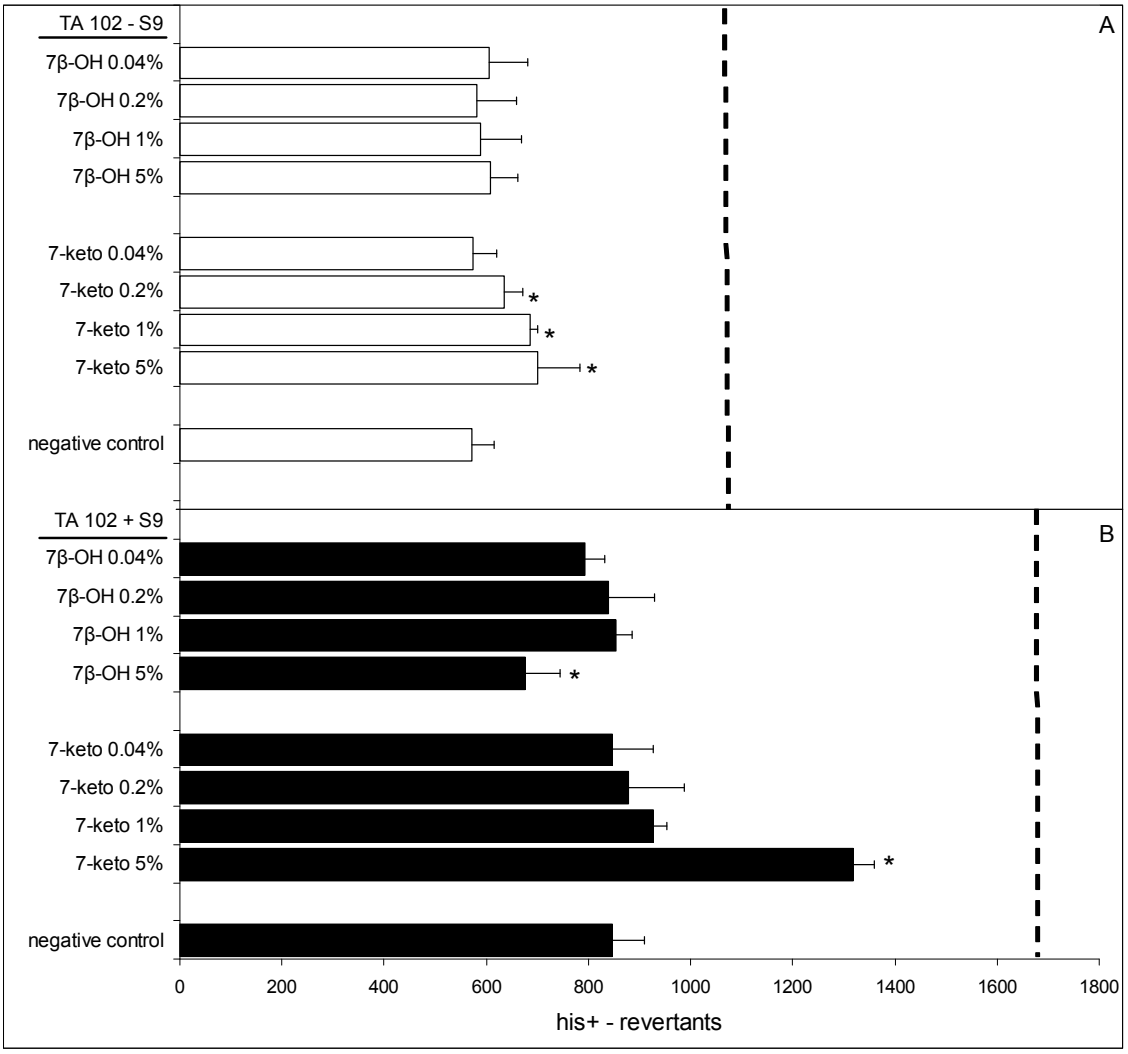


Fig 4.4 Effects of 7-ketositosterol and 7β-OH-sitosterol in TA102 without (A) or with (B) metabolic activation. The dotted lines represent the doubled negative control. Results are expressed as mean + SD (* $p < 0.05$ to negative control).

Publications on the mutagenic properties of plant sterol oxides other than that of Lea et al. (2004) are lacking. However, some evidence exists that mixtures of COPs have mutagenic effects towards Salmonella typhimurium strains (Ansari et al., 1982; Smith et al., 1979). Due to the structural similarity of POPs and COPs analogues biological effects are assumed.

Sevanian and Peterson (1986) investigated pure oxidation products of cholesterol and showed α-epoxide and β-epoxide to be mutagenic in V79 Chinese lung fibroblasts. Both epoxide epimers can be hydrolysed to triol which was not found to be mutagenic but highly cytotoxic. More recently Cheng et al. (2005) investigated three pure COPs, 7-ketocholesterol, 5,6α-epoxy-cholesterol and triol compounds, in the Ames and in the chromosome aberration test. Contrary to the results cited above the mutagenic response of triol was clearly demonstrated, whereas 7-keto- and 5,6α-epoxy-cholesterol were not active. Similar to Smith et al. (1986) the authors suggested ROS to be involved in the mutagenic action since antioxidant enzymes diminished the mutagnic response.

compound	concentration (mg/plate)	TA102 revertants/ plate -S9	TA102 revertants/ plate +S9	TA102+tBOOH revertants/ plate -S9	TA102+tBOOH revertants/ plate +S9
negative control		571 ±44[b)]	847 ±63[b)]	747 ±99[b)]	1589 ±349[b)]
7keto-sitosterol	5	**701 ±82[a)b)]**	**1318 ±41[a)b)]**	**866 ±24[a)b)]**	2038 ±239[b)]
	1	**685 ±16[a)b)]**	926 ±28[b)]	**936 ±66[a)b)]**	1714 ±137[b)]
	0.2	**635 ±36[a)b)]**	879 ±108[b)]	**940 ±75[a)b)]**	1891 ±334[b)]
	0.04	575 ±45[b)]	846 ±81[b)]	**963 ±52[a)b)]**	1974 ±123[b)]
7β-OH-sitosterol	5	607 ±55[b)]	**675 ±68[a)b)]**	**525 ±45[a)b)]**	1374 ±60[b)]
	1	589 ±79[b)]	853 ±32[b)]	776 ±58[b)]	1770 ±146[b)]
	0.2	582 ±76[b)]	840 ±91[b)]	840 ±65[b)]	1679 ±146[b)]
	0.04	606 ±74[b)]	793 ±37[b)]	804 ±71[b)]	1591 ±103[b)]
7α-OH-sitosterol	1	597 ±101[b)]	744 ±97[b)]	684 ±56[b)]	1222 ±156[b)]
	0.2	**510 ±27[a)b)]**	802 ±16[b)]	812 ±36[b)]	1252 ±164[b)]
	0.04	**452 ±103[a)b)]**	**652 ±34[a)b)]**	686 ±111[b)]	1317 ±93[b)]
6α-OH-3-keto/ 6β-OH-3-keto-sitosterol	2.5	**684 ±88[a)b)]**	**1051 ±28[a)b)]**	809 ±51[b)]	**2119 ±90[a)b)]**
	1	**644 ±51[a)b)]**	**979 ±12[a)b)]**	**1031 ±42[a)b)]**	**2082 ±32[a)b)]**
	0.2	609 ±40[b)]	**990 ±29[a)b)]**	801 ±121[b)]	**2338 ±29[a)b)]**
	0.04	**644 ±62[a)b)]**	**953 ±2[a)b)]**	814 ±32[b)]	2065 ±70[b)]
mixture	10	**713 ±60[a)b)]**	**1079 ±107[a)b)]**	**891 ±14[a)b)]**	1501 ±35[b)]
	5	**648 ±94[a)b)]**	**1008 ±54[a)b)]**	797 ±12[b)]	1879 ±158[b)]
	1	598 ±55[b)]	**967 ±82[a)b)]**	842 ±102[b)]	**1997 ±5[a)b)]**
	0.2	556 ±67[b)]	829 ±41[b)]	**902 ±76[a)b)]**	2003 ±19[b)]
	0.04	**625 ±27[a)b)]**	731 ±143 [b)]	851 ±113[b)]	2057 ±175[b)]
positive control		2353 ±422	3480 ±165	2412 ±71	3780 ±271

Tab 4.4 Overview on the data obtained in the preincubation assay with the strain TA102. Numbers of his$^+$ - revertants are listed as means ± SD ([a)] $p< 0.05$ to negative control, [b)] $p< 0.05$ to positive control). In addition values significantly different to the negative control are coloured in orange.

4.2.2 Antioxidant testing

To strengthen the information on oxidative stress challenge tests with the pro- oxidant tertiary-butylhydroperoxide (tBOOH) and the strain TA102 were conducted. tBOOH is a known initiator of lipid peroxidation, where it leads to the formation of alkoxyl and alkyl radicals (figure 4.5). By the use of tBOOH a possible antioxidative action of the respective test compounds due to a radical scavenging mechanism is determined (Grey and Adlercreutz, 2003). Per definition a reduction of the revertant colony number below 50% of the negative control level is necessary for an antioxidative effect (Mortelmans and Zeiger, 2000).

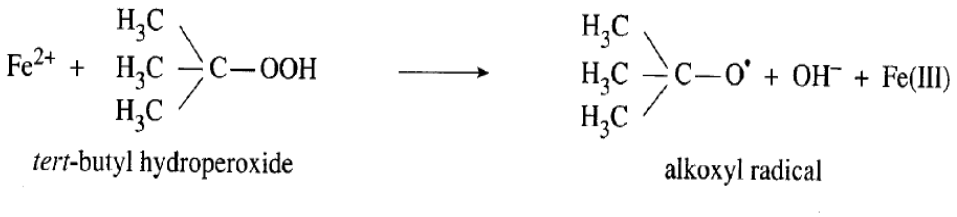


Fig 4.5 Decomposition of tBOOH in the presence of Fe^{2+} (Halliwell and Gutteridge, 1999a)

After treatment with 7-ketositosterol number of his^+ - revertants of all tested concentrations differed significantly from those obtained for the solvent control. Lowest number of revertants was obtained after incubation with the highest concentrated fraction (5%). However, since revertant numbers were still higher than the negative control no indication for an antioxidative effect was seen. In the test approach with metabolic activation an increase in the number of his^+ - revertants for all concentrations of 7-ketositosterol, but in particular for the highest concentrated fraction (5%), was observed. Yet no significant results were obtained (figure 4.6).

The significant reduction of the number of his^+ - revertants of 7β-OH-sitosterol (5%) observed in the preincubation assay with TA102 was even more pronounced by the addition of tBOOH. However, no dose response was found. Therefore no antioxidative effect could be proven, even though an indication for a weak antioxidative behaviour

can be presumed. However, with the addition of S9 this antioxidative property was attenuated (figure 4.6).

Results for 7α-OH-sitosterol, the mixture of 6β-OH-3-ketositosterol/6α-OH-3-ketositosterol and the mixture containing polar β-sitosterol oxides were, despite some significantly increased values, rather inconclusive.

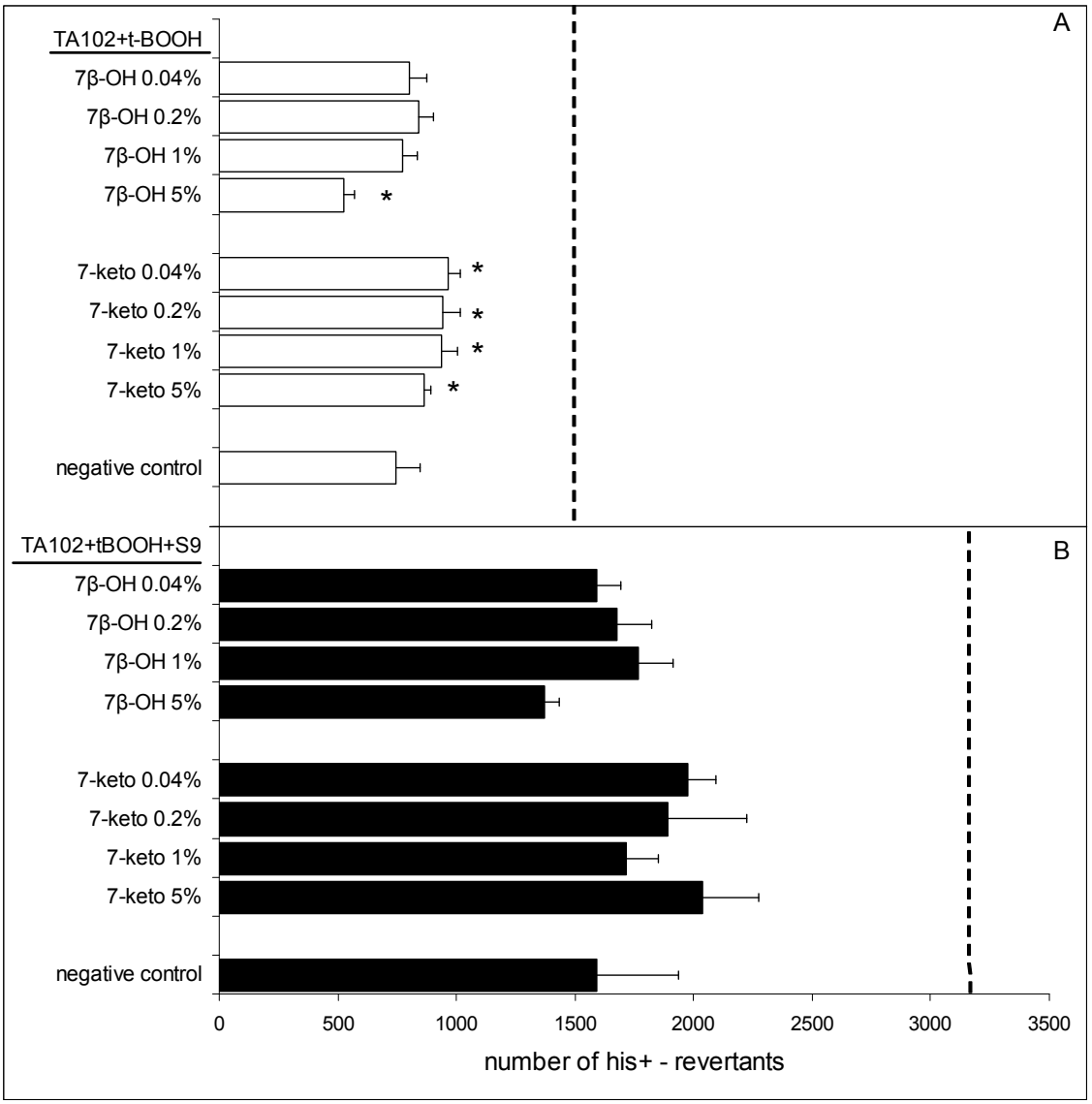


Fig 4.6 Effects of 7-ketositosterol and 7β-OH-sitosterol in the challenge test using tBOOH and TA102 without (A) or with (B) metabolic activation. The dotted lines represent the doubled negative control. Results are expressed as mean + SD (* $p < 0.05$ to negative control).

4.3 Cell culture assays

Cytotoxicity of the phytosterol oxides 7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol, a mixture of 6β-OH-3-keto-sitosterol/6α-OH-3-keto-sitosterol (ratio 4:3) and a mixture of polar oxides was examined by the use of HepG2-cells. 3 different concentrations (30 µM, 60 µM und 120 µM) of each oxidation products as well as an incubation period of 24 hours were chosen for all experiments in order to guarantee uniformity and comparability.

4.3.1 Viability assays

For evaluation of cell viability after treatment with oxide samples the trypan blue exclusion assay and the MTT test were applied, respectively. Similar results for both test approaches were obtained.

4.3.1.1 Trypan blue exclusion assay

Using the trypan blue exclusion assay incubation with the test compounds resulted in a significant decrease in cell numbers relative to the negative control ($p \leq 0.001$, except 7β-OH-sitosterol). Significant concentration dependencies were found for 7α-OH-sitosterol ($p \leq 0.001$), 7-ketositosterol ($p \leq 0.01$) and the mixture ($p \leq 0.05$). The order of cytotoxicity at the highest concentration tested (120 µM) was 7α-OH-sitosterol > 7-ketositosterol > 6α-OH-3-keto/6β-OH-3-keto-sitosterol = mixture > 7β -OH-sitosterol (figure 4.7).

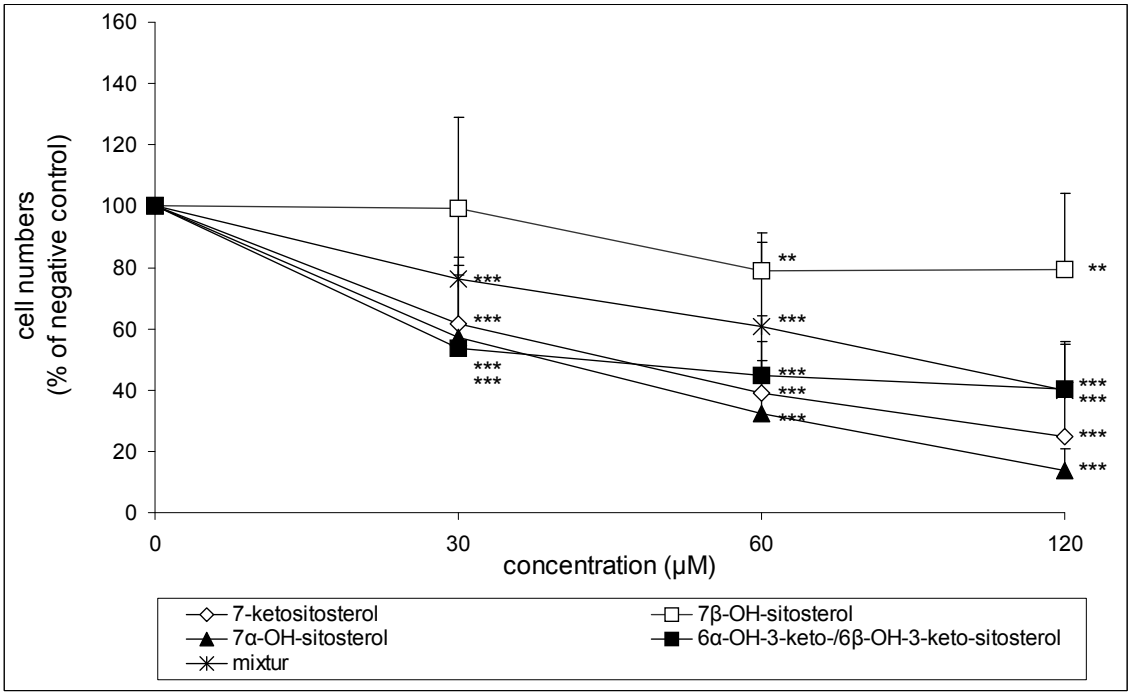


Fig 4.7 Cell numbers measured by the TB-assay after 24 h of incubation with β-sitosterol oxides, expressed as percentage of negative control. Results are expressed as mean + SD (* $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ to negative control).

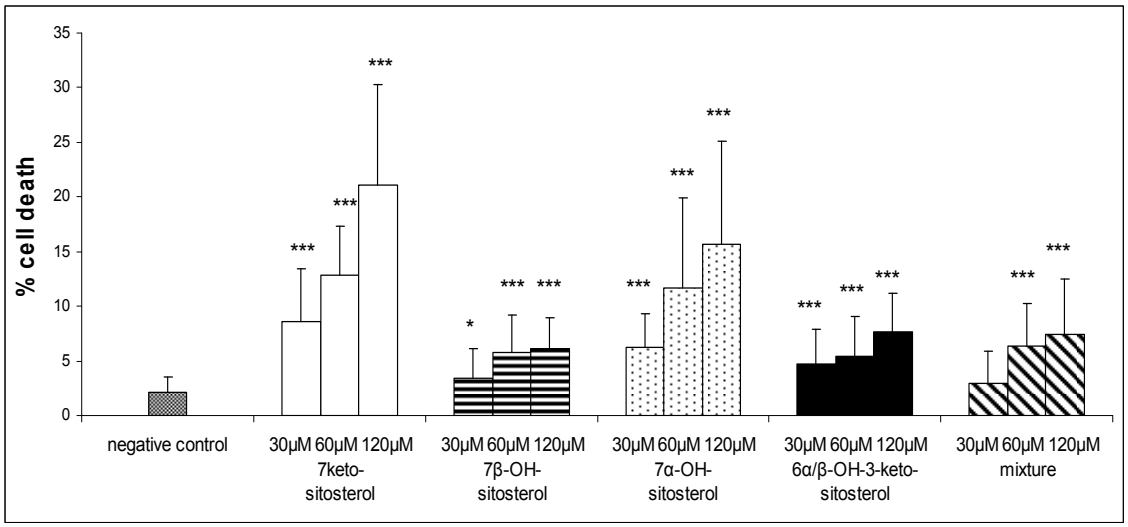


Fig 4.8 Percentage of cell death measured by the TB-assay after 24h of incubation with β-sitosterol oxides. Results are expressed as mean + SD (* $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ to negative control).

When the percentage of cell death in control and treated cells was assessed a significant ($p \leq 0.001$, for 7β -OH-sitosterol (30 μM): $p \leq 0.05$) increase for all compounds, except for the mixture at 30 μM ($p = 0.298$), could be seen. Significant dose response was only observed for 7-ketositosterol ($p \leq 0.01$). At all three concentrations tested the increase in cell death was particularly pronounced for 7-keto-sitosterol and 7α-OH-sitosterol (figure 4.8).

4.3.1.2 MTT- assay

Applying the MTT assay a significant reduction of metabolically active cells compared to the negative control was observed for all sterol oxides ($p \leq 0.001$, for 7β -OH-sitosterol $p \leq 0.01$) tested (figure 4.9). Further concentration dependencies ($p \leq 0.001$) were found for 7α-OH-sitosterol, 6α-OH-3-keto/6β-OH-3-keto-sitosterol and the mixture. Also for 7β -OH-sitosterol and 7-ketositosterol cell viability after treatment with 30 μM of the respective oxides differed significantly ($p \leq 0.001$) from that obtained after incubation with concentrations of 60 μM or 120 μM. However, no significant differences were observed between the results of 60 μM and 120 μM treatment.

Similar to the results of the trypan blue exclusion assay 7α-OH-sitosterol appeared to be the most toxic compound, followed by 6α-OH-3-keto/6β-OH-3-keto-sitosterol and 7-ketositosterol. At the highest concentration tested (120 μM) 7α-OH-sitosterol reduced viability of the cells to 23 % of the control level. The order of cytotoxicity at this concentration was 7α-OH-sitosterol > 6α-OH-3-keto/6β-OH-3-keto-sitosterol > 7-ketositosterol > mixture > 7β -OH-sitosterol.

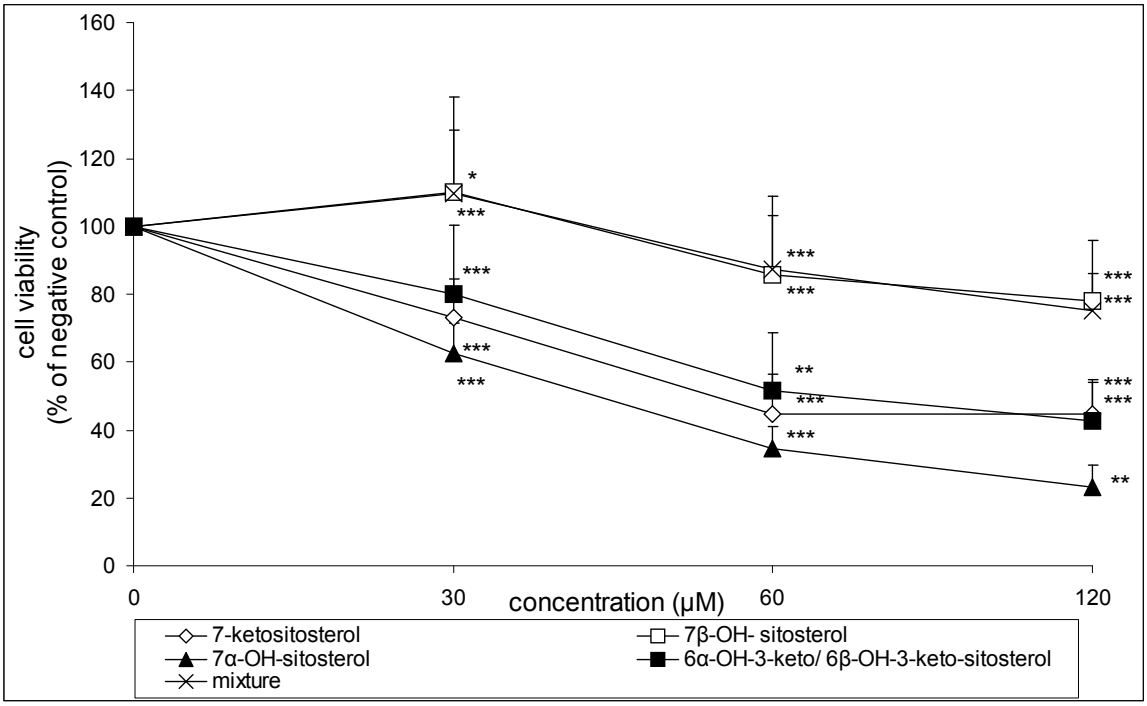


Fig 4.9 Cell viability measured by the MTT-assay after 24h of incubation with β-sitosterol oxides, expressed as percentage of negative control. Results are expressed as mean + SD (* $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ to negative control).

Thus results of the trypan blue assay were consistent with those obtained by the MTT assay (7-ketositosterol: $r = 0.609$, $p \leq 0.001$; 7β -OH-sitosterol: $r = 0.389$, $p \leq 0.001$; 7α-OH-sitosterol: $r = 0.637$, $p \leq 0.001$; 6α-OH-3-keto/6β-OH-3-keto-sitosterol: $r = 0.235$, $p \leq 0.05$; mixture: $r = 0.466$, $p \leq 0.001$). In both test approaches viability of the cells was significantly reduced after incubation with increasing concentrations of β-sitosterol oxides, which corresponds to earlier studies on phytosterol oxides (Maguire et al., 2003; Adcox et al., 2001; Ryan et al., 2005; Roussi et al., 2005).

Ryan et al. (2005) investigated several β-sitosterol oxidation products (7β-OH, 7-keto, sitostanetriol, 5α, 6α-epoxide, 5β, 6β-epoxide and a mixture of 5α, 6α-epoxide/5β, 6β-epoxide (6:1)) and their corresponding oxycholesterols in three cell lines including HepG2-cells. In contrast to our results none of the tested phytosterol oxides caused significant cell damage at a concentration of 30 µM. Further 7β-OH- and 7-ketositosterols turned out to be the compounds exerting the highest cytotoxicity.

Conversely, in the present study 7β-OH-sitosterol appeared to have the lowest effect on the viability of cells. This corresponds to the investigation of Roussi et al. (2005), who found a reduction in cell numbers through treatment with 7β-OH-sitosterol only after incubation periods longer than 24 h.

Regarding both viability assays conducted the mixture had the second lowest impact on HepG2-cells. Based on several investigations on COPs (Leonarduzzi et al., 2001; Aupeix et al., 1995; Biasi et al., 2004), mixtures of oxysterols are thought to be less toxic towards cells than isolated oxides.

No information on the cytotoxic properties of 6α-OH-3-keto- and 6β-OH-3-keto-compound of phytosterols and cholesterol towards cell lines exist. Compared to the other oxides tested moderate inhibition of cell proliferation was observed. Due to the lack of comparative data no further conclusion can be drawn.

Consistently 7-ketositosterol and 7α-OH-sitosterol emerged as those compounds exerting the strongest reduction of cell viability. The toxic potential of 7-ketositosterol, was already confirmed in various in vitro assays (Ryan et al., 2005), whereas so far data on 7α-OH-sitosterol is lacking. Contrary to the present findings its cholesterol counterpart was found to exhibit less than or equal toxicity to 7β-OH-cholesterol (Clare et al., 1995). However, individual 7α-OH-products of plant sterols have never been tested before.

4.3.2 Detection of apoptosis

To elucidate whether apoptosis was involved in the reduction of cell numbers flow cytometry was conducted. Apoptosis is characterised by morphological changes as shrunk cell volume, condensed chromatin and finally the formation of apoptotic bodies (Samali et al., 1999). These morphological alterations affect the light scattering properties of cells. Therefore the analysis of scattered signals as done by flow cytometry allows information on cell size and structure. Decreased forward scatter (cell size) and increased side scatter (granularity) are characteristic of apoptotic cells (Koopman et al., 1994; Tuschl and Schwab, 2003). Such subpopulations were observed after treatment of cells with 60 and 120 µM of 7-ketositosterol, 7α-OH-sitosterol and 6α-OH-3-keto/6β-OH-3-keto-sitosterol, thus indicating apoptotic cell death.

To further clarify the mode of cell death double staining with PE-labelled AnnexinV and 7-AAD was conducted. In the presence of Ca^{2+} ions Annexin V attaches specifically to phosphatidylserine, which is translocated from the inner to the outer cell surface during the early phase of apoptosis (Vermes et al., 2000).

Of all tested compounds only 7-ketositosterol (30 µM) was able to increase (+ 45 %) the proportion of AnnexinV positive cells compared to the negative control (figure 4.10). This increase, however, was not significant. Treatment with higher concentrations of 7-ketositosterol led to a reduction in AnnexinV positive cells. Also for all other oxides a decrease in the proportions of AnnexinV positive cells with increasing concentration of test samples was found. The present observation indicates chosen incubation conditions being too intense for detecting early apoptotic events. This corresponds to findings of Leonarduzzi et al. (2002) who observed that 7-ketocholesterol induced apoptosis at low concentrations whereas at higher sample amounts necrosis was the predominate mode of cell death.

At the time point investigated higher percentages of necrotic or late apoptotic than early apoptotic cells were detected. Therefore it could either be that apoptosis was already initialised at earlier timepoints and the detected results display post apoptotic necrosis or that tested oxides induced generally necrosis in HepG2-cells.

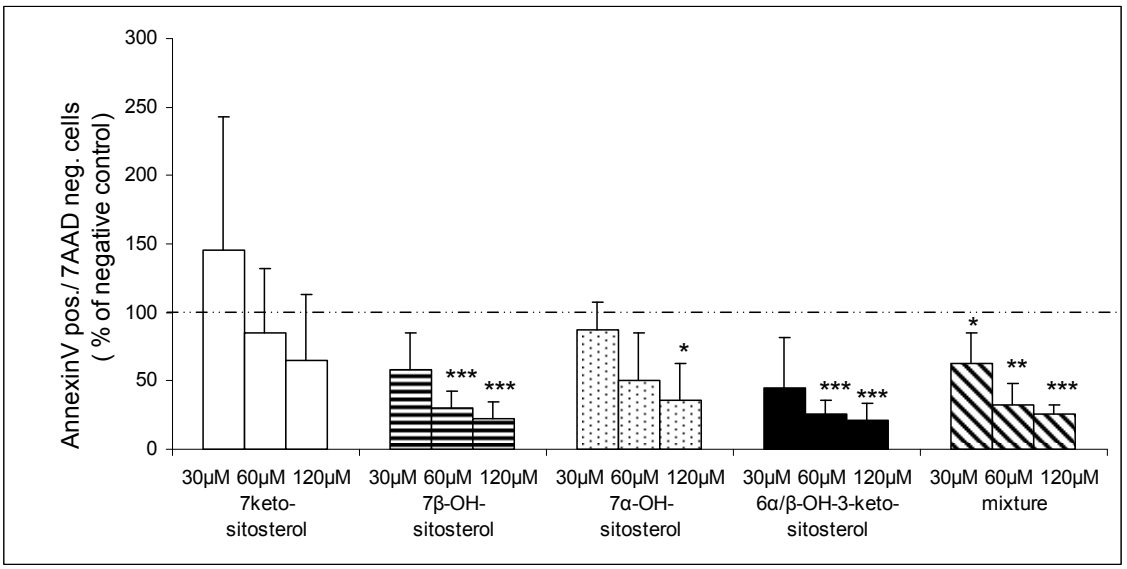


Fig 4.10 Effects of β-sitosterol oxides on phosphatidlyserine externalization in HepG2-cells. Values are mean ± SD of 3 separate experiments and expressed as percentage of

negative control (dotted line). Columns significantly different to control levels are marked with * for $p \leq 0.05$, ** for $p \leq 0.01$ or *** for $p \leq 0.001$.

The induction of apoptosis by POPs has already been determined previously (Roussi et al., 2005; Ryan et al., 2005; Maguire et al., 2003). Increase of apoptotic cells, assessed by morphological analysis and determination of DNA fragmentation, was found for 7-keto- and 7β-OH-sitosterol in U937 cells, while no signs of apoptosis were ascertained in HepG2 and CaCo2-cells, respectively (Ryan et al., 2005).
In contrast, Roussi et al. (2005) observed apoptosis, as measured by caspase activation and DNA fragmentation, in CaCo2-cells after treatment with 7β-OH-sitosterol using same sample concentrations.
In our study increase in apoptotic cells was observed only after incubation with 7-ketositosterol, but not with 7β-OH-sitosterol. However, considering the different methods applied, no firm comparison can be drawn.
Phosphatidylserine exposure has not been tested before with phytosterol oxides. Concerning cholesterol, 7-keto- and 7β-OH- and 5,6β-epoxy-cholesterol were found to induce an increase in AnnexinV positive cells (Lemaire-Ewing et al., 2005). In contrast to our study in the case of cholesterol oxides U937 cells were used. Differences in the toxicity of oxysterols according to the applied cell lines have already been reported before (Miguet et al., 2001).

4.4 Detection of oxidative stress

Oxidation products of cholesterol were found to rapidly increase $O_2^{\bullet-}$ production (Monier et al., 2003; Miguet-Alfonsi et al., 2002; Lemaire-Ewing et al., 2005), which in general induces cell death. Therefore collected β-sitosterol oxides were tested for their oxidative properties. As $O_2^{\bullet-}$ constitutes only one type of ROS the measurement of MDA as a second maker for oxidative stress was included.

4.4.1 Measurement of superoxide anions ($O_2^{\bullet-}$) production

The generation of $O_2^{\bullet-}$ was quantified by flow cytometry after staining with HE. For all tested compounds a significant overproduction of $O_2^{\bullet-}$ was observed (figure 4.11). Highest activity, however, was assessed for 7-ketositosterol (+56 %) and the mixture (+54 %) at a concentration of 30 µM. No significant concentration dependency was found.

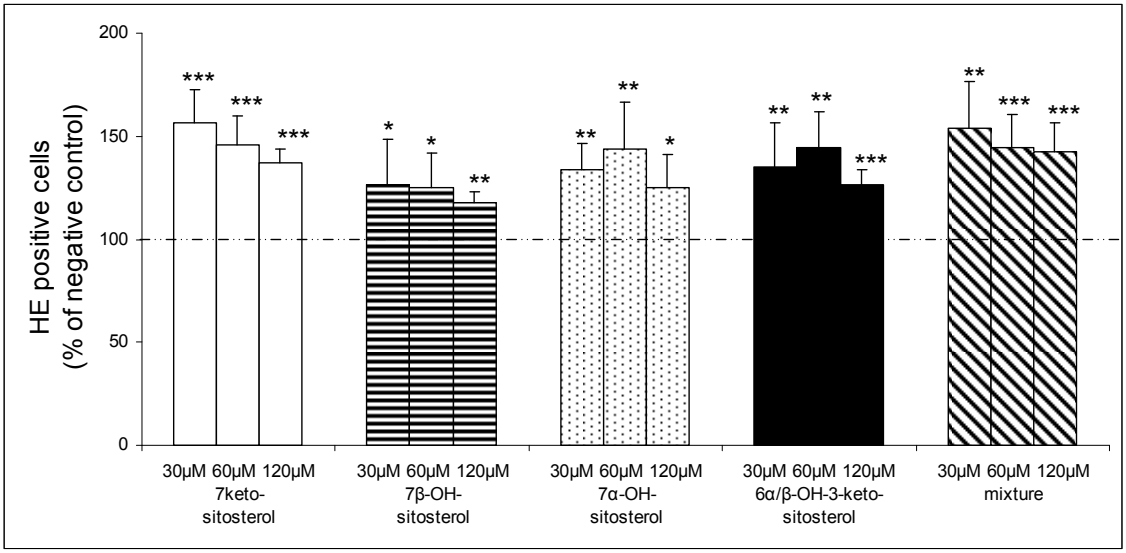


Fig 4.11 Effects of β-sitosterol oxides on superoxide anion production in HepG2-cells. Values are mean ± SD of 3 separate experiments and expressed as percentage of negative control (dotted line). Columns significantly different to control levels are marked with * for $p \leq 0.05$, ** for $p \leq 0.01$ or *** for $p \leq 0.001$.

4.4.2 Measurement of lipid peroxidation (MDA)

The effect of oxysterols on lipid peroxidation was investigated by HPLC-detection of MDA.

A concentration dependent increase in MDA-levels was observed after treatment of cells with 7-ketositosterol, 7α-OH-sitosterol and 6α-OH-3-keto-/6β-OH-3-keto-sitosterol (figure 4.12). At the highest concentration tested (120µM) MDA levels of 194 % (7-ketositosterol), 298 % (7α-OH-sitosterol) and 140 % (6α-OH-3-keto/6β-OH-3-keto-sitosterol) of the negative control were found. On the other hand 7β -OH-sitosterol

and the mixture had no effect on lipid peroxidation, as MDA levels in the range of the negative control were detected.

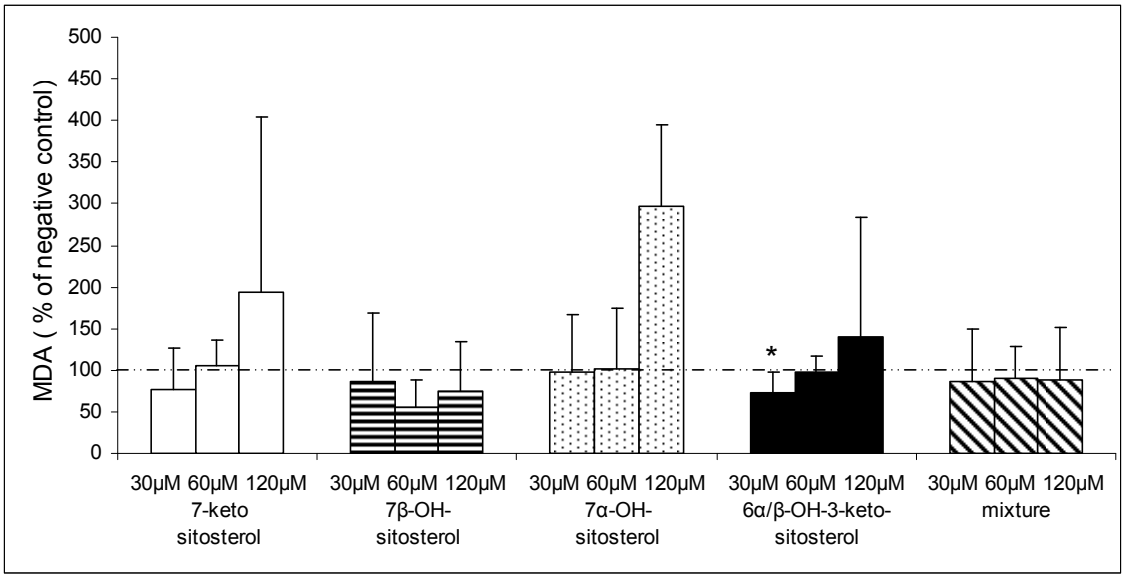


Fig 4.12 Effects of β-sitosterol oxides on Malondialdehyde formation in HepG2-cells. Values are mean ± SD of 3 separate experiments and expressed as percentage of negative control (dottet line). Columns significantly different to control levels are marked with * for $p \leq 0.05$.

Only limited data exist concerning the oxidative properties of plant sterol oxides. Depletion of glutathione levels, but no effect on catalase activity was found in U937 cells after incubation with a mixture of β-sitosterol oxides (Maguire et al., 2003). Similar Ryan et al. (2005) observed a significant decrease in glutathione after treatment with 7β -OH-sitosterol (120 µM), but neither β-carotene nor α- and γ-tocopherol could protect against POP induced damage. Comparing the oxidative effects of 7β-OH-sitosterol and -cholesterol in CaCo2-cells, in contrast to the oxycholesterol the sitosterol derivate was found to diminish the intracellular production of reactive oxygen species (Roussi et al., 2005).

Intracellular $O_2^{\bullet-}$ production induced by phytosterol oxides has never been measured before. In line with previous results on cholesterol oxides (Lemaire-Ewing et al., 2005; Miguet-Alfonsi et al., 2002; Monier et al., 2003) all tested oxidation products showed a significant increase in $O_2^{\bullet-}$.

Data on lipid peroxidation caused by POPs are lacking. Concerning cholesterol oxides an increase in MDA levels was found for 7-keto- and 7β-OH-cholesterol, while 7α-OH-cholesterol showed to be rather ineffective (Miguet-Alfonsi et al., 2002). In contrast to the mentioned publication and in order to improve the accuracy of the results in the present study MDA was detected by HPLC rather than by the more commonly used TBARS assay, which has already been criticized for insufficient specificity and validity (Halliwell and Whiteman, 2004). Nevertheless, the increase in MDA levels induced by 7-ketocholesterol was in line with our results. Data for 7α-OH- and 7β-OH- sitosterol, however, differed from those observed for its cholesterol counterparts, as in our case 7α-OH-sitosterol, in particular at a concentration of 120 µM, proved to strongly increase MDA levels.

However, in line with earlier publications no correlation could be found between the increase in lipid peroxidation and the generation of $O_2^{\bullet-}$ (Hall, 2006). $O_2^{\bullet-}$, contrary to many other ROS, seems to be relatively unreactive with lipids (Halliwell and Gutteridge, 1999b).

Investigations on plant sterol and cholesterol oxides have shown that cytotoxicity was not necessarily associated with enhanced oxidative stress (Roussi et al., 2007; Lemaire-Ewing et al., 2005). Also in our study differences in the cytotoxic effects of the single oxides couldn't entirely be explained by their ability to induce oxidative stress. Yet 7-keto- and 7α-OH-sitosterol, as those compounds displaying the strongest effect on cell viability could enhance both $O_2^{\bullet-}$ and levels of lipid peroxidation. Though it is also possible that lipid peroxidation was induced through necrosis as a secondary event.

4.5 Detection of DNA damage

Since ROS have the potential to interact with cellular components including DNA the capability of phytosterol oxides to induce DNA strand breaks was also assessed using the alkaline single-cell gel electrophoresis assay.

As presented in figure 4.13 applied oxidation products were rather inactive. For all compounds tested measured % DNA in tail were in the range of the negative control. Therefore no significant increase of DNA damage could be observed. Further standard derivations were found to be very high. Accordingly, in line with reports on the

genotoxic action of COPs (Maguire et al., 2003) no increased DNA damage was observed.

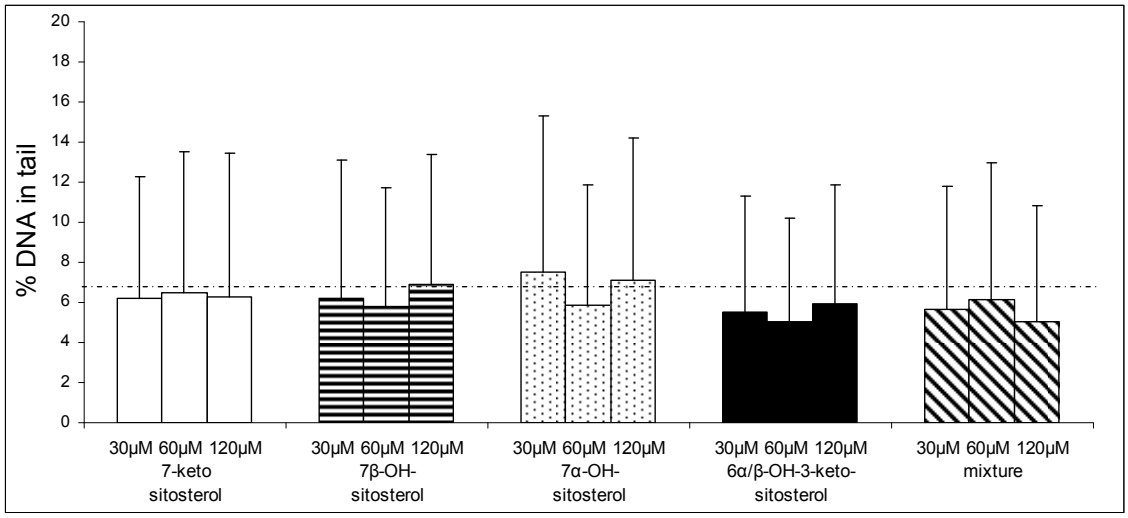


Fig 4.13 Effects of β-sitosterol oxides on DNA in HepG2-cells. Values are mean ± SD of 2 separate experiments. Dotted line represents the level of solvent control.

4.6 Assessment of phytosterol oxidation products' uptake in HepG2-cells

For a better discussion of the obtained results it was of utmost importance to know whether the employed oxidation products were absorped by the cells. A confirmation of their accumulation would substantiate the observed cytotoxic actions of the tested POPs.

4.6.1 Extraction of phytosterol oxides

Extraction of oxides from cells and media was done according to Tian et al. (2006). In our case, however, diethyl ether was used instead of chloroform/methanol.

In agreement with Palozza et al. (2007) no time consuming sample pre-treatment other than dissolving and filtration was needed as no interfering peaks were present in the subsequent HPLC analysis. Hence, unwanted sample losses caused by several purification steps could be prevented (Abidi, 2001).

4.6.2 Optimization of the HPLC conditions for the separation of oxidized β-sitosterol compounds

Complete separation of sterol oxidation products and other cell lipid residues was achieved on a silica column by an isocratic elution system of n-heptane/2-propanol. Different mobile phase systems with changing percentages of 2-propanol were tested based on former investigations by Kemmo et al. (2005; 2007; Säynäjoki et al., 2003). Best resolution within 20 min was achieved with a mobile phase of n-heptane/2-propanol (97/3, v/v) at a flow rate of 0.6 mL/min. Standard solutions of 7-ketocholesterol, 7β-OH-cholesterol and 7α-OH-cholesterol were used since standards of β-sitosterol oxidation products do not exist. Their separation is given in figure 4.14.

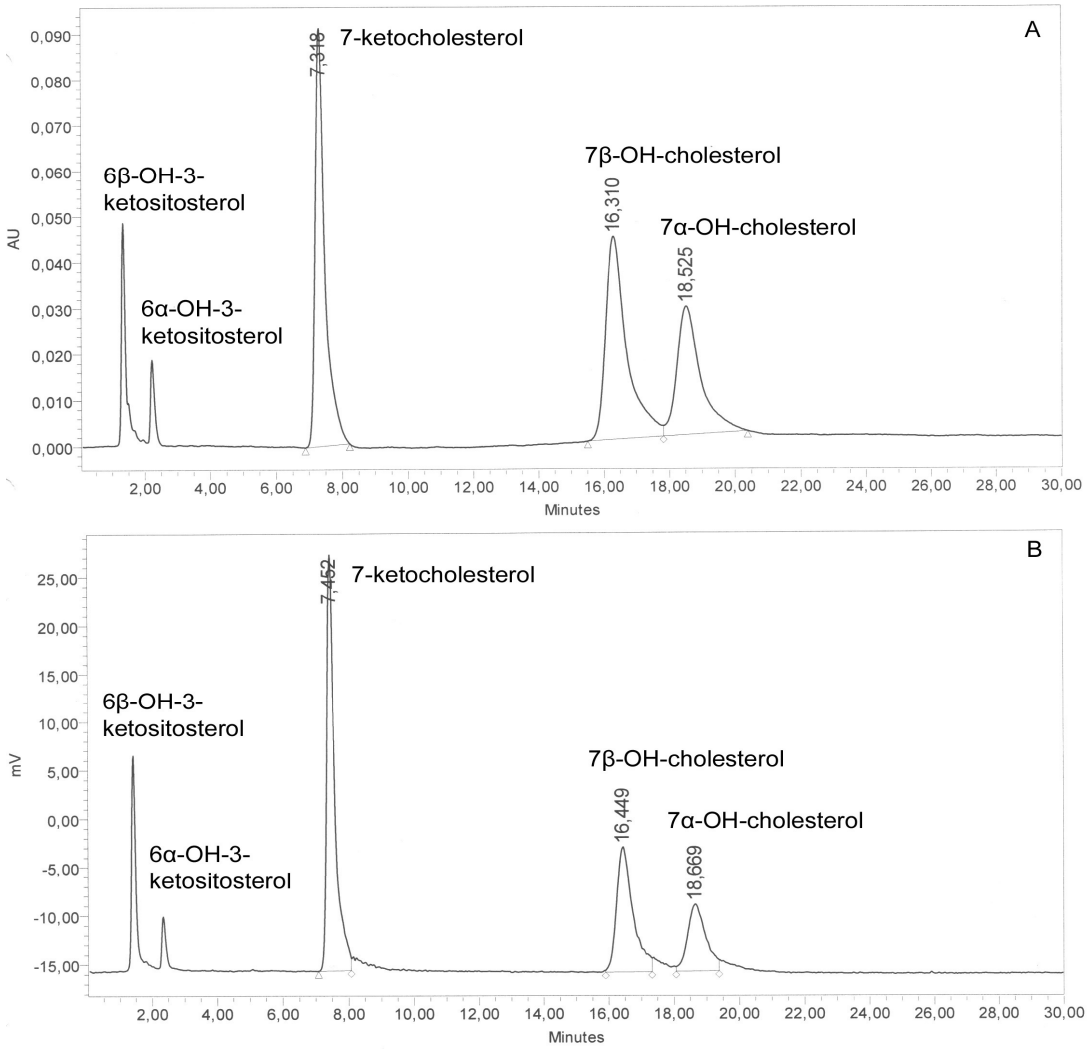


Fig 4.14 NP-HPLC chromatogram of cholesterol standards, 6β-OH-3-ketositosterol and 6α-OH-3-ketositosterol with (A) UV detection at 206nm and (B) ELS-detection.

As neither cholesterol nor plant sterol 6β-OH-3-keto- and 6α-OH-3-ketoproducts are available, laboratory prepared sitosterol compounds, used for the cell incubation procedure, were employed. In order to ascertain coelutions of oxysterols and other lipids of the cell membrane, lipid extracts derived from HepG2-cells were injected together with standard solutions as well.

The elution order in this system was as follows: 6β-OH-3-ketositosterol, 6α-OH-3-ketositosterol, 7-ketocholesterol, 7β-OH-cholesterol and 7α-OH-cholesterol.

4.6.3 UV-Detection

Although for single oxysterols their maximum UV absorption was observed at different wavelengths (Osada et al., 1999; Kermasha et al., 1994), 206nm is the common wavelength employed for sterol oxidation products (Csallany et al., 1989). Therefore UV detection performed at 206nm was used for quantification of sterol oxides.

To enable highly accurate results identification and quantification of the sample peaks was done with cholesterol standards of the corresponding oxidation products. As 6β-OH-3-keto- and 6α-OH-3-ketocholesterol standards were not available amounts had to be estimated with the help of 7-ketocholesterol. In general UV absorption depends on the number of double bonds in the structure of the analyte. Hence, inaccuracies are possible when standards with a different number of double bonds are used (Kemmo et al., 2005). As β-sitosterol and cholesterol oxides contain the same number of double bonds, misinterpretations were prevented.

4.6.4 ELS-Detection

For optimal signal to noise ratio, both gas flow rate and drift tube temperature have to be adjusted on the respective analyte (Moreau, 2005). Therefore different gas pressures (20, 30, 40 and 50 PSI) and temperatures (45 °C/36 °C, 50 °C/42 °C, 55 °C/42 °C, 60 °C/42 °C, 55 °C/48 °C and 60 °C/48 °C for drift tube/nebulising temperature) were tested. Lower gas pressures led to slightly bigger peak areas, but as too low pressures could cause contamination of the detector 20 PSI were chosen for the subsequent analysis. In accordance with Lakritz and Jones (1997) only minor changes were

observed by increasing the temperature. Therefore the initial adjustment, a nebulising temperature of 42 °C and a drift tube temperature of 50 °C, were maintained.
For the analysis of cholesterol and its oxidation products also higher temperatures have been applied (Lakritz and Jones, 1997; Osada et al., 1999), but here mobile phases with higher proportions of polar solvents were used, which require higher evaporating temperatures (Nair et al., 2006). In general the lowest temperature that allows acceptable low noise baseline response and the detection of all analytes of interest should be chosen (Young and Dolan, 2004).

4.6.5 Linearity of UV and ELSD responses

For assessment of linearity triplicate injections of the standard solution containing 7-ketocholesterol, 7β-OH-cholesterol and 7α-OH-cholesterol were processed. As shown in table 4.5 good linearity was observed for UV-detection with a determination coefficient (r^2) between 0.996 and 0.998 at a concentration range of 0.3-9.4 µg (6 calibration points).
The response of the ELSD has previously been described as sigmoidal or exponential (Lakritz and Jones, 1997). Correspondingly, a non linear respond, especially at lower concentrations, was found for the here tested standard mixture. After log transformation of peak area versus standard concentration a linear respond of r^2 = 0.997 - 0.998 was observed for the same concentration range as tested in UV mode (table 4.5). Described data transformation is well established in literature (Lakritz and Jones, 1997; Heron et al., 2004).

4.6.6 Limits of detection

LOD were calculated on the basis of a signal to noise ratio of 3 by the use of standard dilutions. With UV detection the LODs were in the range of 5-20.8 ng, with 7-ketocholesterol being the most sensitive (Csallany et al., 1989). In accordance with earlier publications (Osada et al., 1999) ELS-detection was less sensitive. The LODs for the single oxysterols were found to be about 12 times higher, between 62.5 and 250 ng (table 4.5).

	compounds	retention times (min)	linearity range (µg)	determination coefficient (r^2)	LOD (ng)
UV	7-ketocholesterol	7.28	0.29 - 10.1	0.998 ± 0.002	5
	7β-OH-cholesterol	16.28	0.35 - 11.9	0.998 ± 0.001	15.6
	7α-OH-cholesterol	18.61	0.28 - 9.4	0.996 ± 0.001	20.8
ELSD	7-ketocholesterol	7.41	0.29 - 10.1	0.998 ± 0.001	62.5
	7β-OH-cholesterol	16.4	0.35 - 11.9	0.998 ± 0.001	250
	7α-OH-cholesterol	18.74	0.28 - 9.4	0.997 ± 0.002	250

Tab 4.5 Retention times, linearity ranges, determination coefficients and detection limits assessed for HPLC - UV and - ELS-detection.

4.6.7 Detection and quantification of β-sitosterol oxides in cells and corresponding media

Due to the higher sensitivity of UV detection and the relatively low concentration of oxidation products applied for cell assays only UV results were used for quantification. To our knowledge so far no information on the uptake of oxyphytosterols in cells is published, even though uptake data is necessary to support information on a dose-response. However, similar to investigations on COPs (Biasi et al., 2004) a concentration dependent increase in sterol compounds inside the cells after incubation with increasing sample concentrations could be clearly demonstrated (figure 4.15).

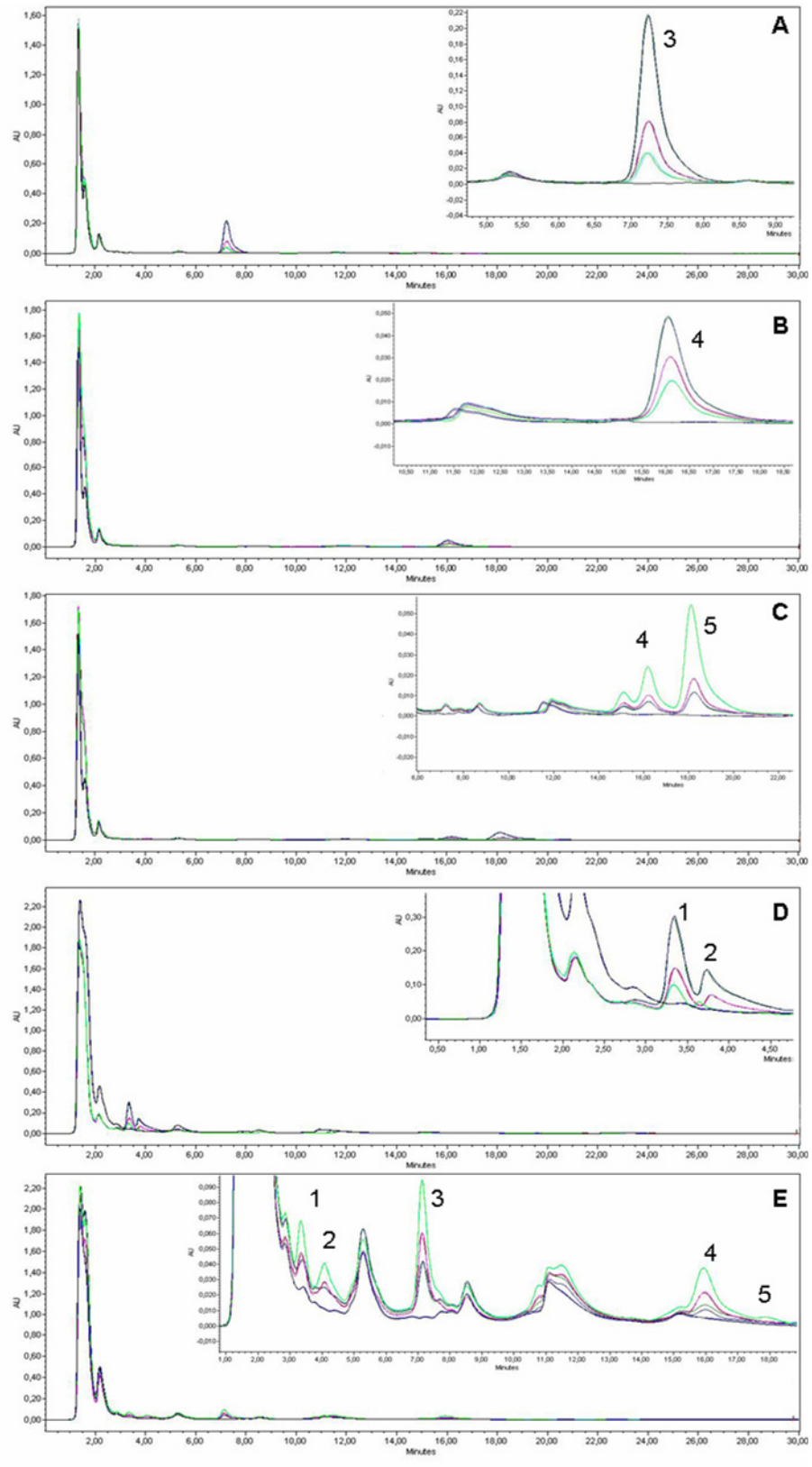


Fig 4.15 HPLC-UV profiles of β-sitosterol oxidation products extracted from HepG2-cells after incubation with 0, 30, 60 and 120 µM of 7-ketositosterol (A), 7β-OH-

sitosterol (B), 7α-OH-sitosterol (C), 6α-OH-3-keto/6β-OH-3-keto-sitosterol (D) and the mixture of polar oxides (E). Peaks: 1=6β-OH-3-keto-sitosterol, 2=6α-OH-3-keto-sitosterol, 3=7-ketositosterol, 4=7β-OH-sitosterol, 5=7α-OH-sitosterol.

Further for 7-ketositosterol, 7β-OH-sitosterol and 7α-OH-sitosterol a significant ($p \leq 0.05$) raise in intracellular concentrations of the single oxidation products was found. Similar to Miguet et al. (2001) for oxycholesterols different absorption affinities of the single oxyphytosterols were observed. Considering the distribution pattern of the POPs between cell and media compartments corresponding trends could be found irrespective of the added concentration of the oxidation product. Whereas for 7-ketositosterol and 7α-OH-sitosterol after 24 hours the majority of the detected oxides (~ 70 %) was found inside the cells, for 7β-OH-sitosterol no definite tendency was observed. For the mixture of 6α-OH-3-keto- and 6β-OH-3-keto-sitosterol only 30 % of the α-compound was detected inside the cells, whereas for the β-counterpart similarly 50 % were assessed in the cells and the media, respectively. Considering the combined uptake as a mixture of these two compounds, larger parts (55-60 %) of the oxidation products were located in the media (for details see figure 4.16).
Interestingly, for those compounds exhibiting a higher reactivity in the various cell assays also higher amounts of oxidation products inside the cells than in the corresponding media were detected (7-keto- and 7α-OH-sitosterol), whereas for the rather inactive 7β-OH-sitosterol no specific uptake trend was observed.
Due to the very small amount of the single oxides present in the mixture a precise quantification was not possible.

Further the obtained uptake data correlates negatively with the outcomes of the MTT- ($r = -0.667$, $p \leq 0.05$ for 7-ketositosterol; $r = -0.874$, $p \leq 0.01$ for 7β-OH-sitosterol; $r = -0.781$, $p \leq 0.01$ for 7α-OH-sitosterol; $r = -0.655$, $p \leq 0.05$ for 6α-OH-3-keto-/6β-OH-3-keto-sitosterol) and the TB-test ($r = -0.689$, $p \leq 0.05$ for 7-ketositosterol; $r = -0.823$, $p \leq 0.01$ for 7α-OH-sitosterol), respectively. Accordingly, a link between differences in absorption levels of the single oxides and their diverse toxic effects may be possible.

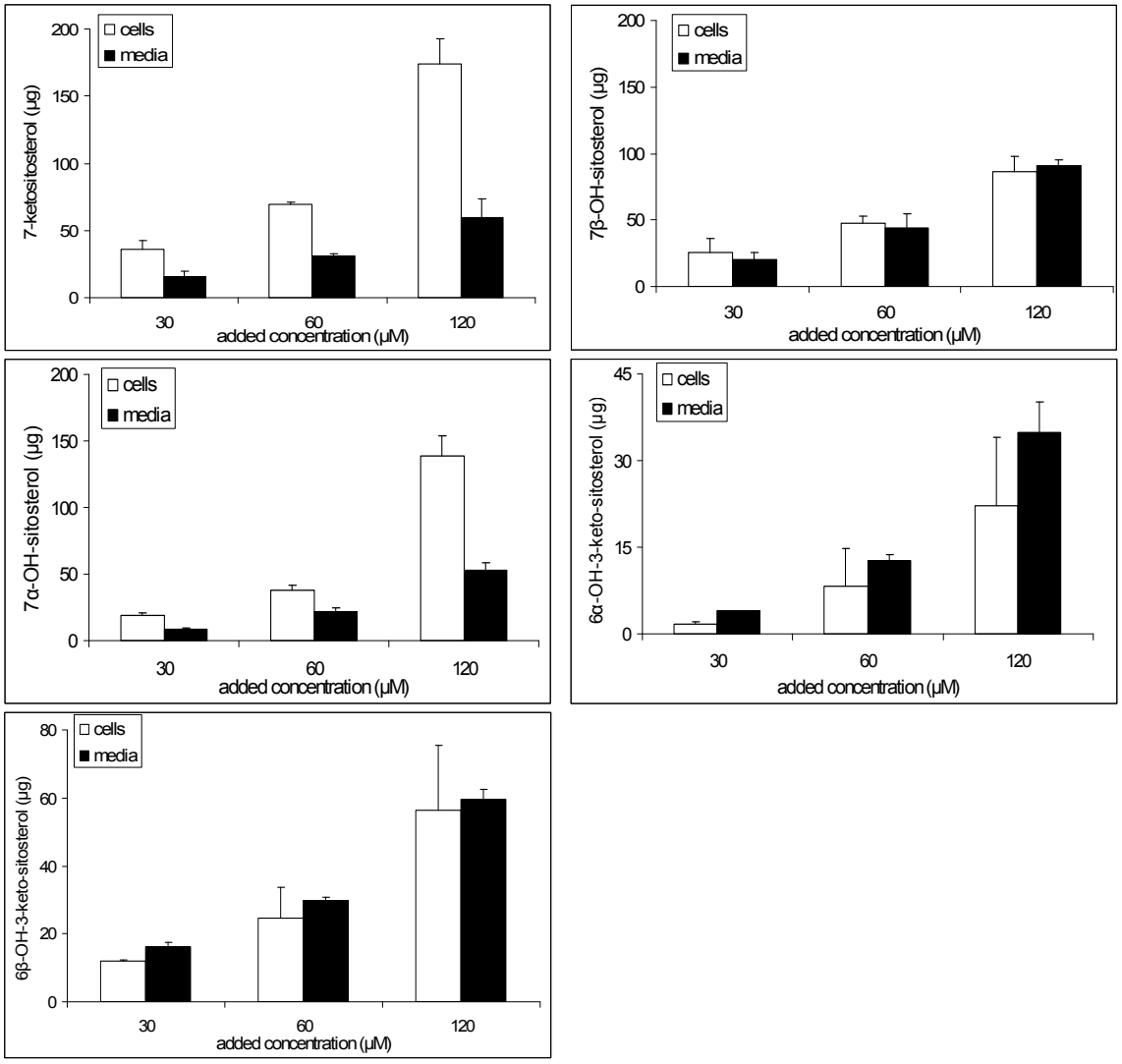


Fig 4.16 Detected amounts (µg) of β-sitosterol oxides (7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol, 6α-OH-3-keto-sitosterol and 6β-OH-3-keto-sitosterol) in HepG2-cells and corresponding media after 24 hours of incubation.

5. Conclusion

Since the variety of products fortified with phytosterols has increased rapidly during the last decade, investigations for a better understanding of the biological significance of their oxidation products are needed.

However, POPs are not commercially available. Therefore the first objective of our project was to develop a method for the isolation of common phytosterol oxides. The method based on heat treatment (130°C, 24h) in a ventilated oven, purification steps and a preparative HPLC-UV system showed to be simple and effective for the isolation of four different oxide fractions (7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol and a mixture of 6β-OH-3-keto-sitosterol/6α-OH-3-keto-sitosterol (ratio 4:3)). In addition a mixture of all polar oxidation products was generated. Sufficient amounts of the single oxides could be obtained for the subsequent test approaches, as yields in the range of several milligrams per fraction were obtained by one single HPLC run.

The following safety assessment was based on various bacterial and cell culture assays which are in accordance with the OECD guidelines. The Salmonella microsome assay was performed as preincubation method with and without metabolic activation using three different indicator strains (TA98, TA100 and TA102). The concentrations of the samples tested ranged from 0.04 to 5 mg/plate. To further investigate their anti-/pro-oxidative effects, challenge tests with the pro-oxidant tBOOH were conducted. Especially in test setups using strain TA102 number of his^+ - revertants significantly different to that of the solvent control were observed for individual oxides. However, in general none of the tested compounds could increase the revertant colony numbers beyond the doubled negative control, which was set as threshold for mutagenic activity. In addition no dose dependent increase could be observed. Since these two criteria must be fulfilled in order to identify a compound as a possible mutagen our tests showed no increased risk by the investigated POPs.

Further the effects of the collected oxidation products towards HepG2-cells were investigated. To enable overall uniformity and comparability for all conducted cell culture applications 3 different concentrations (30 µM, 60 µM and 120 µM) of each oxidation product and an incubation period of 24 hours was chosen. Corresponding results were found when cell viability was evaluated using two different types of test

approaches. Whereas in the Typan blue exclusion assay the term vital cells refers to cells with intact cell membranes, in the MTT-assay metabolically active cells are determined. Incubation with increasing concentrations of oxide samples induced a significant ($p<0.01$) reduction of cell viability, for most oxides also in a concentration dependent way. 7-ketositosterol and 7α-OH-sitosterol were those compounds with the highest toxic potential, whereas cytotoxicity of 7β-OH-sitosterol was far less pronounced.

The mode of cell death was assessed by flow cytometry using PE-labelled AnnexinV. It specifically binds to phosphatidylserine, which is translocated to the outer cell membrane during the early phase of apoptosis. 7-ketositosterol (30µM) was the only compound inducing an increase in apoptotic cells. However, as the proportion of AnnexinV positive cells decreased with increasing concentrations of oxidation products, the applied incubation conditions seemed to be too intense for detecting early events of apoptosis. At the time point investigated higher percentages of necrotic or late apoptotic than early apoptotic cells were detected.

Intracellular generation of super oxide anions was significantly increased after treatment with all sample compounds, as measured by staining with hydroethidium. To include a second marker of oxidative stress malondialdehyde concentration, as an endproduct of lipid peroxidation, was assessed by HPLC. A concentration dependent increase was observed for 7keto-, 7αOH- and 6β-OH-3-keto-/6α-OH-3-keto-sitosterol. Similar to other publications (Hall, 2006) no correlation between $O2^{\bullet-}$ and MDA concentrations was found. Apparently, differences in cytotoxicity of the single fractions do not necessarily depend on their ability to induce oxidative stress.

When samples were investigated in the alkaline single-cell gel electrophoresis assay no increase in DNA strand breaks was observed.

In order to estimate the outcomes of the toxicity assays it was of utmost importance to know whether the tested compounds were absorbed by the cell lines applied. Therefore the different oxidation products were extracted from both the cells and the corresponding media and analysed using an HPLC-UV-ELSD system. Due to the lower sensitivity of ELS-detection, only UV data were used for quantification of oxides. Uptake of all applied compounds could be clearly demonstrated. Obtained results correlated negatively with the outcomes of the MTT and TB assay. Accordingly,

differences in the levels of oxides accumulated in the cells seem to take a responsible part for their diverse toxic effects observed.

6. Summary

The aim of the present study was to assess the effects of common phytosterol oxidation products in various bacterial and cell culture assays.

As POPs so far are not commercially available, the first challenge of this work was to scale up a method for the isolation of adequate amounts of different β-sitosterol oxides. Several milligrams of 7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol, a mixture of 6β-OH-3-keto-sitosterol/6α-OH-3-keto-sitosterol (ratio 4:3) and a mixture of polar oxidation products were obtained after heat treatment of β-sitosterol (130 °C, 24 h) followed by chromatographic purification steps and preparative HPLC.

Salmonella typhimurium strains TA98, TA100 and TA102 and the pro-oxidant tertiary-butyl hydroperoxide (tBOOH) were used to examine the collected oxides in the Salmonella microsome assay. However, the samples showed no mutagenic or pro-oxidative properties.

Further their cytotoxic effects towards HepG2-cells were evaluated and particularly 7-keto- and 7α-OH-sitosterol showed to be highly active. All tested oxides caused a significant reduction ($p<0.05$) in cell viability as determined by two different viability assays. Although treatment with 7-ketositosterol induced an increase in early apoptotic cells, at the time point investigated the prevailing mode of cell death was late apoptosis or necrosis. While a significant increase in intracellular superoxide anion concentration ($O2^{\bullet-}$) was detected, malondialdehyde (MDA) concentration was enhanced by 7-keto-, 7α-OH- and 6β-OH-3-keto- /6α-OH-3-keto-sitosterol only. Therefore differences in the reduction of cell viability couldn't entirely be explained by their ability to induce oxidative stress. Further no induction of DNA strand breaks was observed.

The uptake of oxidation products in HepG2-cells could be clearly demonstrated. Different absorption affinities of the single oxyphytosterols were found. Accordingly, a link between different uptake levels and diverse cytotoxic effects may be possible.

Although not being mutagnic towards Salmonella typhimurium strains, isolated oxids showed different cytotoxic effects in cell culture asssays using HepG2-cells. Varieties could be explained by their diverse uptake affinities into cells. While oxide mixtures are often thought to be less toxic than isolated compounds, in the present study 7β-OH-sitosterol displayed the lowest reduction of cell viability.

7. Zusammenfassung

Ziel der vorliegenden Studie war es die Wirkung bekannter Phytosterinoxidationsprodukte (POPs) in verschiedenen Bakterien- und Zellversuchen zu bestimmen.

Da POPs bislang kommerziell nicht erhältlich sind, war die erste Herausforderung dieser Arbeit die Hochskalierung einer Methode zur Isolierung einer ausreichenden Menge an verschiedenen β-Sitosterinoxiden. Mehrere Milligramm an 7-Ketositosterin, 7β-OH-Sitosterin, 7α-OH-Sitosterin, einer Mischung aus 6β-OH-3-Keto-Sitosterin/6α-OH-3-Keto-Sitosterin (Verhältnis 4:3) und einer Mischung polarer Oxidationsprodukte wurden durch Erhitzung von β-Sitosterin (130 °C, 24 h) gefolgt von chromatographischen Reinigungsschritten und präparativer HPLC isoliert.

Die Salmonella typhimurium Stämme TA98, TA100 and TA102 sowie das Pro-Oxidant tertiär-Butyl-hydroperoxid (tBOOH) wurden zur Untersuchung der gesammelten POPs im Salmonella microsome assay eingesetzt. Allerdings zeigten die Probesubstanzen keine mutagenen oder pro-oxidativen Eigenschaften.

Weiters wurden ihre zytotoxischen Effekte gegenüber HepG2-Zellen evaluiert und hier zeigten besonders 7-Ketositosterin und 7α-OH-Sitosterin hohe Aktivitäten. Alle getesteten Oxide führten zu einer signifikanten Reduktion ($p<0.05$) der Zellviabilität, was in zwei unterschiedlichen Viabilitätstests bestimmt wurde. Obwohl eine Belastung mit 7-Ketositosterin zu einem Anstieg an früh apoptotischen Zellen führte, war zum untersuchten Zeitpunkt späte Apoptose oder Nekrose die vorherrschende Art des Zelltods. Während eine signifikante Steigerung an intrazellulärer Superoxidanionenkonzentration ($O2^{\bullet -}$) festgestellt werden konnte, wurde die Malondialdehydkonzentration (MDA) nur durch 7-Keto-, 7α-OH- und 6β-OH-3-keto-/6α-OH-3-keto-Sitosterin erhöht. Somit kann die unterschiedlich starke Reduktion der Zellviabilität nicht ganzheitlich durch die Fähigkeit oxidativen Stress zu induzieren erklärt werden. Weiters wurde keine Induktion von DNA Strangbrüchen festgestellt.

Die Aufnahme von Oxidationsprodukten in HepG2-Zellen konnte klar demonstriert werden. Unterschiedliche Absorptionsaffinitäten der einzelnen Phytosteroloxide konnten nachgewiesen werden. Demzufolge wäre ein Zusammenhang zwischen den

verschiedenen Aufnahmelevels und den unterschiedlichen zytotoxischen Effekten möglich.

Obwohl die getesteten Oxidationsprodukte keine Mutagenität gegenüber Salmonella typhimurium Stämmen zeigten, konnten in Zellkulturversuchen mit HepG2-Zellen unterschiedliche zytotoxische Effekte der einzelnen isolierten Oxide beobachtet werden. Diese Unterschiede könnten durch ihre verschiedenen Aufnahmeaffinitäten in die Zellen erklärt werden. Während Mischungen an Oxidationsprodukten häufig für weniger toxisch als isolierte Einzelsubstanzen gehalten werden, zeigte in der vorliegenden Studie 7β-OH-Sitosterin die geringste Beeinträchtigung der Zellviabilität.

8. References

Abidi, S.L. (2001): Chromatographic analysis of plant sterols in foods and vegetable oils. Journal of Chromatography A 935 (1-2): 173-201.

Abramsson-Zetterberg, L., Svensson, M., Johnsson, L. (2007): No evidence of genotoxic effect in vivo of the phytosterol oxidation products triols and epoxides. Toxicology Letters 173 (2): 132-139.

Adcox, C., Boyd, L., Oehrl, L., Allen, J., Fenner, G. (2001): Comparative effects of phytosterol oxides and cholesterol oxides in cultured macrophage-derived cell lines. Journal of Agricultural and Food Chemistry 49 (4): 2090-2095.

Aden, D.P., Fogel, A., Plotkin, S., Damjanov, I., Knowles, B.B. (1979): Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 282 (5739): 615-616.

Ames, B.N., Lee, F.D., Durston, W.E. (1973): An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proceedings of the National Academy of Sciences of the United States of America 70 (3): 782-786.

Ansari, G.A., Walker, R.D., Smart, V.B., Smith, L.L. (1982): Further investigations of mutagenic cholesterol preparations. Food and Chemical Toxicology 20 (1): 35-41.

Ansari, G.A.S., Smith, L.L. (1979): High-performance liquid chromatography of cholesterol Autoxidation products. Journal of Chromatography 175 (2): 307-315.

Apprich, S., Ulberth, F. (2004): Gas chromatographic properties of common cholesterol and phytosterol oxidation products. Journal of Chromatography, A 1055 (1-2): 169-176.

Aringer, L., Eneroth, P. (1973): Studies on the formation of C7-oxygenated cholesterol and beta-sitosterol metabolites in cell-free preparations of rat liver. Journal of Lipid Research 14 (5): 563-572.

Aupeix, K., Weltin, D., Mejia, J.E., Christ, M., Marchal, J., Freyssinet, J.M., Bischoff, P. (1995): Oxysterol-induced apoptosis in human monocytic cell lines. Immunobiology 194 (4-5): 415-428.

Ayesh, R., Weststrate, J.A., Drewitt, P.N., Hepburn, P.A. (1999): Safety evaluation of phytosterol esters. Part 5. Faecal short-chain fatty acid and microflora content, faecal bacterial enzyme activity and serum female sex hormones in healthy normolipidaemic volunteers consuming a controlled diet either with or without a phytosterol ester-enriched margarine. Food and Chemical Toxicology 37 (12): 1127-1138.

Baker, V.A., Hepburn, P.A., Kennedy, S.J., Jones, P.A., Lea, L.J., Sumpter, J.P., Ashby, J. (1999): Safety evaluation of phytosterol esters. Part 1. Assessment of oestrogenicity using a combination of in vivo and in vitro assays. Food and Chemical Toxicology 37 (1): 13-22

Berger, A., Jones, P.J.H., Abumweis, S.S. (2004): Plant sterols: Factors affecting their efficacy and safety as functional food ingredients. Lipids in health and disease 3

Biasi, F., Leonarduzzi, G., Vizio, B., Zanetti, D., Sevanian, A., Sottero, B., Verde, V., Zingaro, B., Chiarpotto, E., Poli, G. (2004): Oxysterol mixtures prevent

proapoptotic effects of 7-ketocholesterol in macrophages: implications for proatherogenic gene modulation. FASEB Journal 18 (6): 693-695.

Boberg, K.M., Einarsson, K., Bjorkhem, I. (1990): Apparent lack of conversion of sitosterol into C24-bile acids in humans. Journal of Lipid Research 31 (6): 1083-1088.

Bortolomeazzi, R., Cordaro, F., Pizzale, L., Conte, L.S. (2003): Presence of phytosterol oxides in crude vegetable oils and their fate during refining. Journal of Agricultural Food Chemistry 51 (8): 2394-2401.

Bortolomeazzi, R., De Zan, M., Pizzale, L., Conte, L.S. (1999): Mass spectrometry characterization of the 5alpha-, 7alpha-, and 7beta-hydroxy derivatives of beta-sitosterol, campesterol, stigmasterol, and brassicasterol. Journal of Agricultural and Food Chemistry 47 (8): 3069-3074.

Bosner, M.S., Lange, L.G., Stenson, W.F., Ostlund, R.E., Jr. (1999): Percent cholesterol absorption in normal women and men quantified with dual stable isotopic tracers and negative ion mass spectrometry. Journal of Lipid Research 40 (2): 302-308.

Brufau, G., Canela, M.A., Rafecas, M. (2008): Phytosterols: physiologic and metabolic aspects related to cholesterol-lowering properties. Nutrition Research 28 (4): 217-225.

Caboni, M.F., Costa, A., Rodriguez-Estrada, M.T., Lercker, G. (1997): High-performance liquid chromatographic separation of cholesterol oxidation products. Chromatographia 46 (3/4): 151-155.

Chen, B.H., Chen, Y.C. (1994): Evaluation of the analysis of cholesterol oxides by liquid chromatography. Journal of Chromatography A 661 (1-2): 127-136.

Chen, Z.Y., Jiao, R., Ma, K.Y. (2008): Cholesterol-lowering nutraceuticals and functional foods. Journal of Agricultural and Food Chemistry 56 (19): 8761-8773.

Cheng, Y.W., Kang, J.J., Shih, Y.L., Lo, Y.L., Wang, C.F. (2005): Cholesterol-3-beta, 5-alpha, 6-beta-triol induced genotoxicity through reactive oxygen species formation. Food and Chemical Toxicology 43 (4): 617-622.

Chien, J.T., Wang, H.C., Chen, B.H. (1998): Kinetic Model of the Cholesterol Oxidation during Heating. Journal of Agricultural and Food Chemistry 46 (7): 2572-2577.

Christiansen, L., Karjalainen, M., Serimaa, R., Lonnroth, N., Paakkari, T., Yliruusi, J. (2001a): Phase behavior of b-sitosterol-cholesterol and b-sitostanol-cholesterol co-precipitates. S.T.P. Pharma Sciences 11 167-173.

Christiansen, L.I., Lahteenmaki P, L.A., Mannelin, M.R., Seppanen Laakso, T.E., Hiltunen R, V.K., Yliruusi, J.K. (2001b): Cholesterol-lowering effect of spreads enriched with microcrystalline plant sterols in hypercholesterolemic subjects. European Journal of Nutrition 40 (2): 66-73.

Clare, K., Hardwick, S.J., Carpenter, K.L., Weeratunge, N., Mitchinson, M.J. (1995): Toxicity of oxysterols to human monocyte-macrophages. Atherosclerosis 118 (1): 67-75.

Clifton, P.M., Noakes, M., Sullivan, D., Erichsen, N., Ross, D., Annison, G., Fassoulakis, A., Cehun, M., Nestel, P. (2004): Cholesterol-lowering effects of plant sterol esters differ in milk, yoghurt, bread and cereal. European Journal of Clinical Nutrition 58 (3): 503-509.

Conchillo, A., Cercaci, L., Ansorena, D., Rodriguez-Estrada, M.T., Lercker, G., Astiasarán, I. (2005): Levels of Phytosterol Oxides in Enriched and Nonenriched

Spreads: Application of a Thin-Layer Chromatography-Gas Chromatography Methodology. Journal of Agricultural and Food Chemistry 53 7844-7850.

Csallany, A.S., Kindom, S.E., Addis, P.B., Lee, J.H. (1989): HPLC method for quantitation of cholesterol and four of its major oxidation products in muscle and liver tissues. Lipids 24 (7): 645-651.

Daly, G.G., Finocchiaro, E.T., Richardson, T. (1983): Characterization of some oxidation products of b-sitosterol. Journal of Agricultural and Food Chemistry 31 (1): 46-50.

De Jong, A., Plat, J., Bast, A., Godschalk, R.W., Basu, S., Mensink, R.P. (2008): Effects of plant sterol and stanol ester consumption on lipid metabolism, antioxidant status and markers of oxidative stress, endothelial function and low-grade inflammation in patients on current statin treatment. European Journal of Clinical Nutrition 62 (2): 263-273.

De Jong, A., Plat, J., Mensink, R.P. (2003): Metabolic effects of plant sterols and stanols. Journal of Nutritional Biochemistry 14 362-369.

De Jong, A., Plat, J., Mensink, R.P. (2006): Plant sterol or stanol consumption does not affect erythrocyte osmotic fragility in patients on statin treatment. European Journal of Clinical Nutrition 60 985-990.

De Jong, N., Pijpers, L., Bleeker, J.K., Ocke, M.C. (2004): Potential intake of phytosterols/-stanols: results of a simulation study. European Journal of Clinical Nutrition 58 (6): 907-919.

Dinan, L., Harmatha, J., Lafont, R. (2001): Chromatographic procedures for the isolation of plant steroids. Journal of Chromatography A 935 (1-2): 105-123.

Doornbos, A.M., Meynen, E.M., Duchateau, G.S., van der Knaap, H.C., Trautwein, E.A. (2006): Intake occasion affects the serum cholesterol lowering of a plant sterol-enriched single-dose yoghurt drink in mildly hypercholesterolaemic subjects. European Journal of Clinical Nutrition 60 (3): 325-333.

Dutta, P.C. Chemistry, Analysis and Occurence of Phytosterol Oxidation Products in Food. In: Phytosterols as Functional Food Components and Neutraceuticals. Ed. Dutta, P.C. New York: Marcel Dekker, Inc., 2004, 397-417.

Dutta, P.C. Determination of phytosterol oxidation products in foods and biological samples. In: Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence and Biological Effects. Eds. Guardiola, F., Dutta, P.C., Codony, R. and Savage, G.P. Champaign, Illinois: AOCS Press, 2002, 335-374.

Dutta, P.C. Phytosterol oxides in some samples of pure phytosterols mixture and in a few tablet supplement preparations in Finland. In: Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease. Eds. Kumpulainen, J.T. and Salonen, J.T. Cambridge, UK: The Royal Society of Chemistry, 1999, 316-319.

Dutta, P.C. (1997): Studies on phytosterol oxides. II: Content in some vegetable oils and in French fries prepare in these oils. Journal of the American Oil Chemists' Society 74 (6): 659-666(658).

Dutta, P.C., Appelqvist, L.A. (1997): Studies on phytosterol oxides. I: Effect of storage on the content in potato chips prepared in different vegetable oils. Journal of the American Oil Chemists' Society 74 (6): 647-657.

Dutta, P.C., Savage, G.P. Formation and Content of Phytosterol Oxidation Products in Foods. In: Cholesterol and Phytosterol Oxidation Products: Analysis, Occurence and Biological Effects. Eds. F. Guardiola, P.C. Dutta, R. Codony and Savage, G.P. Champaign: AOCS Press, 2002, 319-334.

EFSA. (2008): Consumption of Food and Beverages with Added Plant Sterols in the European Union. The Efsa Journal 133 1-21.

Ellegard, L., Bosaeus, I., Andersson, H. (2000): Will recommended changes in fat and fibre intake affect cholesterol absorption and sterol excretion? An ileostomy study. European Journal of Clinical Nutrition 54 (4): 306-313.

Frankel, E.N. Lipid Oxidation. Dundee, Scotland: Oily Press, 1998.

Fransen, H.P., de Jong, N., Wolfs, M., Verhagen, H., Verschuren, W.M., Lutjohann, D., von Bergmann, K., Plat, J., Mensink, R.P. (2007): Customary use of plant sterol and plant stanol enriched margarine is associated with changes in serum plant sterol and stanol concentrations in humans. Journal of Nutrition 137 (5): 1301-1306.

García-Llatas, G., Cercaci, L., Rodriguez-Estrada, M.T., Lagarda, M.J., Farré, R., Lercker, G. (2008): Sterol Oxidation in Ready-to-Eat Infant Foods During Storage. Journal of Agricultural and Food Chemistry 56 469–475.

Geoffroy, P., Julien-David, D., Marchioni, E., Raul, F., Aoude-Werner, D., Miesch, M. (2008): Synthesis of highly pure oxyphytosterols and (oxy)phytosterol esters Part I. Regioselective hydrogenation of stigmasterol: an easy access to oxyphytosterols. Steroids 73 (7): 702-707.

Göggelmann, W. Die Erfassung von Genmutationen in Bakterien. In: Mutationsforschung und genetische Toxikologie. Ed. Fahrig, R. Darmstadt: Wissenschaftliche Buchgesellschaft, 1993, 207-216.

Grandgirard, A. Biological Effects of Phytosterol Oxidation Products, Future Research Areas and Concluding Remarks. In: Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence and Biological Effects. Eds. Guardiola, F., Dutta, P.C., Codony, R. and Savage, G.P. Champaign, Illinois: AOCS Press, 2002, 375-382.

Grandgirard, A., Bretillon, L., Martinen, L., Beaufrere, B. (1999a): Oxysterols in human plasma. Chemistry and Physics of Lipids 101 198.

Grandgirard, A., Demaison-Meloche, J., Cordelet, C., Demaison, L. (2004a): Incorporation of oxyphytosterols in tissues of hamster. Reproduction, Nutrition, Development 44 (6): 599-608.

Grandgirard, A., Martine, L., Demaison, L., Cordelet, C., Joffre, C., Berdeaux, O., Semon, E. (2004b): Oxyphytosterols are present in plasma of healthy human subjects. British Journal of Nutrition 91 (1): 101-106.

Grandgirard, A., Martine, L., Joffre, C., Juaneda, P., Berdeaux, O. (2004c): Gas chromatographic separation and mass spectrometric identification of mixtures of oxyphytosterol and oxycholesterol derivatives. Application to a phytosterol-enriched food. Journal of Chromatography A 1040 (2): 239-250.

Grandgirard, A., Martine, L., Juaneda, P., Cordelet, C. (2004d): Sitostanetriol is not formed in vivo from sitosterol in the rat. Reproduction Nutrition Development 44 (6): 609-616.

Grandgirard, A., Sergiel, J.P., Nour, M., Demaison-Meloche, J., Ginies, C. (1999b): Lymphatic absorption of phytosterol oxides in rats. Lipids 34 (6): 563-570.

Grey, C.E., Adlercreutz, P. (2003): Ability of antioxidants to prevent oxidative mutations in Salmonella typhimurium TA102. Mutation Research 527 (1-2): 27-36.

Guardiola, F., Boatella, J., Codony, R. Determination of Cholesterol Oxidation Products by Gas Chromatography. In: Cholesterol and Phytosterol Oxidation Products:

Analysis, Occurence and Biological Effects. Eds. Guardiola, F., Dutta, P.C., Codony, R. and Savage, G.P. Champaign, Illinois: AOCS Press, 2002, 50-65.

Guardiola, F., Bou, R., Boatella, J., Codony, R. (2004): Analysis of sterol oxidation products in foods. Journal of AOAC International 87 (2): 441-460.

Guardiola, F., Codony, R., Addis, P.B., Rafecas, M., Boatella, J. (1996): Biological effects of oxysterols: current status. Food and Chemical Toxicology 34 (2): 193-211.

Hall, M.C. (2006): The effect of oxysterols, individually and as a representative mixture from food, on in vitro cultured bovine ovarian granulosa cells. Molecular and Cellular Biochemistry 292 (1-2): 1-11.

Hallikainen, M.A., Sarkkinen, E.S., Gylling, H., Erkkila, A.T., Uusitupa, M.I. (2000a): Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. European Journal of Clinical Nutrition 54 (9): 715-725.

Hallikainen, M.A., Sarkkinen, E.S., Uusitupa, M.I. (2000b): Plant stanol esters affect serum cholesterol concentrations of hypercholesterolemic men and women in a dose-dependent manner. Journal of Nutrition 130 (4): 767-776.

Halliwell, B., Gutteridge, J. Peroxidation of microsomes. In: Free radicals in biology and medicine. Eds. Halliwell, B. and Gutteridge, J. Oxford: Oxford Univ. Press, 1999a, 310.

Halliwell, B., Gutteridge, J.M. Free Radicals in Biology and Medicine. Oxford, U.K.: Oxford University Press, 1999b.

Halliwell, B., Whiteman, M. (2004): Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? British Journal of Pharmacology 142 (2): 231-255.

Heinemann, T., Leiss, O., von, B.K. (1986): Effect of low-dose sitostanol on serum cholesterol in patients with hypercholesterolemia. Atherosclerosis 61 (3): 219-223.

Hendriks, H.F., Brink, E.J., Meijer, G.W., Princen, H.M., Ntanios, F.Y. (2003): Safety of long-term consumption of plant sterol esters-enriched spread. European Journal of Clinical Nutrition 57 (5): 681-692.

Hendriks, H.F.J., Weststrate, J.A., Van Vliet, T., Meijer, G.W. (1999): Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolemic and mildly hypercholesterolemic subjects. European Journal of Clinical Nutrition 53 (4): 319-327.

Heron, S., Dreux, M., Tchapla, A. (2004): Post-column addition as a method of controlling triacylglycerol response coefficient of an evaporative light scattering detector in liquid chromatography-evaporative light-scattering detection. Journal of Chromatography A 1035 (2): 221-225.

Hovenkamp, E., Demonty, I., Plat, J., Lutjohann, D., Mensink, R.P., Trautwein, E.A. (2008): Biological effects of oxidized phytosterols: a review of the current knowledge. Progress in Lipid Research 47 (1): 37-49.

Ikeda, I., Tanaka, K., Sugano, M., Vahouny, G.V., Gallo, L.L. (1988): Discrimination between cholesterol and sitosterol for absorption in rats. Journal of Lipid Research 29 (12): 1583-1591.

Jessup, W., Herman, A., Chapman, M.J. (2008): Phytosterols in cardiovascular disease: innocuous dietary components, or accelerators of atherosclerosis? Future Lipidology 3 (3): 301-310.

Johnsson, L., Andersson, R.E., Dutta, P.C. (2003): Side-chain autoxidation of stigmasterol and analysis of a mixture of phytosterol oxidation products by chromatographic and spectroscopic methods. Journal of the American Oil Chemists' Society 80 (8): 777-783.

Johnsson, L., Dutta, P.C. (2003): Characterization of side-chain oxidation products of sitosterol and campesterol by chromatographic and spectroscopic methods. Journal of the American Oil Chemists' Society 80 (8): 767-776.

Johnsson, L., Dutta, P.C. (2006): Determination of phytosterol oxides in some food products by using an optimized transesterification method. Food Chemistry 97 606-613.

Johnsson, L., Dutta, P.C. (2005): Separation of phytosterol oxidation products by combination of different polarity gas chromatography capillary columns. Journal of Chromatography A 1064 213-217.

Jones, P.J., Raeini-Sarjaz, M., Ntanios, F.Y., Vanstone, C.A., Feng, J.Y., Parsons, W.E. (2000): Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. Journal of Lipid Research 41 (5): 697-705.

Jones, P.J., Vanstone, C.A., Raeini-Sarjaz, M., St-Onge, M.P. (2003): Phytosterols in low- and nonfat beverages as part of a controlled diet fail to lower plasma lipid levels. Journal of Lipid Research 44 (9): 1713-1719.

Jones, P.J.H., MacDougall, D.E., Ntanios, F., Vanstone, C.A. (1997): Dietary phytosterols as cholesterol-lowering agents in humans. Canadian Journal of Physiology and Pharmacology 75 (3): 217-227.

Julien-David, D., Geoffroy, P., Marchioni, E., Raul, F., Aoude-Werner, D., Miesch, M. (2008): Synthesis of highly pure oxyphytosterols and (oxy)phytosterol esters. Part II. (Oxy)-sitosterol esters derived from oleic acid and from 9,10-dihydroxystearic acid [1]. Steroids 73 (11): 1098-1109.

Katan, M.B., Grundy, S.M., Jones, P., Law, M.F.R.C.P., Miettinen, T., Paoletti, R. (2003): Efficacy and Safety of Plant Stanols and Sterols in the Management of Blood Cholesterol Levels. Mayo Clinic Proceedings 78 (8): 965-978.

Kemmo, S., Ollilainen, V., Lampi, A.-M., Piironen, V. (2007): Determination of stigmasterol and cholesterol oxides using atmospheric pressure chemical ionization liquid chromatography/mass spectrometry. Food Chemistry 101 (4): 1438-1445.

Kemmo, S., Ollilainen, V., Lampi, A.-M., Piironen, V. (2008): Liquid chromatography mass spectrometry for plant sterol oxide determination in complex mixtures. European Food Research and Technology 226 1325-1334.

Kemmo, S., Ollilainen, V., Lampi, A.M., Piironen, V. (2004): Determination of stigmasterol hydroperoxides using high-performance liquid chromatography-mass spectrometry with atmospheric pressure chemical ionization. Czech Journal of Food Sciences 22 (Spec. Iss.): 144-146.

Kemmo, S., Soupas, L., Lampi, A.-M., Piironen, V. (2005): Formation and decomposition of stigmasterol hydroperoxides and secondary oxidation products during thermo-oxidation. European Journal of Lipid Science and Technology 107 (11): 805-814.

Kermasha, S., Kubow, S., Goetghebeur, M. (1994): Comparative high-performance liquid chromatographic analyses of cholesterol and its oxidation products using diode-array ultraviolet and laser light-scattering detection. Journal of Chromatography A 685 (2): 229-235.

Kim, J.C., Kang, B.H., Shin, C.C., Kim, Y.B., Lee, H.S., Kim, C.Y., Han, J., Kim, K.S., Chung, D.W., Chung, M.K. (2002): Subchronic toxicity of plant sterol esters administered by gavage to Sprague-Dawley rats. Food and Chemical Toxicology 40 (11): 1569-1580.

Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pals, S.T., van Oers, M.H. (1994): Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84 (5): 1415-1420.

Korpela, R., Tuomilehto, J., Hogstrom, P., Seppo, L., Piironen, V., Salo-Vaananen, P., Toivo, J., Lamberg-Allardt, C., Karkkainen, M., Outila et, a. (2006): Safety aspects and cholesterol-lowering efficacy of low fat dairy products containing plant sterols. European Journal of Clinical Nutrition 60 (5): 633-642.

Lakritz, L., Jones, K.C. (1997): Separation and quantitation of cholesterol oxides by HPLC with an evaporative light scattering detector in a model system. Journal of the American Oil Chemists' Society 74 (8): 943-946.

Lampi, A.-M., Juntunen, L., Toivo, J., Piironen, V. (2002): Determination of thermo-oxidation products of plant sterols. Journal of Chromatography B 777 (1-2): 83-92.

Law, M.R., Wald, N.J., Thompson, S.G. (1994): By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease? British Medical Journal 308 367-372.

Lea, L.J., Hepburn, P.A. (2006): Safety evaluation of phytosterol-esters. Part 9: Results of a European post-launch monitoring programme. Food And Chemical Toxicology: An International Journal Published For The British Industrial Biological Research Association 44 (8): 1213-1222.

Lea, L.J., Hepburn, P.A., Wolfreys, A.M., Baldrick, P. (2004): Safety evaluation of phytosterol esters. Part 8. Lack of genotoxicity and subchronic toxicity with phytosterol oxides. Food and Chemical Toxicology 42 (5): 771-783.

Lebovics, V.K. Determination of Cholesterol Oxidation Products by Thin-Layer Chromatography. In: Cholesterol and Phytosterol Oxidation Products: Analysis, Occurence and Biological Effects. Eds. Guardiola, F., Dutta, P.C., Codony, R. and Savage, G.P. Champaign, Illinois: AOCS Press, 2002, 101-113.

Lee, M.H., Lu, K., Patel, S.B. (2001): Genetic basis of sitosterolemia. Current Opinion in Lipidology 12 (2): 141-149.

Lemaire-Ewing, S., Prunet, C., Montange, T., Vejux, A., Berthier, A., Bessede, G., Corcos, L., Gambert, P., Neel, D., Lizard, G. (2005): Comparison of the cytotoxic, pro-oxidant and pro-inflammatory characteristics of different oxysterols. Cell Biology and Toxicology 21 (2): 97-114.

Leonarduzzi, G., Sevanian, A., Sottero, B., Arkan, M.C., Biasi, F., Chiarpotto, E., Basaga, H., Poli, G. (2001): Up-regulation of the fibrogenic cytokine TGF-beta1 by oxysterols: a mechanistic link between cholesterol and atherosclerosis. FASEB Journal 15 (9): 1619-1621.

Leonarduzzi, G., Sottero, B., Poli, G. (2002): Oxidized products of cholesterol: dietary and metabolic origin, and proatherosclerotic effects (review). Journal of Nutritional Biochemistry 13 (12): 700-710.

Lercker, G., Rodriguez-Estrada, M.T. Cholesterol Oxidation Mechanisms. In: Cholesterol and Phytosterol Oxidation Products. Eds. Guardiola, F., P.C, D., Codony, R. and G.P., S. Champaign, Illinois: AOCS Press, 2002, 1-25.

Ling, W.H., Jones, P.J.H. (1995): Dietary phytosterols: a review of metabolism, benefits and side effects. Life Sciences 57 (3): 195-206.

Lutjohann, D. (2004): Sterol autoxidation: from phytosterols to oxyphytosterols. British Journal of Nutrition 91 (1): 3-4.

Maerker, G., Nungesser, E.H., Zulak, I.M. (1988): HPLC separation and quantitation of cholesterol oxidation products with flame ionization detection. Journal of Agricultural and Food Chemistry 36 (1): 61-63.

Maguire, L., Konoplyannikov, M., Ford, A., Maguire, A.R., O'Brien, N.M. (2003): Comparison of the cytotoxic effects of beta-sitosterol oxides and a cholesterol oxide, 7beta-hydroxycholesterol, in cultured mammalian cells. British Journal of Nutrition 90 (4): 1-10.

Manini, P., Andreoli, R., Careri, M., Elviri, L., Musci, M. (1998): Atmospheric pressure chemical ionization liquid chromatography/mass spectrometry in cholesterol oxide determination and characterization. Rapid Communications in Mass Spectrometry 12 (13): 883-889.

Maron, D.M., Ames, B.N. Revised Methods for the Salmonella Mutagenicity Test. In: Handbook of Mutagenicity Test Procedures. Eds. Kilbey, B.J., Legator, M. and Ramel, C. Second Edition ed: Elsevier Science Publishers BV, 1984, 93-140.

Matvienko, O.A., Lewis, D.S., Swanson, M., Arndt, B., Rainwater, D.L., Stewart, J., Alekel, D.L. (2002): A single daily dose of soybean phytosterols in ground beef decreases serum total cholesterol and LDL cholesterol in young, mildly hypercholesterolemic men. American Journal of Clinical Nutrition 76 (1): 57-64.

Mazalli, M.R., Sawaya, A.C.H.F., Eberlin, M.N., Bragagnolo, N. (2006): HPLC Method for Quantification and Characterization of Cholesterol and Its Oxidation Products in Eggs. Lipids 41 (2): 615-622.

Mensink, R.P., Ebbing, S., Lindhout, M., Plat, J., van Heugten, M.M.A. (2002): Effects of plant stanol esters supplied in low-fat yoghurt on serum lipids and lipoproteins, non-cholesterol sterols and fat soluble antioxidant concentrations. Atherosclerosis 160 (1): 205-213.

Meyer, W., Jungnickel, H., Jandke, M., Dettner, K., Spiteller, G. (1998): On the cytotoxicity of oxidized phytosterols isolated from photoautotrophic cell cultures of Chenopodium rubrum tested on mealworm Tenebrio molitor. Phytochemistry 47 (5): 789-797.

Miettinen, T.A., Gylling, H. Prospects of Increasing Nutritional Phytosterol Levels in Plants. In: Phytosterols as Functional Food Components and Neutraceuticals. Ed. Dutta, P.C. New York: Marcel Dekker, 2004, 431-439.

Miettinen, T.A., Puska, P., Gylling, H., Vanhanen, H., Vartiainen, E. (1995): Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. New England Journal of Medicine 333 (20): 1308-1312.

Miettinen, T.A., Vuoristo, M., Nissinen, M., Jarvinen, H.J., Gylling, H. (2000): Serum, biliary, and fecal cholesterol and plant sterols in colectomized patients before

and during consumption of stanol ester margarine. American Journal of Clinical Nutrition 71 (5): 1095-1102.

Miguet-Alfonsi, C., Prunet, C., Monier, S., Bessede, G., Lemaire-Ewing, S., Berthier, A., Menetrier, F., Neel, D., Gambert, P., Lizard, G. (2002): Analysis of oxidative processes and of myelin figures formation before and after the loss of mitochondrial transmembrane potential during 7beta-hydroxycholesterol and 7-ketocholesterol-induced apoptosis: comparison with various pro-apoptotic chemicals. Biochemical Pharmacology 64 (3): 527-541.

Miguet, C., Monier, S., Bettaieb, A., Athias, A., Bessede, G., Laubriet, A., Lemaire, S., Neel, D., Gambert, P., Lizard, G. (2001): Ceramide generation occurring during 7beta-hydroxycholesterol- and 7-ketocholesterol-induced apoptosis is caspase independent and is not required to trigger cell death. Cell Death and Differentiation 8 (1): 83-99.

Min, D.B., Boff, J.M. (2002): Chemistry and Reaction of Singlet Oxygen in Foods. Comprehensive Reviews in Food Science and Food Safety 1 58-72.

Moghadasian, M.H. (2000): Pharmacological properties of plant sterols. In vivo and in vitro observations. Life Sciences 67 605-615.

Monier, S., Samadi, M., Prunet, C., Denance, M., Laubriet, A., Athias, A., Berthier, A., Steinmetz, E., Jurgens, G., Negre-Salvayre, A., Bessede, G., Lemaire-Ewing, S., Neel, D., Gambert, P., Lizard, G. (2003): Impairment of the cytotoxic and oxidative activities of 7 beta-hydroxycholesterol and 7-ketocholesterol by esterification with oleate. Biochemical and Biophysical Research Communications 303 (3): 814-824.

Moreau, R. The Evaporative Light-Scattering Detector as a Tool for the Analysis of Lipids by HPLC. In: HPLC of Acy Lipids. Eds. Lin, J.-T. and McKeon, T.A. New York: HNB Publishing, 2005, 93-116.

Moreau, R. Plant sterols in Functional Foods. In: Phytosterols as Functional Food Components and Neutraceuticals. Ed. P.C, D. New York: Marcel Dekker, Inc., 2004, 317-345.

Moreau, R.A., Whitaker, B.D., Hicks, K.B. (2002): Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Progress in Lipid Research 41 (6): 457-500.

Mortelmans, K., Zeiger, E. (2000): The Ames Salmonella/microsome mutagenicity assay. Mutation Research 455 (1-2): 29-60.

Morton, G.M., Lee, S.M., Buss, D.H., Lawrance, P. (1995): Intakes and major dietary sources of cholesterol and phytosterols in the british diet. Journal of Human Nutrition and Dietetics 8 429-440.

Mosmann, T. (1983): Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65 (1-2): 55-63.

Moss, G.P. (1989): The nomenclature of steroids. European Journal of Biochemistry 186 (3): 429-458

Nair, V.D., Kanfer, I., Hoogmartens, J. (2006): Determination of stigmasterol, beta-sitosterol and stigmastanol in oral dosage forms using high performance liquid chromatography with evaporative light scattering detection. Journal of Pharmaceutical and Biomedical Analysis 41 (3): 731-737.

Nestel, P., Cehun, M., Pomeroy, S., Abbey, M., Weldon, G. (2001): Cholesterol-lowering effects of plant sterol esters and non-esterified stanols in margarine,

butter and low-fat foods. European Journal of Clinical Nutrition 55 (12): 1084-1090.

Nguyen, T.T. (1999): The cholesterol-lowering action of plant stanol esters. Journal of Nutrition 129 (12): 2109-2112.

Noakes, M., Clifton, P., Ntanios, F., Shrapnel, W., Record, I., McInerney, J. (2002): An increase in dietary carotenoids when consuming plant sterols or stanols is effective in maintaining plasma carotenoid concentrations. American Journal of Clinical Nutrition 75 (1): 79-86.

Normen, A.L., Brants, H.A., Voorrips, L.E., Andersson, H.A., van den Brandt, P.A., Goldbohm, R.A. (2001): Plant sterol intakes and colorectal cancer risk in the Netherlands Cohort Study on Diet and Cancer. American Journal of Clinical Nutrition 74 (1): 141-148.

Normen, L., Dutta, P., Lia, A., Andersson, H. (2000): Soy sterol esters and b-sitostanol ester as inhibitors of cholesterol absorption in human small bowel. American Journal of Clinical Nutrition 71 (4): 908-913.

Normen, L., Shaw, C.A., Fink, C.S., Awad, A.B. (2004): Combination of phytosterols and omega-3 fatty acids: a potential strategy to promote cardiovascular health. Curr Med Chem Cardiovasc Hematol Agents 2 (1): 1-12.

Nourooz-Zadeh, J., Appelqvist, L.A. (1992): Isolation and quantitative determination of sterol oxides in plant-based foods: soybean oil and wheat flour. Journal of the American Oil Chemists' Society 69 (3): 288-293.

Ntanios, F., Duchateau, G.S. (2002): A healthy diet rich in carotenoids is effective in maintaining normal blood carotenoid levels during the daily use of plant sterol-enriched spreads. International Journal for Vitamin and Nutrition Research 72 (1): 32-39.

O'Neill, F.H., Sanders, T.A.B., Thompson, G.R. (2005): Comparison of Efficacy of Plant Stanol Ester and Sterol Ester: Short-Term and Longer-Term Studies. The American journal of cardiology 96 (1A): 29D-36D.

O'Sullivan A, J., O'Callaghan Y, C., O'Brien N, M. (2005): Differential effects of mixtures of cholesterol oxidation products on bovine aortic endothelial cells and human monocytic U937 cells. International Journal of Toxicology 24 (3): 173-179.

Oehrl, L.L., Hansen, A.P., Rohrer, C.A., Fenner, G.P., Boyd, L.C. (2001): Oxidation of phytosterols in a test food system. Journal of the American Oil Chemists' Society 78 (11): 1073-1078.

Olive, P.L. (1989): Cell Proliferation as a requirement for development of the contact effect in the Chinese Hamster V79 spheroids. Radiation Research 117 79-92.

Osada, K. Cholesterol oxidation products: other biological effects. In: Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence and Biological Effects. Eds. Dutta, P.C., Guardiola, F., Codony, R. and Savage, G.P. Champaign, Illinois: AOCS Press, 2002, 278-318.

Osada, K., Kodama, T., Yamada, K., Sugano, M. (1993): Oxidation of cholesterol by heating. Journal of Agricultural and Food Chemistry 41 (8): 1198-1202.

Osada, K., Ravandi, A., Kuksis, A. (1999): Rapid analysis of oxidized cholesterol derivatives by high-performance liquid chromatography combined with diode-array ultraviolet and evaporative laser light-scattering detection. Journal of the American Oil Chemists' Society 76 (7): 863-871.

Ostling, o., Johanson, K.J. (1984): Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochemical and Biophysical Research Communications 123 (1): 291-298.

Ostlund, R.E., Jr. (2002): Phytosterols in human nutrition. Annual Review of Nutrition 22 533-549.

Ostlund, R.E., Jr., McGill, J.B., Zeng, C.-M., Covey, D.F., Stearns, J., Stenson, W.F., Spilburg, C.A. (2002): Gastrointestinal absorption and plasma kinetics of soy Delta(5)-phytosterols and phytostanols in humans. American Journal Of Physiology, Endocrinology And Metabolism 282 (4): E911-E916.

Ostlund, R.E., Jr., Spilburg, C.A., Stenson, W.F. (1999): Sitostanol administered in lecithin micelles potently reduces cholesterol absorption in humans. The American Journal of Clinical Nutrition 70 (5): 826-831.

Palozza, P., Serini, S., Verdecchia, S., Ameruso, M., Trombino, S., Picci, N., Monego, G., Ranelletti, F.O. (2007): Redox regulation of 7-ketocholesterol-induced apoptosis by beta-carotene in human macrophages. Free Radical Biology & Medicine 42 (10): 1579-1590.

Peterson, D.W. (1951): Effect of soybean sterols in the diet on plasma and liver cholesterol in chicks. Proceedings of the Society for Experimental Biology and Medicine 78 (1): 143-147.

Phillips, K.M., Tarragó -Trani, M.T., Stewart, K.K. (1999): Phytosterol content of experimental diets differing in fatty acid composition. Food Chemistry 64 415-422.

Piironen, V., Lampi, A.-M., Dutta, P.C. Occurrence and levels of phytosterols in foods. In: Phytosterols as Functional Food Components and Nutraceuticals. Ed. Anonymous. New York, NY, USA: Marcel Decker, Inc., 2004, 1-32.

Piironen, V., Lindsay, D.G., Miettinen, T.A., Toivo, J., Lampi, A.-M. (2000): Plant sterols: biosynthesis, biological function and their importance to human nutrition. Journal of the Science of Food and Agriculture 80 (7): 939-966.

Piironen, V., Toivo, J., Lampi, A.M. (2002): Plant sterols in cereals and cereal products. Cereal Chemistry 79 (1): 148-154.

Piironen, V., Toivo, J., Puupponen-Pimia, R., Lampi, A.-M. (2003): Plant sterols in vegetables, fruits and berries. Journal of the Science of Food and Agriculture 83 (4): 330-337.

Plat, J., Brzezinka, H., Lutjohann, D., Mensink, R.P., Von Bergmann, K. (2001): Oxidized plant sterols in human serum and lipid infusions as measured by combined gas-liquid chromatography-mass spectrometry. Journal of Lipid Research 42 (12): 2030-2038.

Plat, J., Mensink, R.P. (2005): Plant Stanol and Sterol Esters in the Control of Blood Cholesterol Levels: Mechanism and Safety Aspects. The American journal of cardiology 96 (1A): 15D-22D.

Plat, J., Mensink, R.P. (2000): Vegetable oil based versus wood based stanol ester mixtures: effects on serum lipids and hemostatic factors in non-hypercholesterolemic subjects. Atherosclerosis 148 (1): 101-112.

Plat, J., van Onselen, E.N., van Heugten, M.M., Mensink, R.P. (2000): Effects on serum lipids, lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines and shortenings enriched with plant stanol esters. European Journal of Clinical Nutrition 54 (9): 671-677.

Pollak, O.J. (1953): Reduction of blood cholesterol in man. Circulation 7 702-706.

Quilez, J., Rafecas, M., Brufau, G., Garcia-Lorda, P., Megias, I., Bullo, M., Ruiz, J.A., Salas-Salvado, J. (2003): Bakery products enriched with phytosterol esters, alpha-tocopherol and beta-carotene decrease plasma LDL-cholesterol and maintain plasma beta-carotene concentrations in normocholesterolemic men and women. Journal of Nutrition 133 (10): 3103-3109.

Ramel, A., Wagner, K.H., Elmadfa, I. (2004): Plasma antioxidants and lipid oxidation after submaximal resistance exercise in men. European Journal of Nutrition 43 (1): 2-6.

Ratnayake, W.M.N., L'Abbe, M.R., Mueller, R., Hayward, S., Plouffe, L., Hollywood, R., Trick, K. (2000): Vegetable oils high in phytosterols make erythrocytes less deformable and shorten life span of stroke-prone spontaneously hypertensive rats. Journal of Nutrition 130 1166-1178.

Ratnayake, W.M.N., Plouffe, L., L'Abbe, M.R., Trick, K., Mueller, R., Hayward, S. (2003): Comparative health effects of margarines fortified with plant sterols and stanols on a rat model for hemorrhagic stroke. Lipids 38 (12): 1237-1247.

Raulio, S., Nurttila, A., Mannonen, L. Adding plant sterols and stanols to food - modelling the amount received by Finnish adults. Publications of National Food Agency of Finland 2001.

Razzazi-Fazeli, E., Kleineisen, S., Luf, W. (2000): Determination of cholesterol oxides in processed food using high-performance liquid chromatography-mass spectrometry with atmospheric pressure chemical ionisation. Journal of Chromatography A 896 (1-2): 321-334.

Rodriguez-Estrada, M.T., Caboni, M.F. Determination of Cholesterol Oxidation Products by High-Performance Liquid Chromatography. In: Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence and Biological Effects. Eds. Guardiola, F., Dutta, P.C., Codony, R. and Savage, G.P. Champaign, Illinois: AOCS Press, 2002, 66-100.

Rose-Sallin, C., Huggett, A.C., Bosset, J.O., Tabacchi, R., Fay, L.B. (1995): Quantification of Cholesterol Oxidation Products in Milk Powders Using [2H7]Cholesterol To Monitor Cholesterol Autoxidation Artifacts. Journal of Agricultural and Food Chemistry 43 (4): 935-941.

Rothe, G., Valet, G. (1990): Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin. Journal of Leukocyte Biology 47 (5): 440-448.

Roussi, S., Gosse, F., Aoude-Werner, D., Zhang, X., Marchioni, E., Geoffroy, P., Miesch, M., Raul, F. (2007): Mitochondrial perturbation, oxidative stress and lysosomal destabilization are involved in 7beta-hydroxysitosterol and 7beta-hydroxycholesterol triggered apoptosis in human colon cancer cells. Apoptosis 12 (1): 87-96.

Roussi, S., Winter, A., Gosse, F., Werner, D., Zhang, X., Marchioni, E., Geoffroy, P., Miesch, M., Raul, F. (2005): Different apoptotic mechanisms are involved in the antiproliferative effects of 7beta-hydroxysitosterol and 7beta-hydroxycholesterol in human colon cancer cells. Cell Death and Differentiation 12 (2): 128-135.

Rozner, S., Garti, N. (2006): The activity and absorption relationship of cholesterol and phytosterols. Colloids and Surfaces A: Physicochemical and Engineering Aspects 282-283 435-456.

Ryan, E., Chopra, J., McCarthy, F., Maguire, A.R., O'Brien, N.M. (2005): Qualitative and quantitative comparison of the cytotoxic and apoptotic potential of

phytosterol oxidation products with their corresponding cholesterol oxidation products. British Journal of Nutrition 94 (3): 443-451.

Saldanha, T., Sawaya, A.C.H.F., Eberlin, M.N., Bragagnolo, N. (2006): HPLC Separation and Determination of 12 Cholesterol Oxidation Products in Fish: Comparative Study of RI, UV, and APCI-MS Detectors. Journal of Agricultural and Food Chemistry 54 (12): 4017-4113.

Salen, G., Shefer, S., Nguyen, L., Ness, G.C., Tint, G.S., Shore, V. (1992): Sitosterolemia. Journal of Lipid Research 33 (7): 945-955.

Salo, P., Wester, I., Hopia, A., Gunstone, F.D. Phytosterols. In: Lipids for functional foods and nutraceuticals. Ed. Anonymous. Vol. 13. Bridgwater, England: The Oily Press, 2003, 183-224.

Samali, A., Nordgren, H., Zhivotovsky, B., Peterson, E., Orrenius, S. (1999): A Comparative Study of Apoptosis and Necrosis in HepG2 Cells: Oxidant-Induced Caspase Inactivation Leads to Necrosis. Biochemical and Biophysical Research Communications 255 6-11.

Sanders, D.J., Minter, H.J., Howes, D., Hepburn, P.A. (2000): The safety evaluation of phytosterol esters. Part 6. The comparative absorption and tissue distribution of phytosterols in the rat. Food and Chemical Toxicology 38 (6): 485-491.

Säynäjoki, S., Sundberg, S., Soupas, L., Lampi, A.M., Piironen, V. (2003): Determination of stigmasterol primary oxidation products by high-performance liquid chromatography. Food Chemistry 80 (3): 415-421.

SCF Opinion of the Scientific Commitee on Food on a request for the safety assessment of the use of phytosterol esters in yellow fat spreads. 2000.

Sevanian, A., Peterson, A.R. (1986): The cytotoxic and mutagenic properties of cholesterol oxidation products. Food and Chemical Toxicology 24 (10-11): 1103-1110.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L. (1988): A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 175 (1): 184-191.

Smith, L.L., Smart, V.B., Ansari, G.A. (1979): Mutagenic cholesterol preparations. Mutation Research 68 (1): 23-30.

Smith, L.L., Smart, V.B., Made Gowda, N. (1986): Mutagenic sterol hydroperoxides. Mutation Research 161 (1): 39-48.

Soupas, L., Huikko, L., Lampi, A.M., Piironen, V. (2005): Esterification affects phytosterol oxidation. European Journal of Lipid Science and Technology 107 (2): 107-118.

Soupas, L., Huikko, L., Lampi, A.M., Piironen, V. (2006): Oxidative stability of phytosterols in some food applications. European Food Research and Technology 222 266-273.

Soupas, L., Juntunen, L., Lampi, A.M., Piironen, V. (2004a): Effects of sterol structure, temperature, and lipid medium on phytosterol oxidation. Journal of Agricultural and Food Chemistry 52 (21): 6485-6491.

Soupas, L., Juntunen, L., Säynäjoki, S., Lampi, A.M., Piironen, V. (2004b): GC-MS method for characterization and quantification of sitostanol oxidation products. Journal of the American Oil Chemists' Society 81 (2): 135-141.

Sugano, M., Kamo, F., Ikeda, I., Morioka, H. (1976): Lipid-lowering activity of phytostanols in rats. Atherosclerosis 24 (1-2): 301-309.

Tabee, E., Azadmard-Damirchi, S., Jägerstad, M., Dutta, P.C. (2008a): Lipids and phytosterol oxidation in commercial French fries commonly consumed in Sweden. Journal of Food Composition and Analysis 21 169-177.

Tabee, E., Jägerstad, M., Dutta, P.C. (2008b): Lipids and phytosterol oxidation products in commercial potato crisps commonly consumed in Sweden. European Food Research and Technology 227 745-755.

Tai, C.-Y., Chien, Y.C., Chen, B.H. (1999): Analysis, Formation and Inhibition of Cholesterol Oxidation Products in Foods: An Overview (Part I). Journal of Food and Drug Analysis 7 (4): 243-257.

Thurnham, D.I. (1999): Functional foods: cholesterol-lowering benefits of plant sterols. British Journal of Nutrition 82 (4): 255-256.

Tian, Q., Failla, M.L., Bohn, T., Schwartz, S.J. (2006): High-performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry determination of cholesterol uptake by Caco-2 cells. Rapid Communications in Mass Spectrometry 20 (20): 3056-3060.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F. (2000): Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis 35 (3): 206-221.

Tice, R.R., Andrews, P.W., Singh, N.P. (1990): The single cell gel assay: a sensitive technique for evaluating intercellular differences in DNA damage and repair. Basic Life Sciences 53 291-301.

Tikkanen, M.J., Hogstrom, P., Tuomilehto, J., Keinanen-Kiukaanniemi, S., Sundvall, J., Karppanen, H. (2001): Effect of a diet based on low-fat foods enriched with nonesterified plant sterols and mineral nutrients on serum cholesterol. The American journal of cardiology 88 (10): 1157-1162.

Tomoyori, H., Kawata, Y., Higuchi, T., Ichi, I., Sato, H., Sato, M., Ikeda, I., Imaizumi, K. (2004): Phytosterol oxidation products are absorbed in the intestinal lymphatics in rats but do not accelerate atherosclerosis in apolipoprotein E-deficient mice. Journal of Nutrition 134 (7): 1690-1696.

Trautwein, E.A., Duchateau, G.S.M.J.E., Lin, Y., Mel'nikov, S.M., Molhuizen, H.O.F., Ntanios, F.Y. (2003): Proposed mechanisms of cholesterol-lowering action of plant sterols. European Journal of Lipid Science and Technology 105 (3-4): 171-185.

Turnbull, D., Frankos, V.H., Leeman, W.R., D., J. (1999a): Short-Term Tests of Estrogenic Potential of Plant Stanols and Plant Stanol Esters. Regulatory Toxicology and Pharmacology 29 (2): 211-215.

Turnbull, D., Frankos, V.H., van Delft, J.H., DeVogel, N. (1999b): Genotoxicity evaluation of wood-derived and vegetable oil-derived stanol esters. Regulatory Toxicology and Pharmacology 2 205-210.

Turnbull, D., Whittaker, M.H., Frankos, V.H., Jonker, D. (1999c): 13-week oral toxicity study with stanol esters in rats. Regul Toxicol Pharmacol. 2 216-226.

Tuschl, H., Schwab, C. (2003): Cytotoxic effects of the herbicide 2,4-dichlorophenoxyacetic acid in HepG2 cells. Food and Chemical Toxicology 41 (3): 385-393.

Ulberth, F., Buchgraber, M. Extraction and Purification of Cholesterol Oxidation Products In: Cholesterol and Phytosterol Oxidation Products: Analysis,

Occurence and Biological Effects. Eds. Guardiola, F., Dutta, P.C., Codony, R. and Savage, G.P. Champaign, Illinois: AOCS Press, 2002, 26-49.

Valsta, L.M., Lemström, A., Ovaskainen, M.L., Lampi, A.M., Toivo, J., Korhonen, T., Piironen, V. (2004): Estimation of plant sterol and cholesterol intake in Finland: Quality of new values and their effect on intake. British Journal of Nutrition 92 (4): 671-678.

van Engeland, M., Nieland, L.J., Ramaekers, F.C., Schutte, B., Reutelingsperger, C.P. (1998): Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 31 (1): 1-9.

van Heyningen, C. (1999): Cholesterol lowering margarine may not be useful in healthy fat modified diet. British Medical Journal 319 (7203): 186.

Vermes, I., Haanen, C., Reutelingsperger, C. (2000): Flow cytometry of apoptotic cell death. Journal of Immunological Methods 243 (1-2): 167-190.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C. (1995): A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. Journal of Immunological Methods 184 (1): 39-51.

Volpe, R., Niittynen, L., Korpela, R., Sirtori, C., Bucci, A., Fraone, N., Pazzucconi, F. (2001): Effects of yoghurt enriched with plant sterols on serum lipids in patients with moderate hypercholesterolaemia. British Journal of Nutrition 86 (2): 233-239.

von Bergmann, K., Sudhop, T., Luetjohann, D. (2005): Cholesterol and Plant Sterol Absorption: Recent Insights. The American journal of cardiology 96 (1A): 10D-14D.

Wagner, K.H., Reichhold, S., Koschutnig, K., Cheriot, S., Billaud, C. (2007): The potential antimutagenic and antioxidant effects of Maillard reaction products used as "natural antibrowning" agents. Molecular Nutrition & Food Research 51 (4): 496-504.

Weihrauch, J.L., Gardner, J.M. (1978): Sterol content of foods of plant origin. Journal of the American Dietetic Association 73 (1): 39-47.

Weingärtner, O., Lütjohann, D., Ji, S., Weisshoff, N., List, F., Sudhop, T., von Bergmann, K., Gertz, K., König, J., Schäfers, H.-J., Endres, M., Böhm, M., Laufs, U. (2008): Vascular Effects of Diet Supplementation With Plant Sterols. Journal of the American College of Cardiology 51 (16): 1553-1561.

Wester, I. (2000): Cholesterol-lowering effect of plant sterols. European Journal of Lipid Science and Technology 102 (1): 37-44.

Weststrate, J.A., Meijer, G.W. (1998): Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. European Journal of Clinical Nutrition 52 (5): 334-343.

Whittaker, M.H., Frankos, V.H., Wolterbeek, A.P., Waalkens- Berendsen, D.H. (1999): Two-generation reproductive toxicity study of plant stanol esters in rats. Regulatory Toxicology and Pharmacology 2 196-204.

Wolfreys, A.M., Hepburn, P.A. (2002): Safety evaluation of phytosterol esters. Part 7. Assessment of mutagenic activity of phytosterols, phytosterol esters and the cholesterol derivative, 4-cholesten-3-one. Food and Chemical Toxicology 40 (4): 461-470.

Wolfs, M., de Jong, N., Ocke, M.C., Verhagen, H., Monique Verschuren, W.M. (2006): Effectiveness of customary use of phytosterol/-stanol enriched margarines on blood cholesterol lowering. Food and Chemical Toxicology: An International Journal Published For The British Industrial Biological Research Association 44 (10): 1682-1688.

Young, C.S., Dolan, J.W. (2004): Success with Evaporative Light-Scattering Detection, Part II: Tips and Techniques LC-GC North America 22 (3): 244-250

Zhang, X., Geoffroy, P., Miesch, M., Julien-David, D., Raul, F., Aoudé-Werner, D., Marchioni, E. (2005a): Gram-scale chromatographic purification of [beta]-sitosterol: Synthesis and characterization of [beta]-sitosterol oxides. Steroids 70 (13): 886-895.

Zhang, X., Julien-David, D., Miesch, M., Geoffroy, P., Raul, F., Roussi, S., Aoudé-Werner, D., Marchioni, E. (2005b): Identification and quantitative analysis of [beta]-sitosterol oxides in vegetable oils by capillary gas chromatography-mass spectrometry. Steroids 70 (13): 896-906.

From the data obtained in the present thesis the following papers were prepared:

Paper I KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, FRITZ-TON C, WAGNER K-H Separation and Isolation of ß-sitosterol oxides and their non-mutagenic potential in the Salmonella microsome assay. *Food Chemistry* – in revision

Paper II KOSCHUTNIG K, HEIKKINEN S, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H Cytotoxic and apoptotic effects of single and mixed oxides of ß-sitosterol on HepG2-cells. *Toxicology in Vitro* - in revision

PAPER I

Separation and Isolation of β-sitosterol oxides and their non-mutagenic potential in the Salmonella microsome assay

Karin Koschutnig[1], Suvi Kemmo[2], Anna-Maija Lampi[2], Vieno Piironen[2], Cornelia Fritz-Ton[1], Karl-Heinz Wagner[1]*

[1]Department of Nutritional Sciences, University of Vienna,
Althanstrasse 14, 1090 Vienna, Austria

[2]Department of Applied Chemistry and Microbiology, University of Helsinki,
Latokartanonkaari 11, P.O.Box 27, FIN-00014 University of Helsinki, Finland

*corresponding author: Karl-Heinz Wagner
Department of Nutritional Sciences
Faculty of Life Sciences, University of Vienna
Althanstraße 14
1090 Vienna, Austria
Ph: +43 1 4277 54930
Fax: +43 1 4277 9549
e-mail: karl-heinz.wagner@univie.ac.at

In revision: *Food Chemistry*

Abstract

The recent increase in phytosterol-enriched functional food provokes questions concerning the safety of their oxidation products. However, most of the existing toxicity studies have been performed with mixtures instead of single compounds, a consequence of the lack of pure phytosterol oxidation product (POP)-standards.

The objectives of this study were to take in use a method for the isolation of β-sitosterol oxidation products and to assess their mutagenic and pro-oxidative potential. Oxides were prepared by thermo-oxidation, purified by column chromatography and separated by a NP-HPLC-UV system. 7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol, a mixture of 6β-OH-3-keto-sitosterol/6α-OH-3-keto-sitosterol (ratio 4:3) and a mixture of polar oxides were fractionated. Yields in the range of several milligrams per fraction were achieved within one HPLC-run. Identification and quantification was done by GC-MS and GC-FID, respectively.

In the Ames test the collected fractions failed to show a mutagenic activity towards Salmonella typhimurium strains TA98, TA100 and TA102.

Key words: phytosterol oxidation products, HPLC, Salmonella microsome assay

Introduction

Recently phytosterols (plant sterols) have become a focus of interest due to their serum cholesterol lowering effect and consequently their protection against cardiovascular diseases (Piironen, Lindsay, Miettinen, Toivo & Lampi, 2000). According to the literature an uptake of 1.5 to 3 g phytosterols per day is recommended in order to achieve a reduction of 10-15 % in serum LDL cholesterol (Katan, Grundy, Jones, Law, Miettinen & Paoletti, 2003). As the daily intake from natural sources is estimated to range from 150 to 440mg a day, a wide variety of products fortified with phytosterols has been introduced to the market in order to reach the advised uptake levels. However, besides their positive aspects concerns in terms of health are emerging.

It is well known that during food preparation and storage cholesterol oxidation products (COPs) are formed from cholesterol through autoxidation, photoxidation and enzymatic oxidation (Boesinger, Luf & Brandl, 1993). Mutagenic, carcinogenic, angiotoxic, cytotoxic and atherogenic properties of the thereby generated COPs are generally accepted and have already been extensively documented (for review see Osada, 2002).

Similar to cholesterol, phytosterols consist of a tetracyclic cyclopenta[α]phenanthrene ring, they mainly differ in their side chain (Piironen et al., 2000). Because of their structural similarities, phytosterols are expected to undergo analogous chemical reactions as cholesterol, including oxidation. But information on phytosterol oxidation products (POPs) is still rather limited.

Existing knowledge on POPs is mainly concentrated on their quantification in food. In first studies concerning their absorption in vivo a significant uptake of POPs was proven (Grandgirard et al., 2004).

Regarding their possible harmful effects only a few studies were conducted and most of them dealt with a potential cytotoxicity. Investigations with different cultured mammalian cells showed comparable damage to that caused by COPs, but higher concentrations of POPs were needed (Adcox, Boyd, Oehrl, Allen & Fenner, 2001; Maguire, Konoplyannikov, Ford, Maguire & O'Brien, 2003; Ryan, Chopra, McCarthy, Maguire & O'Brien, 2005). So far only Lea, Hepburn, Wolfreys and Baldrick (2004) investigated the mutagenic potential of POPs in the Ames test and reported no evidence of genotoxicity. However, in this investigation only a non specified mixture with 30 % of POPs was analysed. Due to the lack of commercial POP-standards in most cases a

blend instead of single oxidation products has been used for toxicity studies. But evidence exists that compared to mixtures individual POPs behave in a different way (Maguire et al., 2003). Therefore the biological and safety aspects of POPs remain rather unclear.

Hence, the aim of the present study was to scale up a method for the isolation of single phytosterol oxidation products. ß-sitosterol served as the model compound as it is the most abundant plant sterol in nature. Gram scale amounts of toxicologically relevant oxidation products with sufficient purity were collected in order to undergo toxicity tests.

The second objective of this study was to investigate the collected oxides regarding their possible mutagenic and pro-oxidative properties in the Ames test. Since it is assumed that the toxicity of oxidation products varies depending on their chemical structure (Osada, 2002) a hierarchy of toxicity of the isolated compounds would be essential. In addition a mixture of all oxidation products was prepared and analysed in the same way, to further investigate whether or not single isolated products react differently from mixtures.

Materials and methods

Chemicals

24β-Ethylcholest-5-en-3β-ol (purity: β-sitosterol ~76 %, sitostanol ~13 %, campesterol~ 8 %, campestanol~ 1 %) was purchased from Fluka Chemie (Buchs, Switzerland). 5-Cholesten-3β-ol (cholesterol), Cholest-5-en-3β-ol-7-one (7-ketocholesterol) and Cholestan-5α,6α-epoxy-3β-ol (5α,6α-epoxycholesterol) were provided by Sigma Chemical Co (St.Louis. MO, USA). Cholest-5-en-3β,7α-diol (7α-hydroxycholesterol), Cholest-5-en-3β,7β-diol (7β-hydroxycholesterol) and 5-Cholesten-3β,19-ol (19-hydroxycholesterol), the latter was used as an internal standard (ISTD) in GC- analysis, were obtained from Steraloids (Wilton, NH, USA). All the other cholesterol oxides were used as reference solutions in TLC and HPLC analysis, as standards of phytosterol oxides are not available.

Silica gel 60 (0.2–0.5 mm) for column chromatography and thin layer chromatography (TLC) plates (silica gel 60, 20 x 20 cm) were purchased from E. Merck (Darmstadt, Germany). Spots were visualized by staining with 10% sulphuric acid (H_2SO_4) (E. Merck) in methanol (Rathburn Chemicals Ltd., Walkerburn, Scotland). Bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were obtained from E. Merck and Fluka Chemie respectively, and were used as a 99:1 (v/v) mixture for silylation of the oxidation products. Analytical grade pyridine (>99 %) from Sigma was also used. Ethyl acetate (E. Merck), diethyl ether (J.T.Baker, Deventer, The Netherlands), *n*-heptane and acetone (Rathburn Chemicals Ltd.), 99,5 % ethanol (Primalco, Rajamäki, Finland) and water (purified by Milli-Q Plus, Millipore, Molsheim, France) all of analytical grade, were used.

All chemicals used for the Ames test were obtained from Sigma (Vienna, Austria), unless otherwise stated. The Salmonella thyphimurium strains TA98, TA100 and TA102 were obtained from Discovery Partners International (San Diego, USA) and from Trinova Biochem GmbH (Giessen, Germany). The S9 liver homogenate (from Sprague–Dawley rats induced with Aroclor 1254 prepared as a KCL homogenate) was obtained from MP Biomedicals (Illkirch, France). Agar no. 1 and Nutrient Broth were obtained from Oxoid/Bertoni (Vienna, Austria), Dulbecco's PBS was from PAA (Pasching, Austria). All mutagens and other reagents were of analytical reagent grade or higher and stored at – 80 °C if necessary.

Production and isolation of phytosterol oxidation products

Preparation of oxides by thermo-oxidation

POPs were formed by thermo-oxidation. Commercial available β–sitosterol was heated in open glass vials (300 mg, 25 mm, I.D.) in a ventilated oven for 24 hours at 130 °C. Optimal heating conditions were evaluated in pre-tests. Different temperatures and time periods (130 °C/24 h, 120 °C/48 h and 130 °C/48 h) were tested and resulting oxidation mixtures were analysed using GC-FID and GC-MS. After the heating period the samples were cooled down in a dessicator, dissolved in 15 mL of *n*-heptane/diethyl-ether (90:10, v/v), solubilised using a sonicator and finally stored in a freezer (-20 °C).

Purification and enrichment of phytosterol oxidation products by column chromatography (CC)

The purification method used was based on former investigations by Lampi, Juntunen, Toivo and Piironen (2002) and Apprich and Ulberth (2004). Briefly, 72 g silica gel (loaded with 10 % distilled water) was dry packed into a glass column and pre-wetted with 150 mL *n*-heptane. The whole sample (300 mg heated β–sitosterol/15 mL *n*-heptane/diethyl-ether (90:10, v/v)) was applied to the column and first nonoxidized sterols and apolar components were eluted with 150 mL *n*-heptane/diethyl-ether (90:10, v/v) followed by 450 mL *n*-heptane/diethyl-ether (50:50, v/v). Thereafter POPs were extracted with 150 mL acetone. The acetone fraction was evaporated to dryness. To get rid of the water ethanol was added during the evaporation step. The residue was dissolved in 550 µL *n*-heptane/2-propanol (93:7, v/v).

To confirm the performance of the CC TLC was conducted. 100 µL of the purified sample were applied to silica gel G 60 TLC plates (0.5 mm layer thickness). The identification of the single oxidation products was done using a standard solution, which contained 5α,6α-epoxy-, 7-hydroxy- and 7-ketocholesterol. As eluent *n*-heptane/ethyl-acetat (50:50, v/v) was used. Components were visualized by spraying with 10 % sulphuric acid in methanol followed by heating for a few minutes at 100 ˚C.

Separation of single oxidation products by a NP-HPLC-UV system

For the collection of single oxidation products a preparative normal-phase HPLC-method was used. The method was based on papers published by Kemmo et al. (Kemmo, Soupas, Lampi & Piironen, 2005; Säynäjoki, Sundberg, Soupas, Lampi & Piironen, 2003) with some modifications. A preparative HPLC-instrument (Waters Delta Prep 3000, Milford, USA) equipped with a silica Supelcosil column (250 mm x 21.1 mm, 12 µm; Supelco, Bellefonte, PA, USA) and a UV detector at 206 nm (Waters 484 Milford, USA) was applied. Several mobile phase systems (90:10, 92:8, 93:7, 94:6, 95:5 and 97: 3 *n*-heptane/2-propanol) and flow rates (5, 7, 9.9, 15 and 17 mL/min) were examined. Determination of purity levels and identification of the collected fractions was done by GC-MS. The best separation was achieved at room temperature under isocratic conditions using a mobile phase of *n*-heptane/2-propanol (93:7, v/v) with a

flow rate of 17 mL/min. The injection volume was 1.8 mL. The performance of the separation was checked daily using a cholesterol oxides standard solution (7-ketocholesterol, 7β-hydroxycholesterol and 7α-hydroxycholesterol) by monitoring the retention times. For the generation of the mixture no HPLC separation was performed, therefore it includes all polar β-sitosterol oxidation products.

Identification and quantification

For identification and quantification of the collected fractions GC-mass spectrometry (GC-MS) and GC-flame ionization detection (GC-FID) were used, respectively. Both methods have been developed earlier by co-workers (Lampi et al., 2002; Soupas, Juntunen, Säynäjoki, Lampi & Piironen, 2004) and are routinely used for analysing POPs.

Prior to the GC analysis the samples were converted to TMS-ether derivatives. Therefore 100 µL aliquots of each fraction and 1mL of internal standard solution (19-OH-cholesterol, 18.55 µg/mL) were evaporated to dryness under nitrogen, dissolved in 100 µL of pyridine and subjected to silylation by 100 µL BSTFA/TMCS (100 µL, 99:1, v/v) over night at room temperature. The reagent mixture was then evaporated and the residue was dissolved in 200 µL *n*-heptane before GC analysis.

GC–MS analysis

For identification of the collected oxidation products and verification of the purity of the fractions GC–MS was used as described in Soupas et al. (2004). The GC–MS equipment consisted of a Hewlett Packard 6890 Series gas chromatograph (Wilmington, PA, USA) including a Rtx-5MS w/Integra fused-silica capillary column (60 m x 0.25 mm i.d., crossbond 5 % diphenyl – 95 % dimethyl polysiloxane, 0.1 µm film with 10 m Integra-Guard column; Restek, Bellefonte, PA, USA) and was coupled to an Agilent 5973 mass spectrometer (Palo Alto, CA, USA). Helium was used as carrier gas at 240 kPa. Initial oven temperature was 70 °C, after 1 min the temperature was raised to 280 °C at 40 °C/ min and was then held at 280 °C for 35 min. Interface temperature and ion source were 280 °C and 230 °C, respectively. Spectra were scanned within the mass range of m/z 100-600 using the electron impact mode (70 eV).

GC–FID analysis

The quantification was done by GC- FID measurements using a Hewlett Packard 6890 Series II gas chromatograph equipped with an HP-7673 autosampler (Hewlett-Packard, Karlsruhe, Germany), an automated on-column injection system, a flame ionization detector (FID), ChemStation 3.1 software and a RTX-5w/Integra fused-silica capillary column (60 m x 0.32 mm i.d., 5 % diphenyl–95 % dimethyl polysiloxane, 0.1 μm film with 10m Integra-Guard column; Restek, Bellefonte, PA, USA). Helium (99.996 % AGA, Espoo, Finland) was used as the carrier gas at a constant flow (110 kPa at 200 °C). The initial temperature was 70 °C, which increased after 1min to 245 °C by 60 °C/min. After 1min at 245 °C it raised by 3 °C/min to 275 °C and remained at this temperature for 41 min. The detector temperature was 300 °C. The concentrations of the POPs were quantified by the added internal standard (19-OH-cholesterol).

Salmonella microsome assay

Preparation of reaction mixtures

Three individual oxidation products, 7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol, a mixture of 6β-OH-3-keto-sitosterol/6α-OH-3-keto-sitosterol (ratio 4:3) and a mixture of the polar oxidation products of β-sitosterol were investigated. Considering the results of pre-tests, four concentrations (0.04, 0.2, 1.0 and 5.0 mg per plate (≈ %)) of each compound were analysed. The concentrations range used was a broad spectrum from very low physiological to nonphysiologically high concentrations, all below the solubility range, which is recommended for this test procedure.

Due to precipitation in the highest concentration of 7α-OH-sitosterol, only 3 doses of this compound could be used (0.04 – 1 %). There were similar but minor solubility problems with the mixture of 6β-OH-3-keto-sitosterol/6-α-OH-3-keto-sitosterol, therefore as highest concentration 2.5 mg/plate was tested. On the other hand the mixture of all oxidation products showed very good solubility, so a 10 mg/plate dilution could also be included in the experiments (Table1).

In order to obtain appropriate dilutions, each sample was pre-dissolved in a mixture of acetone/tween80 (3:1, v/v). Afterwards two parts of sterile, distilled water was added to keep the concentration of acetone as low as possible and to avoid potential toxic effects

on the bacterial strains. This procedure was tested to be safe for the strains in pre-experiments.

Metabolic activation

In order to simulate in vivo conditions the oxidation products were treated with a rat liver enzyme mixture (S9, which mainly consists of phase I enzymes) for metabolic activation. The S9 mix was prepared according to the recipes of Maron and Ames (1984) consisting of 19.75 mL destilled water, 25 mL of PBS buffer, 0.5 mL of $MgCl_2$ (0.85 M), 0.5 mL of KCl (1.65 M) and 2 mL of NaDP (90.8 mM), 250 µL of glucose-6-phosphate (1.08 M), and 2 mL of S9. The reagent was stored on ice throughout the whole experiment and discarded after 50 min.

Experimental design

The Ames Salmonella/ microsome mutagenicity assay (Salmonella test, Ames test) was performed according to Maron and Ames (1984). The preincubation assay with an incubation period of 25 min (37 °C) was chosen, as reported earlier (Wagner, Reichhold, Koschutnig, Cheriot & Billaud, 2007). Briefly, 500 µL of PBS or S9 mix, 200 µL of reaction mixture and 100 µL of overnight bacterial culture were added to test tubes. The tubes were shortly vortexed and then placed in an incubator on a rotary shaker and incubated for 25 min at 37 °C. After this period 2 mL of molten top agar were added to each tube. The mixture was vortexed and poured on minimum glucose plates. As soon as the agar had solidified the plates were inverted and stored in an incubator for 48 h at 37 °C. Thereafter his^+- revertants were counted manually.

In the present study three generally recommended tester strains (TA98, TA100 and TA102) were applied. TA98 gives information on frame-shift mutations, TA100 on base-pair substitutions and TA102 detects cross-linking agents, additionally it is specifically used to inform on oxidative stress. To further investigate the anti-/pro-oxidative effects the pro-oxidant tertiary-butyl hydroperoxide (*t*-BOOH, 0.7 mM) was used for a challenge test. The concentration of the oxidant was chosen in order to obtain suitable numbers of revertants on the plates. This test was performed with and without metabolic activation.

Each experiment included a positive control (2,4,7-Trinitro-9-fluorenone, Sodiumazide or 2-Aminofluorene), which confirmed the reversion capacity of the bacterial strains as well as a negative control, the sample solvent. All concentrations and the positive control were tested in triplicate. For the negative control even six plates were prepared. Each test was done at least twice (n = 6 in total).

Statistical analysis and evaluation of the mutagenic experiments

All data are expressed as mean ± SD (standard derivation). Obtained data (n = 6 for each concentration used) were analysed by one-way analysis of variance (ANOVA) and the Student's t-test since they were all normally distributed, using SPSS 15.0 for Windows. Statistical differences were considered significant at a value of $p < 0.05$.

In addition to the statistical, a nonstatistical evaluation was carried out. According to Mortelmans and Zeiger (2000) a compound is considered as "mutagenic" if the total number of his^+- revertants per plate was at least twice as high as the negative control (200 %). Moreover a dose related increase of the number of his^+- revertants has to be shown.

Results and Discussion

As already mentioned POPs are not commercially available, so they have to be laboratory-prepared. Applied methods are based on chemical synthesis (Bortolomeazzi, De Zan, Pizzale & Conte, 1999; Zhang et al., 2005), thermo-oxidation in solid state (Lampi et al., 2002) or aqueous dispersion (Dutta & Appelqvist, 1997). Existing techniques are mostly very complex and time-consuming with the requirement of special equipment. Besides they are usually designed for the production of only small amounts of oxidation products. Therefore the initial target of this study was the development of a fast, simple and effective method for the isolation of common β-sitosterol oxides.

Performance of the method

Preparation of the test compounds by thermo-oxidation

In the present study thermo-oxidation in solid state was used for formation of oxides. Different temperatures and heating periods were tested and generated amounts of

oxysterols were estimated by GC-FID (Figure 1). Heat treatment for 24 h at 130 °C showed to be the best option, yielding to an oxidation rate of nearly 20 %. The total amount of oxidation products decreased throughout longer heat exposure, even at a lower temperature (120 °C compared to 130 °C). This observation can be explained by conversion reactions to other secondary oxidation products as well as further reactions such as polymerisation, leading to non polar compounds, dimers and polymers. The latter occurs further particularly at higher heating conditions. The oxidation products observed in the heating experiments were analogous to those cited in the literature, mainly 7-ketositosterol followed by 7β-OH-sitosterol, 7α-OH-sitosterol, 5,6β-epoxy-sitosterol, 5,6α-epoxy-sitosterol, 6β-OH-3-keto-sitosterol, 6α-OH-3-keto-sitosterol, 6β-OH-sitosterol and 6keto-sitosterol. Our findings supported those of Kemmo et al. (2005), who assessed that at different heating temperatures the same kind of oxidation products were formed, but the amount of the single products varied. Already Caboni, Costa, Rodriguez-Estrada and Lercker (1997) noticed that the ratios of the formed products were influenced by the oxidation conditions.

The high amount of 7-keto-sitosterol and the generation of 7β-OH-sitosterol rather than 7α-OH-sitosterol was in accordance with previous studies (Chien, Wang & Chen, 1998; Kemmo et al., 2005).

Purification and enrichment of the test compounds

When β-sitosterol is heated a mixture of oxidation products, non oxidized material and several unknown by-products is formed. Because of the complexity of the gained blend usually a combination of different separation techniques is applied to isolate single oxidation products. In this work CC was used for the first separation step. In contrast to the often employed solid-phase extraction (SPE) cartridges self prepared glass columns offer a higher loading capacity. Since our interest was focused on POPs with a higher polarity than non oxidized β-sitosterol a stepwise elution order to remove apolar components up to free sitosterol was applied.

Solvent mixtures of *n*-heptane/diethyl-ether in combination with silica gel and a final elution of the desired components with acetone were already successfully applied by others (Apprich et al., 2004; Lampi et al., 2002; Piironen, Toivo & Lampi, 2002). TLC was used to confirm the separation process. No products with a lower polarity than β-

sitosterol were detected. In addition a relevant reduction of non oxidized β-sitosterol could be noted. In other publications preparative TLC was also used to fractionate oxidation products. But this is only appropriate when small amounts of samples for analytical purpose are separated. In addition to its low loading capacity it also allows long exposure of the sample to air, which facilitates the possibility of artefact formation.

Separation, Isolation and Fractionating of the oxidation products by NP-HPLC-UV

The final separation of the oxidation products was achieved by a NP-HPLC-UV system. Due to its mild characteristics preparative HPLC is particularly suitable for the isolation of oxides. Both normal- and reverse phase chromatography has already been successfully used for the analyses of cholesterol (Caboni et al., 1997; Chien et al., 1998; Csallany, Kindom, Addis & Lee, 1989; Mazalli, Sawaya, Eberlin & Bragagnolo, 2006) and plant sterol (Kemmo, Ollilainen, Lampi & Piironen, 2007; Kemmo et al., 2005) oxidation products. In general normal phase chromatography is considered to be the more effective option.

Different mobile phase systems with changing percentages of IPA were tested based on former investigations by Kemmo et al. (2007; 2005; Säynäjoki et al., 2003). Best separation was achieved with a mobile phase of n-heptane /IPA (93:7, v/v). The same solvent system was already used earlier for the separation of COPs (Csallany et al., 1989). The elution order in this system was as followes: 6β-OH-3-ketositosterol/6α-OH-3-ketositosterol, 7-ketositosterol, 6β-OH-sitosterol, 7β-OH-sitosterol and 7α-OH-sitosterol (Figure 2). A rapid separation in one single HPLC-run was achieved within 20 minutes. The application of a big preparative silica column (25 cm x 21.2 mm, 12 µm) allowed an injection volume of 1.8 mL of the obtained oxidation mixture, containing the oxidation products derived from about 1000 mg unoxidized β-sitosterol (collected oxides of several purification steps by CC were combined). This led to yields in the range of several milligrams for the single fractions. Within one HPLC run, for example, 15-20 mg of 7-ketositosterol could be collected. For detailed information see table 2.

However, as already ascertained earlier no full resolution of the entire polarity range of the oxidation products is possible under isocratic conditions (Guardiola, Bou, Boatella & Codony, 2004). 6β-OH-3-ketositosterol and 6α-OH-3-ketositosterol co-eluted, so

they were collected in one fraction. When analysing the collected fractions with GC-FID high proportions of 7-ketositosterol were found in the 6β-OH-sitosterol-sample. Therefore 6β-OH-sitosterol was excluded from subsequent toxicology tests.

Moreover as already observed in earlier investigations (Dutta et al., 1997) it was not possible to separate campesterol oxides from their sitosterol counterparts. Since the applied commercial β-sitosterol included some amount of campesterol (~8 %) also a low amount of campesterol oxides (~10 %) was present in the oxidation mixture. Average values of the purity of the collected compounds are listed in table 3 and GC-FID chromatograms of each isolated oxide fraction are given in figure 3. However, bearing in mind the velocity of our method we put up with these minor impurities.

Among all detection systems available UV detection is still the most frequently applied for sterol analyses. Separation of the oxidation products was monitored using a wavelength of 206 nm. It is the common wavelength employed for oxysterols (Csallany et al., 1989). Since products without double bonds like epoxy- compounds and triols have poor UV absorption they were not included in collected oxides.

Bacterial tests

Although indications for adverse health effects exist, knowledge on biological properties of POPs is rather small. Further almost all of the so far conducted toxicology studies were done with cell lines, among others because of the small sample amounts needed for these assays. As data concerning the mutagenic potential of isolated POPs is lacking, the collected oxidation products were tested in Salmonella typhimurium strains TA98, TA100 and TA102 in the Ames test. To our knowledge it is the first time that single oxides of β-sitosterol are tested on their behaviour towards Salmonella typhimurium strains.

Mutagenicity testing

According to literature the here applied preincubation assay is the most sensitive form of the Ames test. It allows a closer contact of the test compounds and the indicator strain since the bacteria are able to react in a small volume, which is an advantage for detecting short- living mutagens (Mortelmans et al., 2000).

Throughout the whole test period the spontaneous mutation frequency of the strains was in accordance with the control levels published (Mortelmans et al., 2000) and each POP sample was significantly different to the positive control ($p < 0.05$).

In strain TA98 no mutagenic activity could be seen; neither with nor without metabolic activation. Also in TA100 the number of his^+- revertants remained in the range of the negative control and therefore were mutagenic not active (data not shown).

These results were in accordance with those published recently by Lea et al. (2004) who tested a mixture of oxidized and unoxidized products of β-sitosterol by using different Salmonella strains (TA98, TA100, TA102, TA1535 and TA1537). However, the amount of oxidation products in their tested samples was only 30 %.

It is already well known that oxide mixtures react differently from individual oxidation products (Maguire et al., 2003). Also additive, synergistic as well as inhibitory effects of single oxidation products in mixtures have been noted (O'Sullivan A, O'Callaghan Y & O'Brien N, 2005). Therefore, in contrast to our study, the results of Lea et al. (2004) allow no sufficient information about the safety aspects of single oxidation products.

In the aforementioned publication also toxicity and precipitation problems during their work with TA102 were reported. Consequently in these experiments sample amounts had to be reduced. Throughout all of our research work no problems in this regard were observed.

Although in the present study none of the oxidation products were able to increase the number of revertants beyond the doubled negative control, yet for some oxides a certain response was observed in strain TA102 (Table 4). In the preincubation test without metabolic activation the highest concentrated fraction of 7-keto-sitosterol (5 %) increased the number of his^+ - revertants significantly, but not beyond twice of the negative control, which was set as threshold for mutagenic activity. Moreover a concentration- dependent tendency was seen for the lower concentrations but not for the 5 %- fraction, mainly due to its higher standard deviation. With the addition of S9 the increase of 7-keto-sitosterol (5 %) was even more pronounced ($p < 0.05$). On the other hand the highest concentrated fraction of 7β-OH-sitosterol (5 %) reduced the number of his^+-revertants significantly compared to its lower concentrated fractions ($p < 0.05$).

The strain TA102 was chosen as besides its capability of detecting cross-linking agents it additionally informs on oxidative stress (Grey & Adlercreutz, 2003; Mortelmans et

al., 2000). Based on that, the increase in revertants induced by 7-keto-sitosterol (5 %) could be an indication of a pro-oxidant tendency. On the other hand the decline in revertant numbers of 7β-OH-sitosterol (5 %) could be interpreted as a marginal sign of antioxidative effects. However, it could also be the result of increased cytotoxicity (Mortelmans et al., 2000).

To date no papers other than the one by Lea et al.(2004) had been published dealing with possible mutagenic actions of POPs. Hence, more information is available on mutagenic effects of COPs. Due to their structural similarity an analogous mode of action is expected. As early as in 1979 mixtures of COPs have been shown to possess mutagenic effects towards Salmonella typhimurium strains TA98, TA1535 and TA1537 (Smith, Smart & Ansari, 1979). Several studies followed and in spite of contradictory results it seems that particularly epoxy and triol compounds of cholesterol possess mutagenic activity. Epoxy and triol compounds of β-sitosterol had never been tested before in regard of their mutagenicity and they were also not included in the present study. Oxysterols might in general possess rather cytotoxic properties whereas for epoxide epimeres and triols a mutagenic action could also be possible. For the mutagenic reactivity of COPs the authors also suggested ROS to be involved in the mutagenic action (Cheng, Kang, Shih, Lo & Wang, 2005; Smith et al., 1979), as antioxidant enzymes diminished the mutagenic response.

Antioxidant testing

To strengthen the information on oxidative stress challenge tests with the pro-oxidant *t*-BOOH were included in our experiments. *t*-BOOH is commonly used in in vitro assays, as it is known as an initiator of lipid peroxidation, where it leads to the formation of alkoxyl and alkyl radicals (Grey et al., 2003).

A prevention of the thereby caused mutagenicity would indicate possible antioxidant properties of the tested compounds. Per definition a reduction of the revertant colony number below 50 % of the negative control level is necessary for an anti-mutagenic effect (Mortelmans et al., 2000). This was never seen in any of our conducted tests (Table 4). However, the addition of *t*-BOOH 7β-OH-sitosterol (5 %) caused a significant decrease in his^+- revertants ($p < 0.05$); even though not lower than the threshold level. With the addition of S9 the number of his^+-revertants was still lower

compared with the less concentrated fractions, but here this tendency was not significant ($p = 0.196$).

Conclusion

The applied method was proven to be a powerful tool for the collection of oxidation products, as yields in the range of several milligrams per fractions were achieved within only one HPLC-run. Thus the collection of oxysterols for a later application in toxicity tests was possible.

The results of the Ames test demonstrated that the analysed common oxidation products of β-sitosterol were not mutagenic towards Salmonella typhimurium strains TA98, TA100 and TA102. Even though literature on the mutagenic response of COPs is conflicting, for some oxidation products a mutagenic action could be proved. In spite of their similar structure there could still be a difference in the mutagenic behaviour of cholesterol- and plant sterol oxidation products.

Acknowledgement

The work was supported by COST organization as a Cost 927 short term scientific mission, carried out at the University of Helsinki, by the Academy of Finland and by a research grant on behalf of the University of Vienna (F81-B Forschungsstipendium).

References

Adcox, C., Boyd, L., Oehrl, L., Allen, J., & Fenner, G. (2001). Comparative effects of phytosterol oxides and cholesterol oxides in cultured macrophage-derived cell lines. *Journal of Agricultural and Food Chemistry*, *49*(4), 2090-2095.

Apprich, S., & Ulberth, F. (2004). Gas chromatographic properties of common cholesterol and phytosterol oxidation products. *Journal of Chromatography, A*, *1055*(1-2), 169-176.

Boesinger, S., Luf, W., & Brandl, E. (1993). 'Oxysterols': Their occurrence and biological effects. *International Dairy Journal*, *3*, 1-33.

Bortolomeazzi, R., De Zan, M., Pizzale, L., & Conte, L. S. (1999). Mass spectrometry characterization of the 5alpha-, 7alpha-, and 7beta-hydroxy derivatives of beta-sitosterol, campesterol, stigmasterol, and brassicasterol. *Journal of Agricultural and Food Chemistry*, *47*(8), 3069-3074.

Caboni, M. F., Costa, A., Rodriguez-Estrada, M. T., & Lercker, G. (1997). High-performance liquid chromatographic separation of cholesterol oxidation products. *Chromatographia*, *46*(3/4), 151-155.

Cheng, Y. W., Kang, J. J., Shih, Y. L., Lo, Y. L., & Wang, C. F. (2005). Cholesterol-3-beta, 5-alpha, 6-beta-triol induced genotoxicity through reactive oxygen species formation. *Food and Chemical Toxicology*, *43*(4), 617-622.

Chien, J. T., Wang, H. C., & Chen, B. H. (1998). Kinetic Model of the Cholesterol Oxidation during Heating. *Journal of Agricultural and Food Chemistry*, *46*(7), 2572-2577.

Csallany, A. S., Kindom, S. E., Addis, P. B., & Lee, J. H. (1989). HPLC method for quantitation of cholesterol and four of its major oxidation products in muscle and liver tissues. *Lipids*, *24*(7), 645-651.

Dutta, P. C., & Appelqvist, L. A. (1997). Studies on phytosterol oxides. I: Effect of storage on the content in potato chips prepared in different vegetable oils. *Journal of the American Oil Chemists' Society*, *74*(6), 647-657.

Grandgirard, A., Martine, L., Demaison, L., Cordelet, C., Joffre, C., Berdeaux, O., & Semon, E. (2004). Oxyphytosterols are present in plasma of healthy human subjects. *British Journal of Nutrition*, *91*(1), 101-106.

Grey, C. E., & Adlercreutz, P. (2003). Ability of antioxidants to prevent oxidative mutations in Salmonella typhimurium TA102. *Mutation Research*, *527*(1-2), 27-36.

Guardiola, F., Bou, R., Boatella, J., & Codony, R. (2004). Analysis of sterol oxidation products in foods. *Journal of AOAC International*, *87*(2), 441-460.

Katan, M. B., Grundy, S. M., Jones, P., Law, M. F. R. C. P., Miettinen, T., & Paoletti, R. (2003). Efficacy and Safety of Plant Stanols and Sterols in the Management of Blood Cholesterol Levels. *Mayo Clin Proc*, *78*(8), 965-978.

Kemmo, S., Ollilainen, V., Lampi, A.-M., & Piironen, V. (2007). Determination of stigmasterol and cholesterol oxides using atmospheric pressure chemical ionization liquid chromatography/mass spectrometry. *Food Chemistry*, *101*(4), 1438-1445.

Kemmo, S., Soupas, L., Lampi, A.-M., & Piironen, V. (2005). Formation and decomposition of stigmasterol hydroperoxides and secondary oxidation products during thermo-oxidation. *European Journal of Lipid Science and Technology*, *107*(11), 805-814.

Lampi, A.-M., Juntunen, L., Toivo, J., & Piironen, V. (2002). Determination of thermo-oxidation products of plant sterols. *Journal of Chromatography B*, *777*(1-2), 83-92.

Lea, L. J., Hepburn, P. A., Wolfreys, A. M., & Baldrick, P. (2004). Safety evaluation of phytosterol esters. Part 8. Lack of genotoxicity and subchronic toxicity with phytosterol oxides. *Food and Chemical Toxicology*, *42*(5), 771-783.

Maguire, L., Konoplyannikov, M., Ford, A., Maguire, A. R., & O'Brien, N. M. (2003). Comparison of the cytotoxic effects of beta-sitosterol oxides and a cholesterol oxide, 7beta-hydroxycholesterol, in cultured mammalian cells. *British Journal of Nutrition*, *90*(4), 1-10.

Maron, D. M., & Ames, B. N. (1984). Revised Methods for the Salmonella Mutagenicity Test. In: B. J. Kilbey, M. Legator, & C. Ramel, *Handbook of Mutagenicity Test Procedures* (pp. 93-140): Elsevier Science Publishers BV.

Mazalli, M. R., Sawaya, A. C. H. F., Eberlin, M. N., & Bragagnolo, N. (2006). HPLC Method for Quantification and Characterization of Cholesterol and Its Oxidation Products in Eggs. *Lipids*, *41*(2), 615-622.

Mortelmans, K., & Zeiger, E. (2000). The Ames Salmonella/microsome mutagenicity assay. *Mutation Research*, *455*(1-2), 29-60.

O'Sullivan A, J., O'Callaghan Y, C., & O'Brien N, M. (2005). Differential effects of mixtures of cholesterol oxidation products on bovine aortic endothelial cells and human monocytic U937 cells. *Int J Toxicol*, *24*(3), 173-179.

Osada, K. (2002). Cholesterol oxidation products: other biological effects. In: P. C. Dutta, F. Guardiola, R. Codony, & G. P. Savage, *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence and Biological Effects* (pp. 278-318). Champaign, Illinois: AOCS Press.

Piironen, V., Lindsay, D. G., Miettinen, T. A., Toivo, J., & Lampi, A.-M. (2000). Plant sterols: biosynthesis, biological function and their importance to human nutrition. *Journal of the Science of Food and Agriculture*, *80*(7), 939-966.

Piironen, V., Toivo, J., & Lampi, A. M. (2002). Plant sterols in cereals and cereal products. *Cereal Chemistry*, *79*(1), 148-154.

Ryan, E., Chopra, J., McCarthy, F., Maguire, A. R., & O'Brien, N. M. (2005). Qualitative and quantitative comparison of the cytotoxic and apoptotic potential of phytosterol oxidation products with their corresponding cholesterol oxidation products. *British Journal of Nutrition*, *94*(3), 443-451.

Säynäjoki, S., Sundberg, S., Soupas, L., Lampi, A. M., & Piironen, V. (2003). Determination of stigmasterol primary oxidation products by high-performance liquid chromatography. *Food Chemistry*, *80*(3), 415-421.

Smith, L. L., Smart, V. B., & Ansari, G. A. (1979). Mutagenic cholesterol preparations. *Mutation Research*, *68*(1), 23-30.

Soupas, L., Juntunen, L., Säynäjoki, S., Lampi, A. M., & Piironen, V. (2004). GC-MS method for characterization and quantification of sitostanol oxidation products. *Journal of the American Oil Chemists' Society*, *81*(2), 135-141.

Wagner, K. H., Reichhold, S., Koschutnig, K., Cheriot, S., & Billaud, C. (2007). The potential antimutagenic and antioxidant effects of Maillard reaction products used as "natural antibrowning" agents. *Molecular Nutrition & Food Research*, *51*(4), 496-504.

Zhang, X., Geoffroy, P., Miesch, M., Julien-David, D., Raul, F., Aoudé-Werner, D., & Marchioni, E. (2005). Gram-scale chromatographic purification of [beta]-sitosterol: Synthesis and characterization of [beta]-sitosterol oxides. *Steroids*, *70*(13), 886-895.

Figure1: Proportion of collected β-sitosterol secondary oxidation products (mg) after different oxidation conditions (130 °C/24 h, 120 °C/48 h and 130 °C/48 h) measured by a GC-FID system. Results are average values of at least two different heat treatments.

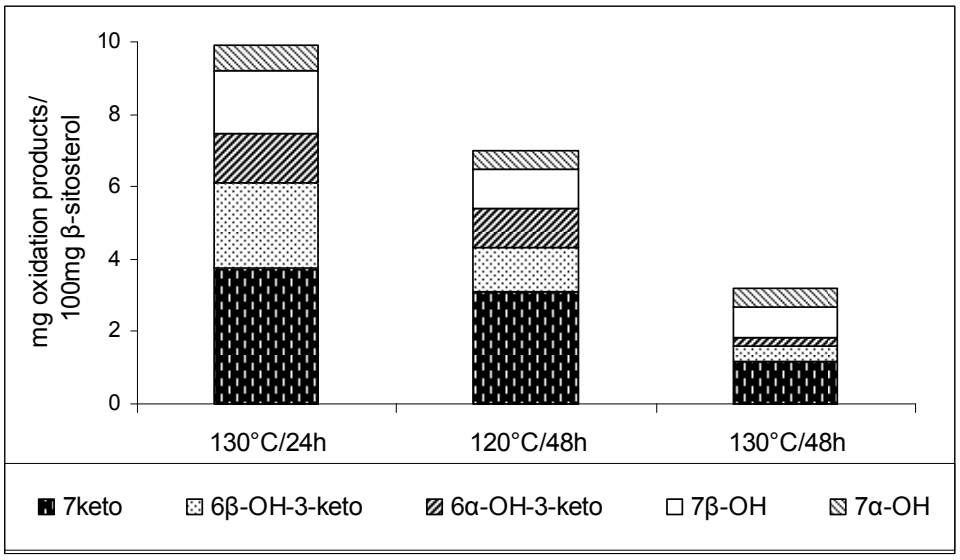


Table 1: Overview of the used concentrations of the sterol oxides in the study

	10 %	5 %	2.5 %	1 %	0.2 %	0.04 %
7-ketositosterol		x		x	x	x
7β-OH-sitosterol		x		x	x	x
7α-OH-sitosterol				x	x	x
6α-OH-3-keto-/ 6β-OH-3-keto-sitosterol			x	x	x	x
mixture	x	x		x	x	x

Figure 2: Preparative NP-HPLC chromatogram of thermo-oxidized (130 °C/24 h) sitosterol with UV detection at 206 nm.

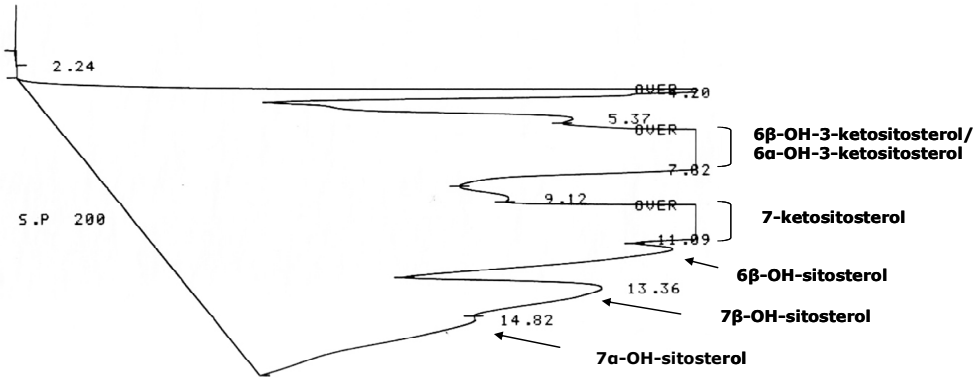


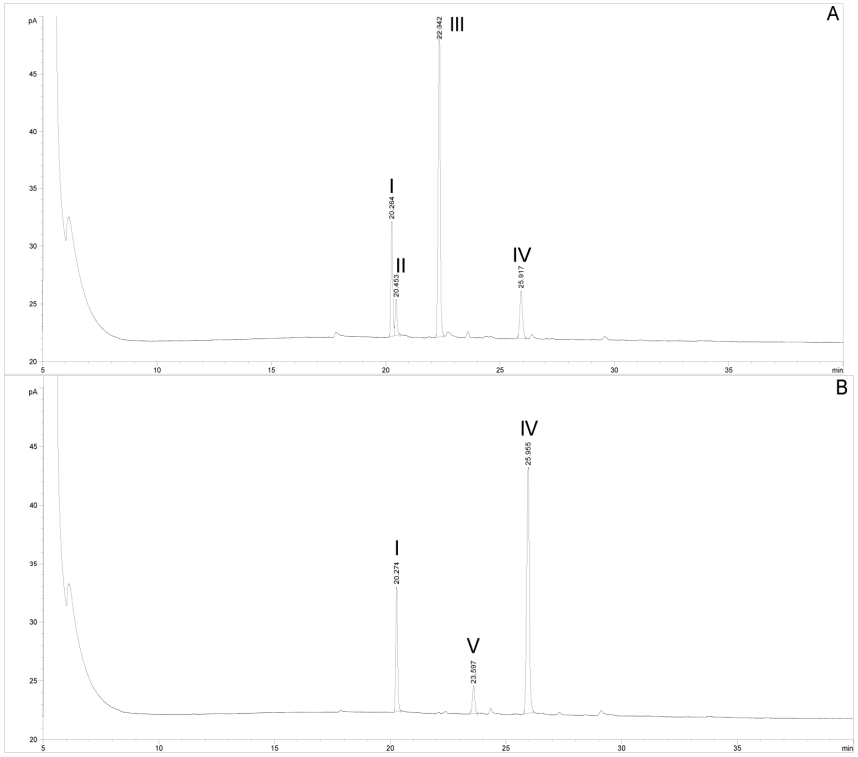
Table 2: Retention times and yield of single oxidation products (mg) within one HPLC-run (average values were obtained in over hundred HPLC-runs).

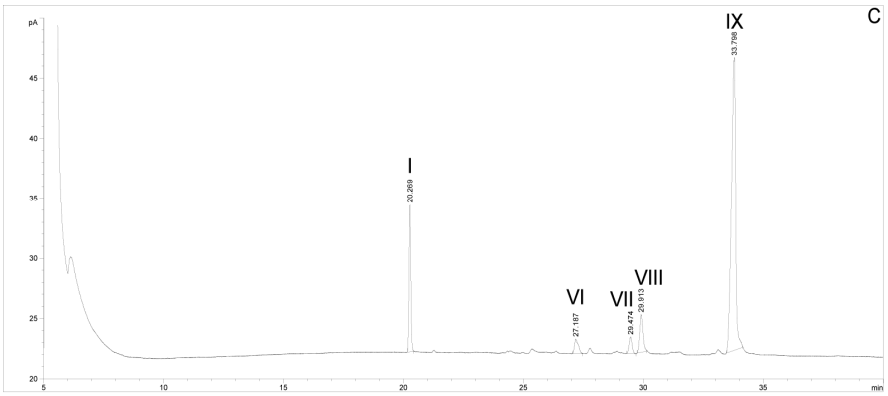
compound	retention time (min)	mg/ injection
6β-OH-3-keto-sitosterol	7.62	5.7
6α-OH-3-keto-sitosterol		4.34
7-ketositosterol	11.09	16.9
7β-OH-sitosterol	13.36	11.03
7α-OH-sitosterol	14.82	6.12

Table 3: Purity of the obtained oxidation products as measured by GC-FID.

fraction	purity (%)	campesterol counterpart (%)	others (%)
6β-OH-3-keto-sitosterol/ 6α-OH-3-keto-sitosterol	88	12	
7-ketositosterol	82	10	8
7β-OH-sitosterol	90	10	
7α-OH-sitosterol	70	10	20

Figure 3: GC-FID chromatograms of isolated β-sitosterol oxide fractions ((A) 7α-hydroxysitosterol; (B) 7β-hydroxysitosterol; (C) 7-ketositosterol; (D) 6α-OH-3-keto-sitosterol/6β-OH-3-keto-sitosterol) as TMS ether derivatives on a RTX-5w/Integra fused-silica capillary column (60m×0.25 mm i.d., 0.1 μm film). Analytical conditions are described in Material and Methods part. Peaks are identified as: (I) 19-hydroxycholesterol (ISTD); (II) 7α-hydroxycampesterol; (III) 7α-hydroxysitosterol; (IV) 7β-hydroxysitosterol; (V) 7β-hydroxycampesterol; (VI) + (VII) unidentified compounds; (VIII) 7-ketocampesterol; (IX) 7-ketositosterol; (X) 6β-OH-3-keto-campesterol; (XI) 6β-OH-3-keto-sitosterol; (XII) 6α-OH-3-keto-campesterol; (XIII) 6α-OH-3-keto-sitosterol.




C
pA
I
20.269
VI
27.187
VII
29.474
VIII
29.913
IX
33.798
min

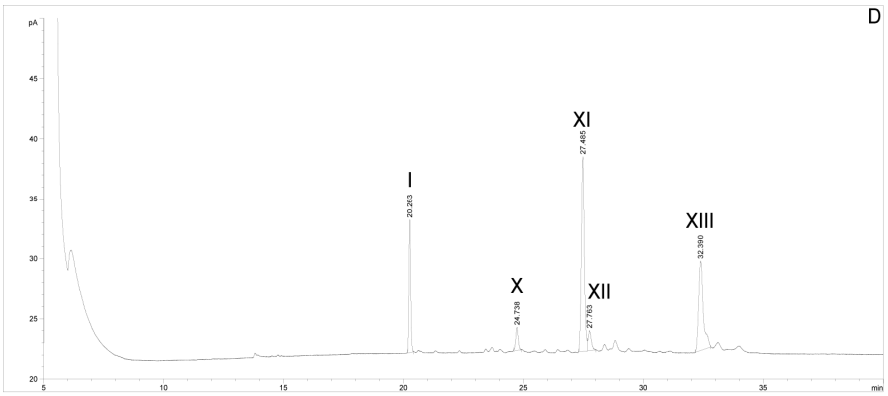

D
pA
I
20.263
X
24.738
XI
27.485
XII
27.763
XIII
32.390
min

Table 4: Overview of all obtained data in the preincubation assay with the strain TA102.

compound	concentration (mg/plate)	TA102 revertants/ plate -S9	TA102 revertants/ plate +S9	TA102+tBOOH revertants/ plate -S9	TA102+tBOOH revertants/ plate +S9
negative control		571 ±44[b)]	847 ±63[b)]	747 ±99[b)]	1589 ±349[b)]
7-ketositosterol	5	701 ±82[a)b)]	1318 ±41[a)b)]	866 ±24[a)b)]	2038 ±239[b)]
	1	685 ±16[a)b)]	926 ±28[b)]	936 ±66[a)b)]	1714 ±137[b)]
	0.2	635 ±36[a)b)]	879 ±108[b)]	940 ±75[a)b)]	1891 ±334[b)]
	0.04	575 ±45[b)]	846 ±81[b)]	963 ±52[a)b)]	1974 ±123[b)]
7β-OH-sitosterol	5	607 ±55[b)]	675 ±68[a)b)]	525 ±45[a)b)]	1374 ±60[b)]
	1	589 ±79[b)]	853 ±32[b)]	776 ±58[b)]	1770 ±146[b)]
	0.2	582 ±76[b)]	840 ±91[b)]	840 ±65[b)]	1679 ±146[b)]
	0.04	606 ±74[b)]	793 ±37[b)]	804 ±71[b)]	1591 ±103[b)]
7α-OH-sitosterol	1	597 ±101[b)]	744 ±97[b)]	684 ±56[b)]	1222 ±156[b)]
	0.2	510 ±27[a)b)]	802 ±16[b)]	812 ±36[b)]	1252 ±164[b)]
	0.04	452±103[a)b)]	652 ±34[a)b)]	686 ±111[b)]	1317 ±93[b)]
6α-OH-3-keto/ 6β-OH-3-keto-sitosterol	2.5	684 ±88[a)b)]	1051±28[a)b)]	809 ±51[b)]	2119 ±90[a)b)]
	1	644 ±51[a)b)]	979 ±12[a)b)]	1031 ±42[a)b)]	2082 ±32[a)b)]
	0.2	609 ±40[b)]	990 ±29[a)b)]	801 ±121[b)]	2338 ±29[a)b)]
	0.04	644 ±62[a)b)]	953 ±2[a)b)]	814 ±32[b)]	2065 ±70[b)]
mixture	10	713 ±60[a)b)]	1079±107[a)b)]	891 ±14[a)b)]	1501 ±35[b)]
	5	648 ±94[a)b)]	1008±54[a)b)]	797 ±12[b)]	1879 ±158[b)]
	1	598 ±55[b)]	967 ±82[a)b)]	842 ±102[b)]	1997 ±5[a)b)]
	0.2	556 ±67[b)]	829 ±41[b)]	902 ±76[a)b)]	2003 ±19[b)]
	0.04	625 ±27[a)b)]	731 ±143 [b)]	851 ±113[b)]	2057 ±175[b)]
positive control		2353 ±422	3480 ±165	2412 ±71	3780 ±271

[a)] $p < 0.05$ to negative control, [b)] $p < 0.05$ to positive control; numbers of his^+- revertants are mean ± SD

PAPER II

Cytotoxic and apoptotic effects of single and mixed oxides of ß-sitosterol on HepG2-cells

K. Koschutnig[1], S. Heikkinen[2], S. Kemmo[2], A.-M.Lampi[2], V. Piironen[2], K.-H.Wagner[1]*

[1]Department of Nutritional Sciences, University of Vienna,
Althanstrasse 14, 1090 Vienna, Austria

[2]Department of Applied Chemistry and Microbiology, University of Helsinki,
P.O.Box 27 (Latokartanonkaari 11), FIN-00014 University of Helsinki, Finland

*corresponding author: Karl-Heinz Wagner
Department of Nutritional Sciences
Faculty of Life Sciences, University of Vienna
Althanstraße 14
1090 Vienna, Austria
Ph: +43 1 4277 54930
Fax: +43 1 4277 9549
e-mail: karl-heinz.wagner@univie.ac.at

Abbreviations

(COPs) cholesterol oxidation products; (FBS) fetal bovine serum; (HE) Hydroethidine; (MDA) Malondialdehyde; (MEM) Minimal Essential Medium Eagle; (MTT) 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromid; ($O_2^{\bullet -}$) Superoxide anion**;** (POPs) phytosterol oxidation products; (ROS) reactive oxygen species; (SCGE) Alkaline single cell gel electrophoresis; (TBA) Thiobarbituric acid**;** (TBARS) Thiobarbituric Acid Reactive Substances;

In revision: *Toxicology in Vitro*

Abstract

While health implications caused by cholesterol oxidation products (COPs) seem to be generally accepted, research on phytosterol oxidation products (POPs) is still limited. Since POPs are commercially not available knowledge on their toxic activities is mainly derived from blends instead of pure compounds.

Therefore the aim of the present study was to examine the cytotoxicity of three individual oxidation products of β-sitosterol, 7-ketositosterol, 7β-OH-sitosterol, 7α-OH-sitosterol, a mixture of 6β-OH-3-keto-sitosterol/6α-OH-3-keto-sitosterol (ratio 4:3) and a mixture of polar oxides towards HepG2-cells. All tested compounds were found to reduce cell viability in a significant and concentration dependent way, particularly 7-keto- and 7α-OH-sitosterol showed to be highly active. Only for 7-ketositosterol an increase in early apoptotic cells was observed. Enhancement of $O_2^{\bullet-}$ production was assessed for all oxides, whereas malondialdehyd (MDA) levels were increased by 7-keto- and 7α-OH-sitosterol only. However, cell death didn't appear to be necessarily dependent on the generation of oxidative stress. Further no DNA strand breaks were observed with the COMET assay. By assessing the accumulation of single oxidation products in the cells a link between higher proportions of oxides inside the cells and their cytotoxic potential could be found.

Key words

phytosterol oxidation products; HepG2; cytotoxicity; apoptosis; superoxide anions; MDA; COMET; cellular uptake

Introduction

Due to their cholesterol lowering effects plant sterols have become important representatives of the broad range of functional foods in many countries. A daily intake of 1.5 to 3 g phytosterols is recommended in order to reduce serum LDL cholesterol levels at 10-15 % (Katan, et al., 2003). Added amounts of phytosterols to the increasing number of enriched products are far higher than in naturally sources. Since phytosterols are susceptible to oxidation, possible negative side-effects of elevated plant sterol consumption have recently started to attract attention (Oehrl and Boyd, 2004).

Similar to cholesterol, their counterpart in animal cells, phytosterols are prone to oxidation during heat treatments and long time storing, thereby forming oxidation products analogous to those derived from cholesterol (Dutta and Savage, 2002).

While health implications, including mutagenic, carcinogenic, angiotoxic, cytotoxic and atherogenic properties of COPs can be considered as generally acknowledged (extensively reviewed by Guardiola, et al., 1996, Osada, 2002), information on POPs is rather scarce. However, in the first reports cholesterol and phytosterol oxides were found to cause similar toxic effects, although in general higher concentrations of POPs were needed (Roussi, et al., 2005, Ryan, et al., 2005).

The lack of commercially available POP-standards is one major reason for the limited research on plant sterol oxides but it also accounts for the use of blends rather than individual oxides in most investigations on their biological effects. Yet it was shown that mixtures of sterol oxidation products act in a different way compared to single purified compounds (Maguire, et al., 2003). Thus biological and safety aspects of POPs remain relatively unclear.

Therefore, the objective of the present study was to investigate the effects of the main oxidation products of β-sitosterol, which represents the most important phytosterol structure, on HepG2 cells, a cell line which is commonly used for toxicity evaluations. In order to assess their toxicological potential, viability of cells as well as the generation of apoptotic cell death, production of superoxide anions ($O_2^{\bullet -}$), lipid peroxidation (MDA) and DNA damage (COMET assay) were determined and compared with the effects of a mixture containing polar β-sitosterol oxides. For the first time the uptake of the single oxides in cells was observed in order to establish whether different absorption affinities are responsible for diverse toxic effects.

Material & Methods

Chemicals and reagents

All chemicals were obtained from Sigma (Vienna, Austria), unless otherwise stated. Minimal Essential Medium Eagle (MEM), Dulbecco's PBS, MEM non-essential Amino Acids, fetal bovine serum (FBS), trypsin (0.05 %)-EDTA (0.02 %) and sodium bicarbonate ($NaHCO_3$) were purchased from PAA (Pasching, Austria). Low melting agarose (LMA) and normal melting agarose (NMA) were taken from Invitrogen Life Technologies (Paisley, Scotland). Annexin V-FITC detection kit was from BD Biosciences (Becton Dickinson, Schwechat, Austria). Cholest-5-en-3β,7α-diol (7α-hydroxycholesterol), Cholest-5-en-3β,7β-diol (7β-hydroxycholesterol) and 5-Cholesten-3β,19-ol (19-hydroxycholesterol) were obtained from Steraloids (Wilton, NH, USA). Cholest-5-en-3β-ol-7-one (7-ketocholesterol) was provided by Sigma Chemical Co (St.Louis. MO, USA). All organic solvents used were HPLC grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). Tissue culture plastics were obtained from Dr. F.Bertoni GmbH (Vienna, Austria).

Cell maintenance

Human hepatoma cells (HepG2) cells were cultured as monolayers in MEM supplemented with 10 % (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 % (v/v) non-essential amino acids in a humidified atmosphere at 37 °C in 5 % CO_2 in the absence of antibiotics. Cultures were allowed to reach 80 % confluence before experiments were performed.

Production of β-sitosterol oxides

Phytosterol oxidation products are not commercially available, so they had to be laboratory-prepared. Oxides were generated by thermo-oxidation of β-sitosterol (130 °C, 24 h), purified by column chromatography and separated by a NP-HPLC-UV system, consisting of a preparative HPLC - instrument (Waters Delta Prep 3000, Milford, USA) equipped with a silica Supelcosil column (250 mm x 21.1 mm, 12 µm; Supelco, Bellefonte, PA, USA) and a UV detector at 206 nm (Waters 484 Milford, USA). Separation was achieved under isocratic conditions using a mobile phase of *n*-heptane/2-propanol (93/7, v/v) and a flow rate of 17 mL/min. Three individual

oxidation products, 7-ketositosterol, 7β-OH-sitosterol 7α-OH-sitosterol, a mixture of 6β-OH-3-keto-sitosterol/6α-OH-3-keto-sitosterol (ratio 4:3) and a mixture of polar oxides were fractionated. For identification and quantification of the collected fractions GC-mass spectrometry (GC-MS) and GC-flame ionization detection (GC-FID) were used, respectively (Lampi, et al., 2002, Soupas, et al., 2004). Purity of the respective compounds is given in table 1.

Cell treatment

For treatment cells were cultured in different multiwell dishes depending on the respective experiment at a density of either $2x10^4$ cells/well in 250 µL (96-well), $2x10^5$ cells/ well in 2 mL (24-well) or $1x\ 10^6$ cells/ well in 3 mL (6-well) of complete medium. After 24 hours medium was removed and cells were incubated with the different β-sitosterol oxide samples. Within all experiments 3 different concentrations (30 µM, 60 µM, 120 µM) and an incubation period of 24 hours were considered. For delivery to the cells all compounds were dissolved in ethanol and added to reduced serum media (2.5 % (v/v) FBS). The final concentration of ethanol in cultures did not exceed 0.4 % (v/v) and did not affect cell proliferation. Equivalent quantities of ethanol were added to control cells. After incubation, both floating and attached cells were collected for analysis.

Cytotoxicity

MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromid) assay

Cell viability was determined by using the MTT assay (Mosmann, 1983), which is based on a reaction catalysed by dehydrogenases of the mitochondria. With this assay the number of metabolic active cells can be established. Briefly, after incubation with sterol oxides the culture medium was discarded, cells were washed with PBS, 200 µL of MTT in culture medium (final concentration 0.5 mg/mL) was added and incubated for 1 hour. The medium was carefully removed, cells were washed with PBS and the formazan crystals were dissolved in DMSO. Absorbance was read at 540 nm with a Fluostar Optima microplate reader (BMG labtechnologies, Germany) and expressed as the number of viable cells as percentage (%) of control cells. Measurements were made in triplicates.

Measurement of trypan blue exclusion

At the end of the incubation period cells were harvested using trypsin/EDTA solution and aliquots of cell suspensions were mixed with equal amounts of trypan blue (0.1 % (v/v)).

For the determination of cell numbers dead (blue) and living cells were counted within a haemocytometer and results were presented as number of viable cells expressed as percentage (%) of negative control. In addition the percentage of dead cells in the total cell population of control and treated cells was calculated. Experiments were performed in triplicates.

Flow cytometric measurments

Detection of apoptosis

For detection of apoptosis an Annexin V-PE detection kit (BD Pharmingen) was used and analysis was done following the manufacturer's instructions. After incubation cells were washed and harvested using trypsin/EDTA solution. Cells were centrifuged (800 RPM, 5 min), washed twice with cold PBS and resuspended in binding buffer (0.1 M HEPES/NaOH (pH 7.4) 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of 1x 10^6 cells/mL. 100 µL of this suspension was transferred to 5 mL culture tubes and stained with 5 µL of Annexin V-PE and 7-ADD, respectively. After 15 min of incubation at RT in the dark 400 µL of binding buffer was added to each tube and cells were analysed by flow cytometry within one hour. For each sample 10.000 cells were acquired and data management was done with CellQuest Pro Software (FACScan, BD Biosciences, USA). Experiments were performed at least in triplicates. Annexin V positive/7-AAD negative cells were defined as early apoptotic, while Annexin V positive/7-AAD positive cells were classified as late apoptotic or necrotic.

Intracellular Superoxide anion ($O_2^{\bullet-}$) generation

The production of superoxide anions was determined by the use of hydroethidine (HE) (Rothe and Valet, 1990). HE is a nonfluorescent compound, which diffuses easily through the cell membrane. Under the action of $O_2^{\bullet-}$ HE is dehydrogenated to ethidium bromide and intercalates DNA. The red fluorescence of ethidium can be measured by flow cytometry (absorption/emission: 518/605 nm).

Briefly, after incubation with oxide samples cells were harvested as described above, centrifuged (800 RPM, 5 min) and resuspended in PBS at a density of $1x10^6$ cells/mL. 5 µL of HE-solution in DMSO (20 µM) were added, cells were incubated at 37 °C for 10 min and then stored on ice until measurement by FACSCalibur flow cytometer (BD Biosciences). For each sample 10.000 cells were analysed and data management was done with CellQuest Pro Software (FACScan, BD Biosciences, USA).

Measurement of Lipid Peroxidation (MDA)

Malondialdehyde (MDA) constitutes an end product of lipid peroxidation and was determined as described earlier (Wagner, et al., 2004), with some modifications. Briefly, at the end of the incubation period, cells were collected, washed with PBS and counted. Cells were centrifuged and resuspended in a mixture of 150 µL of potassium chloride solution (KCl, 75 mM), 700 µL phosphoric acid (44 mM), 250 µL TBA(60 mg/L) and 400 µL acqua bidest. Suspensions were heated in boiling water for 60 min and after cooling, to 100 µL of the cell samples 100 µL of methanol/NaOH (90/10, v/v) was added. An aliquot of 20 µL was injected into the HPLC and MDA was measured by fluorescence detector (553 nm). Data was expressed as nanomoles per 10^9 cells.

Alkaline single cell gel electrophoresis (SCGE)

The SCGE assay (comet assay) was carried out according to the guidelines developed by Tice et al. (2000, 1990) and Singh et al. (1988). After the incubation period cells were harvested using trypsin/EDTA solution and counted by the trypan blue method. Aliquotes of $1x10^5$ cells were mixed with 80 µL 0.5 % LMA and transferred to NMA-coated slides. Slides were covered and cooled (4 °C). As soon as the agarose gel has solidified cover slides were removed and slides were transferred to a lysis buffer solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1 % Triton X, 10% DMSO, pH 10.0) for ≥ 1 hour at 4 °C. After lysis the slides were incubated in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH ≥ 13) at 4 °C for 40 min for DNA unwinding. Electrophoresis was performed at 25 V and 300 mA for 20 min using a horizontal gel electrophoresis (VWR, Vienna, Austria). The slides were neutralized by rinsing them two times for 8 min with cold neutralization buffer (0.4 M trizma base, pH 7.5) and once with cold aqua bidest. Then they were dried at room temperature. For evaluation the slides were stained with 40 µL ethidium bromide

solution (20 µg/mL) and examined using a fluorescence microscope (Axioskop 20, Zeiss, Austria) coupled with a computerized image analysis system (Komet 5.5, Kineting Imaging, Liverpool, UK). For each sample, three replicate gels were analysed and from each replicate slide 50 randomly selected cells were analysed. As parameter of DNA damage percentage of DNA in the tail (% DNA in tail) was determined. Each experiment was done twice.

Measurement of the uptake of oxidation products in HepG2-cells

Extraction of the oxidation products

At the end of the incubation period media was removed and collected separately. Cells were harvested using trypsin/EDTA solution, centrifuged (800 RPM, 5 min), washed twice with PBS and resuspended in 5 mL of KCl (75 mM). After 20 min of incubation the lipid fraction was extracted 3 times from cells and media with 5 mL of diethyl ether. The ether phase was evaporated, extracts were dissolved in 1ml of *n*-heptane/ isopropanol (95/5, v/v), filtered through 0.45 µm GHP membrane filters (PALL, Gelman laboratory, USA) and stored in a freezer (-20 °C) until measurement.

Quantification of the extracted oxidation products by a HPLC-UV-ELSD system

For quantification of the extracted compounds an HPLC-UV-ELSD system was applied. The method used was based on papers by Kemmo et al. [(2005), (2004) and (Säynäjoki, et al., 2003)] with some modifications.

The HPLC instrument consisted of an Waters 717 plus Autosampler (Milford, USA), a silica Supelcosil column (250 mm x 2.1 mm i.d., 5 µm; Supelco, Bellefonte, PA, USA), a Waters pump (model 515) and a Photodiode Array Detector (model Waters 996) as well as an ELS-detector (model Waters 2420, Milford, USA). The separation was achieved at room temperature with a mobile phase of *n* - heptane/ isopropanol (97/3, v/v) at a flow rate of 0.6 mL/min. The injection volume was 20 µL and each sample was injected twice.

Detection was performed simultaneously by PDA detection at 206 nm and ELSD (nebulization temperature 42 ˚C, drift tube temperature 50 ˚C, pressurised air 20 psi), whereas quantification was done based on UV data only.

The concentrations of the oxidation products were estimated by cholesterol standards of the corresponding oxidation products (7-ketocholesterol, 7α-OH-cholesterol and 7β-OH-cholesterol), whereas 7-ketocholesterol was also used for the quantification of 6α-OH-3-keto-sitosterol and 6β-OH-3-keto-sitosterol. Concentration and purity of the standards was confirmed by gas chromatography–flame ionization detection (GC–FID) as described by Lampi et al. (2002). Standard curves were analysed at the beginning and at the end of each compound, concentration of the oxides was calculated using the mean standard curve. Detection Limits (LOD) were calculated on the basis of a signal to noise ratio of 3 by the use of standard dilutions and for UV detection they were 5, 15.6 and 20.8 ng/injection for 7-ketocholesterol, 7β-OH-cholesterol and 7α-OH-cholesterol, respectively.

Statistical analysis

All data are expressed as mean ± SD (standard derivation). Obtained data were analysed by the Student's t-test since they were all normally distributed, using SPSS 15.0 for Windows. Statistical differences were considered significant at a value of $p < 0.05$ and are reported as $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results

Cytotoxicity of β-sitosterol oxidation products on HepG2 cells

Cytotoxicity of the tested compounds was determined using the MTT assay after 24 hours of incubation. Viability of the control samples was set at 100 %. All sterol oxides induced a significant ($p \leq 0.001$, for 7β -OH-sitosterol $p \leq 0.01$) reduction of cell viability relative to the negative control (Fig 1A). In addition a significant concentration dependency ($p \leq 0.01$) was observed for all samples except for 7β -OH-sitosterol and 7-keto-sitosterol between the concentrations 60 and 120 μM. The most toxic agent was 7α-OH-sitosterol reducing viability to 23 % of the control value at 120 μM. At the highest concentration tested the order of cytotoxicity was 7α-OH-sitosterol > 6α-OH-3-keto/6β-OH-3-keto-sitosterol > 7-keto-sitosterol > mixture > 7β -OH-sitosterol.

Similar results were obtained when counting viable cells in the Trypan blue exclusion assay (Fig 1B). A significant reduction in cell numbers compared to the negative control

($p \leq 0.01$, except 7β-OH-sitosterol) was observed after treatment with the tested oxidation products. Concentration dependencies were found for 7α-OH-sitosterol ($p \leq 0.001$), 7-keto-sitosterol ($p \leq 0.01$) and the mixture ($p \leq 0.05$). Considering the percentage of cell death in control and treated cells a significant ($p \leq 0.001$, for 7β -OH-sitosterol at 30 µM $p \leq 0.05$) increase for all compounds, except for the mixture at 30 µM ($p = 0.272$), could be seen (Fig 1C). Only for 7-keto-sitosterol changes were based on the concentrations ($p \leq 0.01$). The increase in cell death was particularly pronounced for 7-keto-sitosterol and 7α-OH-sitosterol.

Effect of β-sitosterol oxidation products on apoptosis

To elucidate whether apoptosis was involved in the reduction of cell numbers flow cytometry was used. Decreased forward scatter (cell size) and increased side scatter (granularity) are typical signs for apoptotic cells (Koopman, et al., 1994, Tuschl and Schwab, 2003). Described subpopulations were observed in cells incubated with 7-ketositosterol, 7α-OH-sitosterol and 6α-OH-3-keto/6β-OH-3-keto-sitosterol at concentrations of 60 and 120 µM indicating apoptotic cell death (data not shown). To further clarify the mode of cell death double staining with FITC-labeled Annexin V and 7-AAD was conducted. As shown in figure 2A only 7-ketositosterol (30 µM) was able to increase (+ 45 %) the number of Annexin V positive/7AAD negative cells compared to the negative control, even though not in a significant way. For all compounds a decrease in the proportions of Annexin V positive/7AAD negative cells with increasing concentration of test samples was found.

Generation of superoxide anions ($O_2^{\bullet-}$) and lipid peroxidation (MDA) by β-sitosterol oxidation products

The production of reactive oxygen species (ROS) and the resulting oxidative stress precedes cell death. Therefore the generation of $O_2^{\bullet-}$ was quantified by flow cytometry after staining with HE. For all tested compounds a significant ($p \leq 0.05$) overproduction of $O_2^{\bullet-}$ could be noted (Fig 2B). No concentration dependency was found for the single oxides.

The effect of oxysterols on cellular lipid peroxidation was investigated by HPLC-detection of MDA. MDA concentration in control cells was found to be 3.35 nM/10^9

cells. Incubation with 7β -OH-sitosterol and the mixture appeared to have little effect on lipid peroxidation, as MDA levels were found to be in the range of the negative control. For 7-ketositosterol, 7α-OH-sitosterol and 6α-OH-3-keto-/6β-OH-3-keto-sitosterol to some extent a concentration dependent increase of MDA-levels in surviving cells was shown. At 120 µM a remarkable raise in MDA concentration to 194 % (7-ketositosterol), 298 % (7α-OH-sitosterol) and 140 % (6α-OH-3-keto/6β-OH-3-keto-sitosterol) of control levels was observed. However, none of these effects were significant (figure 2C).

Effect of β-sitosterol oxidation products on DNA damage

In order to detect the potential of β-sitosterol oxides to induce DNA-strand breaks the alkaline single-cell gel electrophoresis assay was conducted. Tested compounds showed to be quite inactive, measured % DNA in tail were in the range of the negative control (data not shown). Therefore no significant increase of DNA damage could be observed.

Concentration dependent uptake of β-sitosterol oxidation products by HepG2-cells

To assess the uptake of the individual oxidation products in HepG2 cells, lipid extracts of cell residues and corresponding media were analysed by a HPLC-UV-ELSD system. After incubation with increasing concentrations of sterol compounds amounts inside the cells were increased as a matter of concentration, whereas in control cells no plant sterol oxides were detected.

The raise in the intracellular uptake of the single oxidation products was significant for 7-ketositosterol, 7β-OH-sitosterol and 7α-OH-sitosterol ($p \leq 0.05$) (Fig 3). Considering the distribution pattern of the POPs between cell and media compartments corresponding trends could be found irrespective of the added concentration of the oxidation product. Whereas for 7-ketositosterol and 7α-OH-sitosterol after 24 hours the majority of the detected oxides (~ 70 %) were found in the cell extracts, for 7β-OH-sitosterol no definite tendency was observed. Regarding the mixture of 6α-OH-3-keto- and 6β-OH-3-keto-sitosterol only 30 % of the α-compound was detected inside the cells, whereas for the β-counterpart similarly 50 % were assessed in the cells and the media, respectively. Considering the combined uptake as a mixture of these two compounds larger parts (55-60 %) of the oxidation products were located in the media.

Further the obtained uptake data correlates negatively with the outcomes of the MTT- ($r = -0.667$, $p \leq 0.05$ for 7-ketositosterol; $r = -0.874$, $p \leq 0.001$ for 7β-OH-sitosterol; $r = -0.781$, $p \leq 0.01$ for 7α-OH-sitosterol; $r = -0.783$, $p \leq 0.01$ for 6α-OH-3-keto-/6β-OH-3-keto-sitosterol) and the TB-test ($r = -0.868$, $p \leq 0.001$ for 7-ketositosterol; $r = -0.878$, $p \leq 0.001$ for 7α-OH-sitosterol), respectively.

Discussion

Although consumption of plant sterols is steadily increasing little is known about the biological effects of their oxidation products. So far only a limited number of studies using pure phytosterol oxides had been published. However, to allow a comprehensive evaluation of their safety aspects more information on single oxides is essential.

The present study clearly demonstrates the cytotoxic effect of β-sitosterol oxidation products towards HepG2 cells. In line with earlier results (Adcox, et al., 2001, Maguire, et al., 2003, Roussi, et al., 2005, Ryan, et al., 2005) viability of cells as measured by the trypan blue exclusion – and the MTT assay were significantly decreased by incubation with increasing concentrations of sterol oxides (Fig1). However, in contrast to others (Ryan, et al., 2005) in the present study cytotoxic effects were already observed at low sample concentrations of 30 μM.

Consistently 7-ketositosterol and 7α-OH-sitosterol emerged as those compounds exerting the strongest reduction in cell viability. While the toxic potential of 7-ketositosterol, as the most abundant oxyphytosterol, was already confirmed in various in vitro assays, data on 7α-OH-sitosterol is lacking. Contrary to the present findings its cholesterol counterpart was found to exhibit less than or equal toxicity to 7β-OH-cholesterol (Clare, et al., 1995). However, individual 7α-OH-products of plant sterols have never been tested before.

Interestingly it was 7β-OH-sitosterol that together with the mixture appeared to have little effect on the viability of cells. Whereas Ryan et al. (2005) investigating the impact of phytosterol oxides on various cell lines, including HepG2-cells, reported 7β-OH-sitosterol to exhibit the strongest effects on cell viability.

It is already well known that individual oxidation products and their mixtures react in a different way (Maguire, et al., 2003). Moreover, synergistic as well as inhibitory effects

of single sterol oxides in mixtures have been noted (O'Sullivan A, et al., 2005). In general a reduction of the toxic potential is the predominant effect (Aupeix, et al., 1995, Hall, 2006, Leonarduzzi, et al., 2002), which also contributes to our findings.

To further elucidate the mode of cell death Annexin V binding was assessed. Annexin V attaches specifically to phosphatidylserine which is translocated from the inner to the outer cell surface during the early phase of apoptosis (Vermes, et al., 1995). Only 7-ketositosterol (30 μM) was able to increase the proportion of Annexin V positive/7AAD negative cells relative to control levels (Fig 2A). For all other compounds early apoptotic cells were in the range of the negative control or lower. The reduction of the amount of Annexin V positive/7AAD negative cells with increasing concentrations of oxidation products indicates chosen incubation conditions being too intense for detecting early apoptotic events. This corresponds to findings of Leonarduzzi et al.(2002) who observed that 7-ketocholesterol induced apoptosis at low concentrations whereas at higher sample amounts necrosis was the predominate mode of cell death. At the time point investigated higher percentages of necrotic or late apoptotic than early apoptotic cells were detected. It may be that apoptosis occurred at an earlier time point, after 24 hours showing mainly late apoptotic cells. However, also necrosis as principal mode of cell death is possible. In order to fully exclude a β-sitosterol induced apoptotic cell death further experiments at earlier time points would be necessary.

Apoptosis induced by POPs has already been observed previously (Ryan, et al., 2005) (Maguire, et al., 2003) (Roussi, et al., 2005). In contrast to our findings in the major part of studies investigating phytosterol and cholesterol oxidation products 7β-OH-compounds appeared to be the highly potent inducers of apoptotic cell death. These diverse findings could be explained by the cell specific effect of oxysterols to induce apoptosis (Miguet, et al., 2001). While β-sitosterol oxides caused apoptosis in U937 cells, no indication was found instead in HepG2 and CaCo2 cells (Ryan, et al., 2005). However, reports on the cell death mode of oxidation products seem to be conflicting, as in contrast to Ryan et al. (2005) Roussi et al. (2005) observed apoptosis for 7β-OH-sitosterol in CaCo2 cells using same sample concentrations. In general induction of apoptosis was mainly analysed in non adherent cell lines (Wielkoszynski, et al., 2006).

Oxysterols were found to rapidly increase $O_2^{\bullet-}$ production (Lemaire-Ewing, et al., 2005, Miguet-Alfonsi, et al., 2002, Monier, et al., 2003), which in general induces cell death.

Also phytosterol oxides were reported to induce an upregulation of several antioxidant enzyme activities (Ryan, et al., 2005), POP induced intracellular $O_2^{\bullet-}$ production however, has never been measured before. Consistently with previous results on COPs all tested oxidation products showed a significant increase in $O_2^{\bullet-}$ (Fig 2B). Interestingly, although being not reactive in all other assays conducted, treatment with the mixture also resulted in a remarkable raise of HE positive cells. As $O_2^{\bullet-}$ constitutes only one type of ROS the measurement of MDA as a second maker for oxidative stress was included. To improve the accuracy of the results in the present study MDA was detected by HPLC and not by the TBARS assay, which has already been criticized for insufficient specificity and validity (Halliwell and Whiteman, 2004). No information is available on lipid peroxidation caused by POPs and investigations on COPs are also rather limited. For 7-keto- and 7α-OH-sitosterol an increase in MDA levels was found (Fig 2C). Again results for 7α-OH-sitosterol differed from those observed for its cholesterol counterpart as 7α-OH-cholesterol appeared to possess only negligible effects on oxidative stress (Miguet-Alfonsi, et al., 2002). However, in line with earlier publications no correlation could be found between the levels of lipid peroxidation and the generation of $O_2^{\bullet-}$ (Hall, 2006). $O_2^{\bullet-}$,contrary to many other ROS, seems to be relatively unreactive with lipids (Halliwell and Gutteridge, 1999).

Previous investigations on plant sterol oxides reported that cytotoxicity was not necessarily associated with enhanced oxidative stress, which was true for 7β-OH-sitosterol and the mixture. On the other hand enhanced levels of lipid peroxidation were found after incubation with 7-keto- and 7α-OH-sitosterol which also emerged as the most potent inducers of cell death. Though it is also possible that lipid peroxidation was induced through necrosis as a secondary event.

As ROS have the potential to interact with cellular components including DNA their capability to induce DNA strand breaks was also assessed. In line with reports on the genotoxic action of COPs (Maguire, et al., 2003) no increased DNA damage was observed.

Throughout the whole study differences in the cytotoxic potential of the single oxidation products, especially α- and β- hydroxy sterols, were prevailing. Considering the uptake of the individual β-sitosterol oxides in respect of their toxicity data may help to explain these differences. To date no information on the uptake of plant sterol oxides

in cell lines is available. Hence, similar to investigations on COPs (Biasi, et al., 2004) in the present study a concentration dependent accumulation of all oxidation products was confirmed (Fig 3).

So far, in contrast to the present study concentration of oxides in the corresponding media remained disregarded. Interestingly, different affinities of the single oxidation products regarding their distribution between media and cell compartment could be found. Higher percentages of oxides incorporated into the cells were observed for those compounds (7-keto-, 7α-OH-sitosterol and 6α-OH-3-keto-/6β-OH-3-keto-sitosterol) exerting the highest reactivity, whereas for the rather inactive 7β-OH-sitosterol no specific uptake trend was found. Further uptake data in cells correlated negatively with the results of the viability assays indicating that different uptake tendencies seem to be responsible for their cytotoxic actions. Similar Miguet et al. (2001) found a twofold higher accumulation of 7α-OH-cholesterol than of 7-keto- and 7β-OH-cholesterol in U937 cells, however 7α-OH- cholesterol displayed no cytotoxic effects.

In conclusion, the present study could demonstrate that individual oxidation products of β-sitosterol exhibit cytotoxic effects towards HepG2-cells. While 7-keto- and 7α -OH -sitosterol proved to be highly active, treatment with 7β -OH -sitosterol and the mixture of polar oxides showed little effects. Reduction in cell viability was found to be significant and concentration dependent. Under the conditions tested only for 7-ketositosterol an increase in early apoptotic cells was detected. Although a significant increase in $O_2^{\bullet-}$ was assessed after treatment with all tested oxides MDA levels were insignificantly enhanced only by 7-keto- and 7α -OH -sitosterol. A link between cell death and oxidative stress didn't seem to exist for all tested compounds. The different levels of oxide uptake in the cells seem to take a responsible part for the diverse toxic effects.

Acknowledgement

The work was supported by COST organization as a Cost 927 short term scientific mission, carried out at the University of Helsinki, by the Academy of Finland and by a research grant on behalf of the University of Vienna (F81-B Forschungsstipendium).

References

Adcox, C., Boyd, L., Oehrl, L., Allen, J., Fenner, G., 2001. Comparative effects of phytosterol oxides and cholesterol oxides in cultured macrophage-derived cell lines. Journal of Agricultural and Food Chemistry 49, 2090-2095.

Aupeix, K., Weltin, D., Mejia, J.E., Christ, M., Marchal, J., Freyssinet, J.M., Bischoff, P., 1995. Oxysterol-induced apoptosis in human monocytic cell lines. Immunobiology 194, 415-428.

Biasi, F., Leonarduzzi, G., Vizio, B., Zanetti, D., Sevanian, A., Sottero, B., Verde, V., Zingaro, B., Chiarpotto, E., Poli, G., 2004. Oxysterol mixtures prevent proapoptotic effects of 7-ketocholesterol in macrophages: implications for proatherogenic gene modulation. FASEB Journal 18, 693-695.

Clare, K., Hardwick, S.J., Carpenter, K.L., Weeratunge, N., Mitchinson, M.J., 1995. Toxicity of oxysterols to human monocyte-macrophages. Atherosclerosis 118, 67-75.

Dutta, P.C., Savage, G.P., 2002. Formation and Content of Phytosterol Oxidation Products in Foods. In: F. Guardiola, P.C. Dutta, R. Codony, Savage, G.P. (Eds.), Cholesterol and Phytosterol Oxidation Products: Analysis, Occurence and Biological Effects. AOCS Press, Champaign, pp. 319-334.

Guardiola, F., Codony, R., Addis, P.B., Rafecas, M., Boatella, J., 1996. Biological effects of oxysterols: current status. Food and Chemical Toxicology 34, 193-211.

Hall, M.C., 2006. The effect of oxysterols, individually and as a representative mixture from food, on in vitro cultured bovine ovarian granulosa cells. Molecular and Cellular Biochemistry 292, 1-11.

Halliwell, B., Gutteridge, J.M.,1999. Free Radicals in Biology and Medicine. Oxford University Press, Oxford, U.K. Pages.

Halliwell, B., Whiteman, M., 2004. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? British Journal of Pharmacology 142, 231-255.

Katan, M.B., Grundy, S.M., Jones, P., Law, M.F.R.C.P., Miettinen, T., Paoletti, R., 2003. Efficacy and Safety of Plant Stanols and Sterols in the Management of Blood Cholesterol Levels. Mayo Clinic Proceedings 78, 965-978.

Kemmo, S., Ollilainen, V., Lampi, A.M., Piironen, V., 2004. Determination of stigmasterol hydroperoxides using high-performance liquid chromatography-mass

spectrometry with atmospheric pressure chemical ionization. Czech Journal of Food Sciences 22, 144-146.

Kemmo, S., Soupas, L., Lampi, A.-M., Piironen, V., 2005. Formation and decomposition of stigmasterol hydroperoxides and secondary oxidation products during thermo-oxidation. European Journal of Lipid Science and Technology 107, 805-814.

Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pals, S.T., van Oers, M.H., 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84, 1415-1420.

Lampi, A.-M., Juntunen, L., Toivo, J., Piironen, V., 2002. Determination of thermo-oxidation products of plant sterols. Journal of Chromatography B 777, 83-92.

Lemaire-Ewing, S., Prunet, C., Montange, T., Vejux, A., Berthier, A., Bessede, G., Corcos, L., Gambert, P., Neel, D., Lizard, G., 2005. Comparison of the cytotoxic, pro-oxidant and pro-inflammatory characteristics of different oxysterols. Cell Biology and Toxicology 21, 97-114.

Leonarduzzi, G., Sottero, B., Poli, G., 2002. Oxidized products of cholesterol: dietary and metabolic origin, and proatherosclerotic effects (review). Journal of Nutritional Biochemistry 13, 700-710.

Maguire, L., Konoplyannikov, M., Ford, A., Maguire, A.R., O'Brien, N.M., 2003. Comparison of the cytotoxic effects of beta-sitosterol oxides and a cholesterol oxide, 7beta-hydroxycholesterol, in cultured mammalian cells. British Journal of Nutrition 90, 1-10.

Miguet-Alfonsi, C., Prunet, C., Monier, S., Bessede, G., Lemaire-Ewing, S., Berthier, A., Menetrier, F., Neel, D., Gambert, P., Lizard, G., 2002. Analysis of oxidative processes and of myelin figures formation before and after the loss of mitochondrial transmembrane potential during 7beta-hydroxycholesterol and 7-ketocholesterol-induced apoptosis: comparison with various pro-apoptotic chemicals. Biochemical Pharmacology 64, 527-541.

Miguet, C., Monier, S., Bettaieb, A., Athias, A., Bessede, G., Laubriet, A., Lemaire, S., Neel, D., Gambert, P., Lizard, G., 2001. Ceramide generation occurring during 7beta-hydroxycholesterol- and 7-ketocholesterol-induced apoptosis is caspase independent and is not required to trigger cell death. Cell Death and Differentiation 8, 83-99.

Monier, S., Samadi, M., Prunet, C., Denance, M., Laubriet, A., Athias, A., Berthier, A., Steinmetz, E., Jurgens, G., Negre-Salvayre, A., Bessede, G., Lemaire-Ewing, S., Neel, D., Gambert, P., Lizard, G., 2003. Impairment of the cytotoxic and oxidative activities of 7 beta-hydroxycholesterol and 7-ketocholesterol by esterification with oleate. Biochemical and Biophysical Research Communications 303, 814-824.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55-63.

O'Sullivan A, J., O'Callaghan Y, C., O'Brien N, M., 2005. Differential effects of mixtures of cholesterol oxidation products on bovine aortic endothelial cells and human monocytic U937 cells. International Journal of Toxicology 24, 173-179.

Oehrl, D.L., Boyd, L.C., 2004. Biological Effects and Safety Aspects of Phytosterol Oxides. In: Dutta, P.C. (Ed.), Phytosterols as Functional Food Components and Nutraceuticals. Marcel Dekker Inc, New York, pp. 419-430.

Osada, K., 2002. Cholesterol oxidation products: other biological effects. In: Dutta, P.C., Guardiola, F., Codony, R., Savage, G.P. (Eds.), Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence and Biological Effects. AOCS Press, Champaign, Illinois, pp. 278-318.

Rothe, G., Valet, G., 1990. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin. Journal of Leukocyte Biology 47, 440-448.

Roussi, S., Winter, A., Gosse, F., Werner, D., Zhang, X., Marchioni, E., Geoffroy, P., Miesch, M., Raul, F., 2005. Different apoptotic mechanisms are involved in the antiproliferative effects of 7beta-hydroxysitosterol and 7beta-hydroxycholesterol in human colon cancer cells. Cell Death and Differentiation 12, 128-135.

Ryan, E., Chopra, J., McCarthy, F., Maguire, A.R., O'Brien, N.M., 2005. Qualitative and quantitative comparison of the cytotoxic and apoptotic potential of phytosterol oxidation products with their corresponding cholesterol oxidation products. British Journal of Nutrition 94, 443-451.

Säynäjoki, S., Sundberg, S., Soupas, L., Lampi, A.M., Piironen, V., 2003. Determination of stigmasterol primary oxidation products by high-performance liquid chromatography. Food Chemistry 80, 415-421.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 175, 184-191.

Soupas, L., Juntunen, L., Säynäjoki, S., Lampi, A.M., Piironen, V., 2004. GC-MS method for characterization and quantification of sitostanol oxidation products. Journal of the American Oil Chemists' Society 81, 135-141.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis 35, 206-221.

Tice, R.R., Andrews, P.W., Singh, N.P., 1990. The single cell gel assay: a sensitive technique for evaluating intercellular differences in DNA damage and repair. Basic Life Sciences 53, 291-301.

Tuschl, H., Schwab, C., 2003. Cytotoxic effects of the herbicide 2,4-dichlorophenoxyacetic acid in HepG2 cells. Food and Chemical Toxicology 41, 385-393.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. Journal of Immunological Methods 184, 39-51.

Wagner, K.-H., Jürß, A., Zarembach, B., Elmadfa, I., 2004. Impact of antiseptics on radical metabolism, antioxidant status and genotoxic stress in blood cells: povidone-iodine versus octenidine dihydrochloride. Toxicology in Vitro 18, 411-418.

Wielkoszynski, T., Gawron, K., Strzelczyk, J., Bodzek, P., Zalewska-Ziob, M., Trapp, G., Srebniak, M., Wiczkowski, A., 2006. Cellular toxicity of oxycholesterols. BioEssays 28, 387-398.

Table 1: Purity of the tested β-sitosterol oxidation products as measured by GC-FID. Campesterol counterpart represents the oxidation product of the respective fraction but derived from campesterol instead of sitosterol.

fraction	purity (%)	campesterol counterpart (%)	others (%)
6β-OH-3-keto-sitosterol/ 6α-OH-3-keto-sitosterol	88	12	
7keto-sitosterol	82	10	8
7β-OH-sitosterol	90	10	
7α-OH-sitosterol	70	10	20

Figure 1: Cell viability measured by the MTT-test (A) as well as number of viable cells (B) and percentage of cell death (C) measured by the TB-assay after 24h of incubation with β-sitosterol oxides. Values are mean ± SD of 3 separate experiments and are marked with * when significantly different to control levels (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

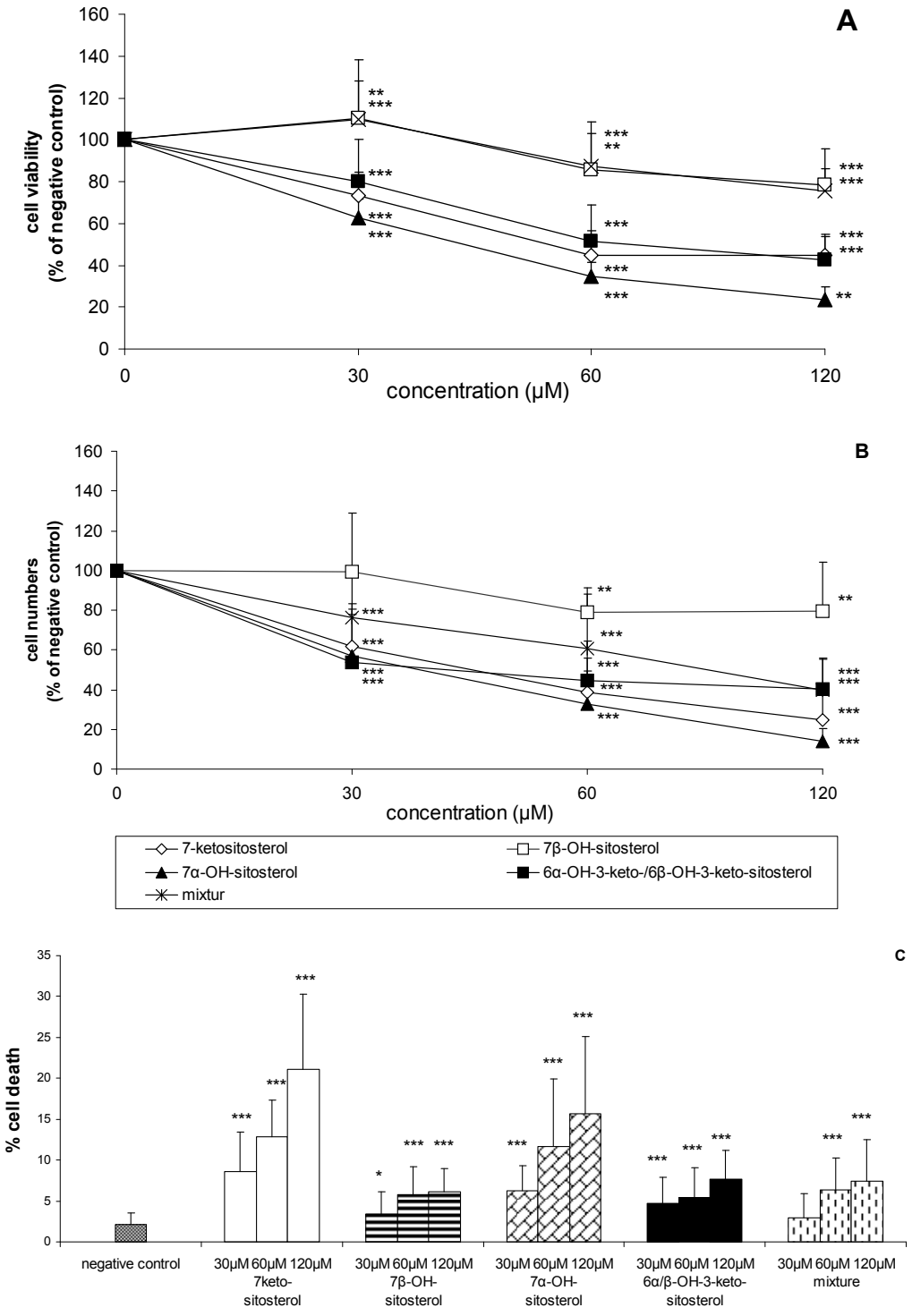
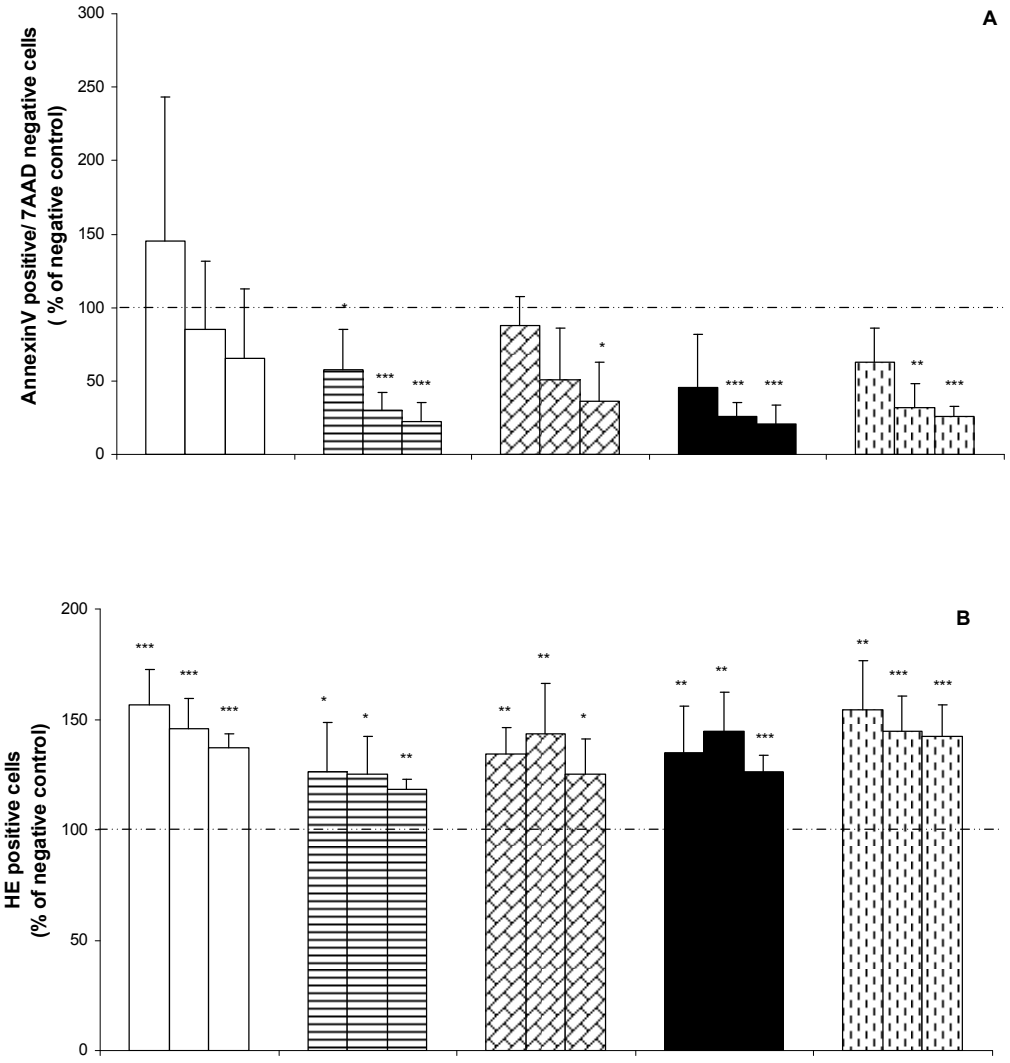

A
cell viability
(% of negative control)
concentration (μM)
B
cell numbers
(% of negative control)
concentration (μM)
7-ketositosterol
7β-OH-sitosterol
7α-OH-sitosterol
6α-OH-3-keto-/6β-OH-3-keto-sitosterol
mixtur
C
% cell death
negative control
30μM 60μM 120μM 7keto-sitosterol
30μM 60μM 120μM 7β-OH-sitosterol
30μM 60μM 120μM 7α-OH-sitosterol
30μM 60μM 120μM 6α/β-OH-3-keto-sitosterol
30μM 60μM 120μM mixture

Figure 2: Effects of β-sitosterol oxides on phosphatidlyserine externalization (A), superoxide anion production (B) and Malondialdehyde (C) formation in HepG2-cells. Values are mean ± SD of 3 separate experiments and expressed as percentage of negative control. Columns significantly different to control levels are marked with * for $p \leq 0.05$, ** for $p \leq 0.01$ or *** for $p \leq 0.001$.



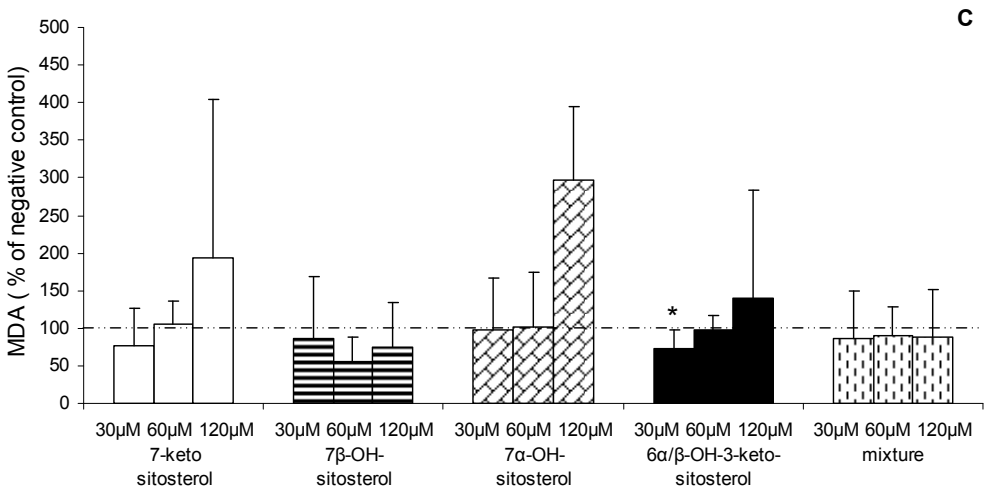
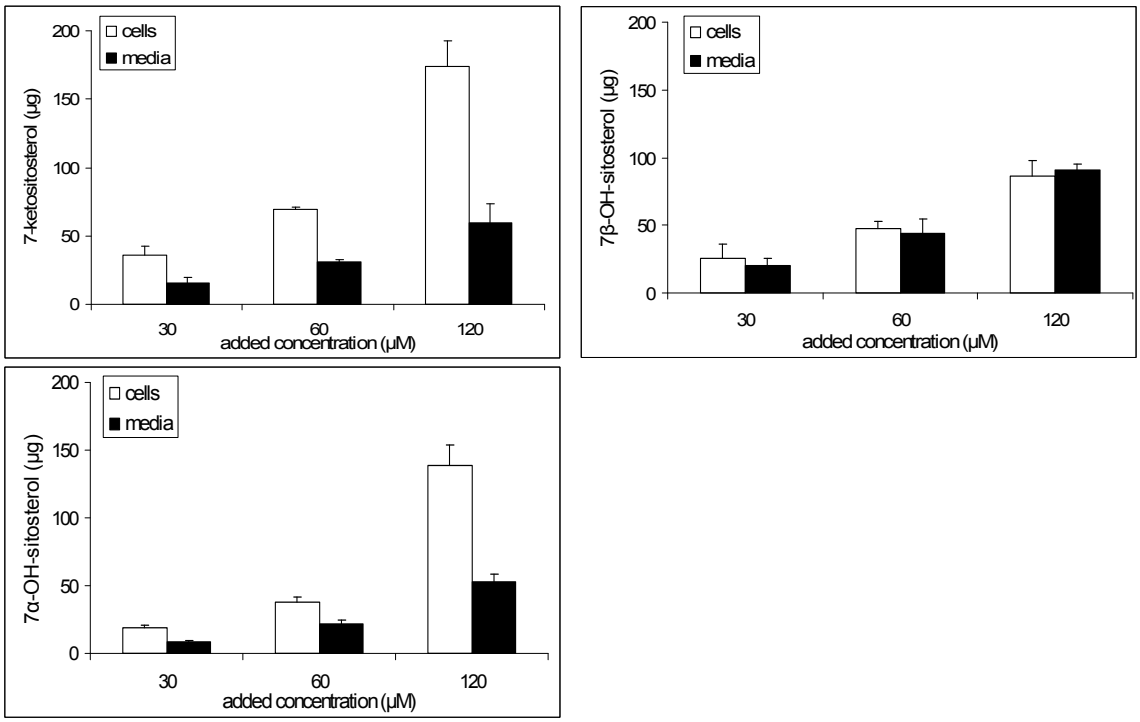


Figure 3: Detected amounts (µg) of β-sitosterol oxides (7-ketositosterol, 7β-OH-sitosterol and 7α-OH-sitosterol) in HepG2-cells and corresponding media after 24 hours of incubation.



The following publications and presentations emerged during the period of this thesis

Publications

KOSCHUTNIG K, HEIKKINEN S, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H Cytotoxic and apoptotic effects of single and mixed oxides of ß-sitosterol on HepG2-cells. *Toxicology in Vitro* - in revision, IF= 2.193

KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, FRITZ-TON C, WAGNER K-H Separation and Isolation of ß-sitosterol oxides and their non-mutagenic potential in the Salmonella microsome assay. *Food Chemistry* - in revision, IF= 3.052

WAGNER K-H, REICHHOLD S, KOSCHUTNIG K, BILLAUD C.: The potential antimutagenic and antioxidant effects of Maillard reaction products used as "natural antibrowning" agents. *Mol. Nutr. Food Res.* 2007, 51(4),497-504, IF= 2.687

Published abstracts

KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H Toxicological effects of oxyphytosterols on HepG2 – cells, *Ann Nutr Metab* 2008;52:128

KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H β – sitosterol oxidation products failed to show mutagenic potential in the Ames test, *BMC Pharmacology* 2007, 7(Suppl 2)**:**A62

REICHHOLD S, KOSCHUTNIG K, WAGNER K-H. The potential antimutagenic and antioxidant effects of Maillard reaction products used as "natural antibrowning" agents, *BMC Pharmacology* 2007, 7(Suppl 2)**:**A72

Presentations

KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H Cytotoxic effects and oxidative stress towards HepG2-cells induced by isolated β – sitosterol oxidation products, 24th GUM-meeting, 17-20. 02. 2008, Vienna, Austria
Abstract: Congress proceedings

KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H Toxicological effects of Phytosterol Oxidation Products on HepG2 –Cells, 6th Euro Fed Lipid Congress 07.09-10.09.2008, Athens, Greece
Abstract: Congress proceedings

KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H Toxikologische Wirkung von Phytosteroloxidationsprodukten auf HepG2-Zellen, Lebensmittelchemikertage 2008, 28-30.05.2008, Eisenstadt, Österreich
Abstract: Congress proceedings

KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H Toxicological effects of oxyphytosterols on HepG2 – cells, 1st Meeting of the Vienna Research Platform of Nutrition and Food Science, April 25 2008, Vienna, Austria
Abstract: Congress proceedings

KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H β – sitosterol oxidation products failed to show mutagenic potential in the Ames test, 13th Scientific Symposium of the Austrian Pharmacological Society (APHAR), Joint Meeting with the Austrian Society of Toxicology (ASTOX), 22.–24. 11. 2007, Vienna, Austria
Abstract: Congress proceedings

KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H No mutagenic potential of 7- keto- sitosterol and 7β- OH- sitosterol in the Ames test, 5th Euro Fed Lipid Congress 16-19.09. 2007, Gothenburg, Sweden
Abstract: Congress proceedings

KOSCHUTNIG K, KEMMO S, LAMPI A-M, PIIRONEN V, WAGNER K-H. Safety assessment of plant sterol oxidation products, COST 927 Workshop, 3.-5. 05. 2007, Sofia, Bulgaria
Abstract: Congress proceedings

KOSCHUTNIG K, REICHHOLD S, WAGNER K-H. Maillard reaction products used as "natural antibrowning" agents and their mutagenic and oxidative effects in the Ames test, SYNTHETIC AND NATURAL COMPOUNDS IN CANCER THERAPY AND PREVENTION, 28.-30. 03. 2007, Bratislava, Slovakia
Abstract: Congress proceedings

REICHHOLD S, KOSCHUTNIG K, WAGNER K-H. The potential antimutagenic and antioxidant effects of Maillard reaction products used as "natural antibrowning" agents, 12th Scientific Symposium of the Austrian Pharmacological Society (APHAR), Joint Meeting with the Austrian Society of Toxicology (ASTOX), 23.–25. 11. 2006, Vienna, Austria
Abstract: Proceedings

KOSCHUTNIG K, REICHHOLD S, WAGNER K-H. The potential antimutagenic and antioxidant effects of Maillard reaction products used as "natural antibrowning" agents, COST 926/927 CONFERENCE, VIENNA 11.-14. 10.2006
Abstract: Congress proceedings

WAGNER K-H, KOSCHUTNIG K, REICHHOLD S, BILLAUD C. Safety assessment of Glucose vs. Fructose based Maillard Reaction Products, Cost 927- IMARS Joint Workshop, May 24-27, Naples, Italy
Abstract: Congress proceedings

Curriculum Vitae

Personal Information

Name	Karin Koschutnig
Date of Birth	Mai 16, 1982
Place of Birth	St Veit/Glan, Kärnten
Citizenship	Austria
Marital Status	single

Education

1992 – 2000	Bundesrealgymnasium Perau, Villach
06/2000	Matura (Austrian advanced matriculation) with special mention
10/2000-01/2006	Study of Nutritional Sciences at the University of Vienna Specialisation in Food production and -technology
01/2006	Second diploma examination passed with distinction Master thesis: Properties of glucose-based Maillard products in the Ames test
since 03/2006	Post graduate study at the Department of Nutritional Sciences, University of Vienna
03-07/2006	Short term scientific mission (STSM) at the Department of Applied Chemistry and Microbiology, University of Helsinki
08-11/2007	Short term scientific mission (STSM) at the Department of Applied Chemistry and Microbiology, University of Helsinki

Work Experience

07/2003	Internship at the sewage-works laboratory in Feldkirchen, Lake Ossiach Water Association
07/2004	Internship at the Food Test Center Kärnten
08/2004	Internship at the brewery in Villach
09/2005	Internship at the brewery in Villach
08-12/2006	Short-time employment at the library for Pharmacies and Nutrition, University Vienna
10/2006-02/2009	Tutor at the Department of Nutritional Sciences, University of Vienna, 'UE zur Ernährungsphysiologie'

10/2008-01/2009	Research assistant at the Department of Nutritional Sciences, University of Vienna
01-04/2009	ABCSG, Vienna: temporary employment in the Data management

Further Education

2006-2008	Advanced training courses at the Department for Human Resources Development at the University of Vienna - SPSS - Presentations and Lectures in the University Context - Das Verfassen von naturwissenschaftlichen Publikationen - English Pronunciation A2-C2
04/2007	Advanced training course: Flow cytometry - FACSCalibur

Scholarships

2001-2006	Performance-based support grant for the study-years 2001/02 - 2005/06
03-05/2006	Scholarship for Brief Scientific Stays Abroad (University of Vienna)
04/2006	STSM-Scholarship of the European COST Action 927
01-12/2007	Research grant on behalf of the University of Vienna (F81-B Forschungsstipendium)

March 2009 Mag. Karin Koschutnig