PHYTOSTEROL OXIDATION PRODUCTS

Formation, Analysis and Occurrence

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Abstract

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The main aim of this thesis was to study the formation of phytosterol oxidation products (POP) and to evaluate and optimize an analytical method for the determination of POP in food products.

The cholesterol lowering effect of phytosterols (PS) and phytostanols has been known for more than half a century and many reports in this area have been published. During the last decade the intake of PS has increased and products enriched with PS and phytostanols have been launched on the commercial market.

Due to their chemical structure, PS are susceptible to oxidation at various sites in their structure. In the present study, oxidation products from the major phytosterols, sitosterol, campesterol and stigmasterol, were generated through autoxidation at elevated temperature. POP from both the ring-structure and side-chain structure were separated by preparative TLC and characterized using GC, GC-MS and NMR spectroscopy. Chromatographic data for many of the products were reported for the first time.

The analytical methodology investigated and optimized in the present study included transesterification of the lipid compounds. The enrichment of POP by amino SPE-technique was reported as efficient and time saving compared with the more commonly used saponification technique. In addition, highly improved separation of a mixture of 29 POP was illustrated when a combination of different polarity GC capillary columns was studied.

The optimized methodology was used for the determination of the levels of POP in some food products. The levels increased in olive oil and maize oil after heated at 180 ± 5 °C for 0-2 h from 8 μg/g to 18 μg/g and 4 μg/g to 12 μg/g, respectively. However, the levels of POP were almost unchanged during heating of peanut oil. The total amount of POP in a commercial available PS ester enriched spread was calculated to around 12 μg/g spread.

Overall, the findings from this thesis demonstrate that numerous oxidation products from PS are formed during heat-facilitated autoxidation. For quantification of all these POP by GC, very high efficient capillary column/columns are necessary. The development of efficient and reliable analytical methods is urgent for the determination of levels of POP in food products, and possibilities to accurately estimate the daily intake of POP.

Keywords: autoxidation, campesterol, capillary GC, phytosterols, phytosterol oxidation products, plant sterols, POP, PS, sitosterol, stigmasterol, thermal oxidation.

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Spendera 80% av Din tid med att fokusera på morgondagens möjligheter istället för gårdagens problem. Om Du försöker köra in i framtiden med blicken fastnaglad i backspegeln så kommer Du att krocka

Okänd

Anyone who has never made a mistake has never tried anything new

Albert Einstein (1879-1955)

Swedish summary
Svensk populärvetenskaplig sammanfattning

Kolesterol bildas naturligt i kroppen men kan även tillföras kroppen genom kosten. Det har visat sig att då kolesterolhalten är hög i blodet ökar risken för hjärt- och kärlsjukdomar. På grund av att växtsteroler och kolesterol kemiskt liknar varandra kan växtsteroler ersätta kolesterol i våra celler och därmed minska koncentrationen av kolesterol i blodet. Denna kolesterolsänkande effekt har varit känd i mer än 50 år och kan uppgå till ca: 15%. Genom att tillsätta höga halter av växtsteroler till vissa produkter så anses vi ha fått hälsosammare livsmedel, s.k. functional foods. Sådana produkter finns idag på den kommersiella marknaden och konsumenterna kan köpa dessa i vanliga livsmedelsaffärer.

Den dagliga konsumtionen av produkter innehållandes växtsteroler har under de senaste årtiondena stadigt ökat. Vid konsumtion av normalt sammansatt kost kommer det mesta av det dagliga intaget av växtsteroler från cerealier, margarinprodukter och vegetabiliska oljor men även grönsaker, frukt och bär är viktiga källor.

På grund av sin kemiska struktur kan växtsteroler oxideras och oxidationsprodukter bildas. Värme, luft samt lagring är faktorer som ökar halten av oxidationsprodukter i matvaror. Ett ökat intag av växtsteroler innebär även ett ökat intag av dess oxidationsprodukter. Trots denna kunskap finns det väldigt lite information som behandlar oxideringen av växtsteroler samt eventuella biologiska effekter som ett ökat intag av dessa produkter skulle kunna utgöra. Studier har visat att upptag av växtsteroler sker i kroppen, dock är uppgifterna om eventuella biologiska effekter motstridiga bland de fåtal studier som utförts.

I denna doktorsavhandling har bildandet av oxidationsprodukter av växtsteroler under värmebehandling studerats. Störst andel oxidationsprodukter bildas i ringstrukturen men det kan även bildas produkter i sidokedjestrukturen av sterolmolekylen. I detta arbete har 29 oxidationsprodukter separerats och karakteriserats med en mängd olika analytiska metoder och av dessa har 11 produkter för första gången beskrivits i detalj. För att i framtiden lättare kunna analysera innehållet av växtsteroloxidationsprodukter i olika matriser och dessutom veta vilka typer av produkter man kan förvänta sig är denna typ av information mycket viktig.

En annan viktig del i detta arbete var att optimera en analysmetod för bestämning av halten växtsteroloxidationsprodukter i olika livsmedel. Oxidationsprodukterna koncentrerades och renades med hjälp av transesterifiering följt av SPE samt kvantifierades med GC. Detta är första gången som denna typ av analysteknik använts inom detta område. Samtliga metodparametrar som undersöktes ansågs vara godtagbara, dock behövs ytterligare optimeringsarbete innan metodiken är helt färdigutvecklad. En stor fördel med den metod som optimerats i detta arbete är en minskad tidsåtgång.

Vidare optimerades separationen av oxidationsprodukter med hjälp av gaskromatografi. En kombination av två kapillärkolonner med olika polaritet undersöktes och en förbättrad separation av 29 oxidationsprodukter uppnåddes jämfört med tidigare redovisade resultat.

Den optimerade analysmetodiken användes för att bestämma halten växtsterol-oxidationsprodukter i tre vegetabiliska oljor samt i en växtsterolberikad margarinprodukt. Samtliga produkter inhandlades i en livsmedelsaffär i Uppsala. De oljor som undersöktes hade olika fettsyrasammansättning och var olivolja, jordnötsolja samt majsolja. Det visade sig att halten oxidationsprodukter var låg i samtliga undersökta oljor men att den ökade under värmning i 180 °C under 0-2 h. Halten av växtsteroloxidationsprodukter i olivolja ökade från 8 μg/g to 18 μg/g och halten i majsolja från 4 μg/g to 12 μg/g. I jordnötsoljan

var koncentrationen tämligen opåverkad. Oxidationsprodukter från växtsteroler detekterades även i den berikade margarinprodukten och en total halt på ca: 12 μg/g margarin rapporterades. När det gäller dessa berikade produkter är informationen väldigt begränsad och ytterligare undersökningar av lagringseffekter samt andra faktorer som kan påverka halten oxidationsprodukter i margarinerna är önskvärda.

Denna studie visar att en mängd olika oxidationsprodukter kan bildas från växtsteroler. För att bibehålla säkerheten och kvaliteten på matprodukter innehållandes växtsteroler behövs nya förbättrade analysmetoder och denna studie är en bit på vägen. Vidare behövs dessa analysmetoder för att bestämma det dagliga intaget av oxidationsprodukter både för de konsumenter som använder de berikade produkterna och för de som konsumerar en "normalt" sammansatt kost. Först efter dessa beräkningar kan eventuella långsiktiga biologiska effekter av oxidationsprodukterna i människa studeras.

CONTENTS

Appendix

The present thesis is based on the following papers, referred to by their Roman numerals.

I. 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.

II. 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.

III. Johnsson, L. & Dutta, P. C. 2004. Separation of phytosterol oxidation products by combination of different polarity gas chromatography capillary columns. *Journal of Chromatography A*. (Submitted)

IV. Johnsson, L. & Dutta, P. C. 2004. Method optimization and determination of levels of phytosterol oxidation products in some food products. (*Manuscript)*

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Authors' contributions to the papers

Papers I-IV: The author, L. Johnsson, was active in the planning of the work, performed the experimental work, evaluated the results and responsible for writing the manuscripts.
The work in all papers was guided and supervised by P. Dutta.

The NMR analyses performed in Paper II were conducted at the Chemistry Department at SLU, Uppsala under the supervision and guidance of R. Andersson. In addition, R. Andersson contributed to the evaluation of the NMR data and the writing of some parts in Paper II.

List of abbreviations

FA	Fatty Acid
FAME	Fatty Acid Methyl Esters
FID	Flame Ionization Detector
FS	Free Phytosterol
GC	Gas Chromatography
GC-MS	Gas Chromatograph-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
LOD	Limit of Detection
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
POP	Phytosterol Oxidation Product(s)
PS	Phytosterols
RT	Room Temperature
SOP	Sterol Oxidation Products
SPE	Solid Phase Extraction
TLC	Thin Layer Chromatography

Introduction

Phytosterols (PS) are compounds present in all plants and in food products with plant origin. In general, PS are thought to stabilize plant membranes and increase membrane rigidification (Guardiola, Dutta, Codony & Savage, 2002; Moreau, Whitaker & Hicks, 2002). PS make up the greatest proportion of the unsaponifiable matter of plant lipids and several different types of PS have been observed.

The first phytosterol (PS) was reported almost 150 years ago by Beneke (Beneke, 1862). Beneke discovered a steroid compound in peas and falsely assumed that it was cholesterol. Due to poor analytical techniques it took more than 50 years until it was established that the compound Beneke had found was in fact sitosterol. Thirty-five years after Beneke reported the separated steroid compound, the term phytosterol was for the first time established. It was H. Thoms that in 1897 stated that all sterols of plant origin should be classified as phytosterols. Nowadays, many PS have been characterized and the group includes more than 200 different sterols and related compounds from various plant materials (Akihisa, Kokke & Tamura, 1991).

The cholesterol lowering effects of PS have been known for a long time and already during the 1950's the first studies were reported. These studies used doses of PS up to 50 g/day in order to decrease the level of cholesterol in serum (Pollak & Kritchevsky, 1981). In the 1980's, Japanese researchers managed to produce the saturated form of PS, known as phytostanols, which were reported to have stronger cholesterol lowering potential. Ten years later the first human trial using phytostanols (1.5 g/day) was performed and it was observed that the use of phytostanols lowered the level of cholesterol in human serum (Miettinen & Gylling, 2003).

During the 1990's the esterification of sterols and stanols was reported and the possibility to include sterols in food matrices increased dramatically. The first commercial product containing esters of phytostanols was a spread in Finland called Benecol® that was launched in 1995. Lately, many products enriched with PS and phytostanols, such as yogurt, snack bar, milk and mayonnaise, have been launched worldwide, but still, Finland is the country with most PS and phytostanol enriched commercial available products (Moreau, 2004).

The chemical nature of PS makes the molecules susceptible to oxidation and many factors such as heat and light increase the oxidation rate. Due to time consuming work, the knowledge in this area is limited and only a few studies have been conducted in the past. However, when fortified products are launched on the market worldwide, the demand for more efficient and accurate analytical techniques increases. Robust and validated methods are crucial for the determination of the levels of phytosterol oxidation products (POP) in food products and also for calculations of the daily intake of POP. During the last decade interest in the oxidation of PS has increased. The oxidation mechanism and

products formed has been studied and some analytical methods have been reported. However, much information in the area still remains to be discovered (Dutta, 2004a).

Reports on POP in food products have mainly been focused on edible vegetable oils and certain products prepared in these oils. However, with more efficient analytical techniques, further investigations regarding POP levels in various foods products and biologically samples can be performed. In addition, more detailed and accurate information can be distributed to consumers, regulatory authorities and health organizations.

Background

Phytosterols are biosynthetically derived from squalene and belong to the group of triterpenes that includes more than 4000 different compounds (Moreau, Whitaker & Hicks, 2002). PS are made up of a tetracyclic cyclopenta(α)phenanthrene ring and a flexible side-chain at C_{17} (Piironen *et al.*, 2000). The most common PS, sitosterol, campesterol and stigmasterol are structurally closely related to cholesterol. The PS have similar backbone structure but differ with an additional methyl or ethyl group in the side chain. PS have a double bond between C_5-C_6 (Δ^5) and a hydroxy group in β-position at C_3 and if the molecule is saturated at C_5-C_6 the compound is referred to as a "stanol" (Figure 1) (Dutta, Przybylski & Appelqvist, 1996).

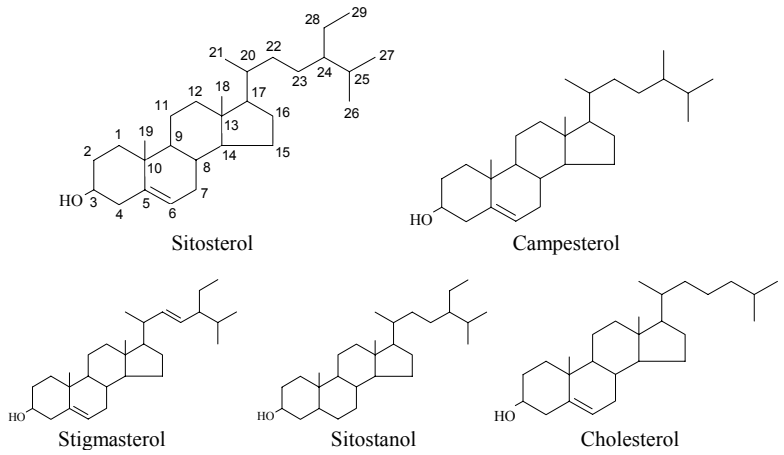

Figure 1. The chemical structures of some common sterols; sitosterol, campesterol, stigmasterol, sitostanol (the saturated form of sitosterol) and cholesterol.

PS can be divided into three major subgroups based on the number of methyl groups on carbon 4, two (4,4'-dimethyl), one (4-monomethyl) and none (4-desmethyl) (Dutta, 2002). 4,4'-dimethyls and 4-monomethyls are intermediates in the biosynthetic pathway that leads to the end product 4-desmethyl PS. However, these intermediates are commonly found at low levels in most plant tissues.

In plants, phytosterols exist as free sterols and in several conjugates (Figure 2).

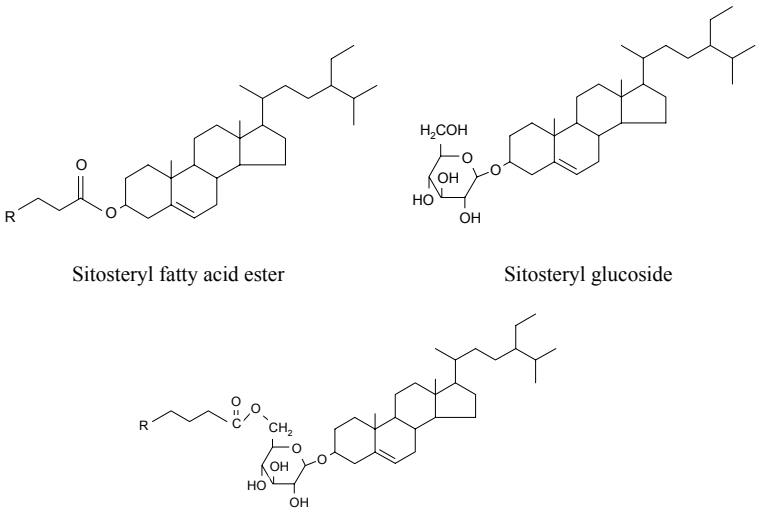

Figure 2. In plants PS exist in several conjugates. These are the chemical structures of the most common phytosterol conjugates (Moreau, Whitaker & Hicks, 2002).

Free sterols (FS) (Figure 1) are a part of the cell wall and have important structural functions (Normén, 2001). The free hydroxy group at C_3 enables the free sterols to interact with phospholipids and proteins in the cell membranes. FS also regulate the fluidity of the cell membranes and play an important role in the temperature adaptation of the membranes (Piironen *et al.*, 2000). Phytosteryl esters are storage products in the cell and can be found in the cytosol and in droplets or vesicles (Lorentz, Haeckler & Fenner, 1989). Besides, phytosteryl glycosides are found in the microsome fraction and acetylated phytosteryl glycosides are found in the mitochondria (Grunwald & Huang, 1989).

Phytosterols in functional food products

The cholesterol lowering effects of PS have been known for more than fifty years, when it was reported from studies on rabbits and chickens (Peterson, 1951; Pollak, 1953). Some years later the first study of the effects of PS on humans was published (Farquhar, Smith & Dempsey, 1956). Fifteen young men were fed 12-18 g/day of PS and reduction in serum cholesterol was reported. However, due to

the high doses of unesterified PS the taste was characterized as chalky (Norman & Wong, 2001). The use of crystalline PS limited the applicability in food products and it was suggested that the esterified form was preferable (Mattson, Grundy & Crouse, 1982). Researchers at Proctor and Gamble were the first to suggest esterification of the PS in order to increase the solubility in fat matrices (Moreau, Whitaker & Hicks, 2002).

The dietary intake of PS ranges from 150-400 mg/day (Ahrens & Boucher, 1978; Miettinen & Kesaniemi, 1989; Morton *et al.*, 1995; Nguyen, 1999; Piironen *et al.*, 2000; Norman & Wong, 2001; Trautwein *et al.*, 2003) and the absorption of PS is below 10% (Grundy, Ahrens & Davignon, 1969; Salen, Ahrens & Grundy, 1970; Heinemann, Axtmann & von Bergmann, 1993; Moreau, Norton & Hicks, 1999; Ostlund, 2002; Trautwein *et al.*, 2003). An increased intake of PS or phytostanols (around 2 g/day) lowers the cholesterol absorption (Miettinen *et al.*, 2000; Plat & Mensink, 2000). The lowered absorption stimulates the endogenous production of cholesterol so the net results of the cholesterol lowering effect caused by increased consumption of PS and phytostanols are often reported to be between 9-15% (Hendriks *et al.*, 1999; Moreau, Norton & Hicks, 1999; Gylling & Miettinen, 2000; Neil, Meijer & Roe, 2001; Ostlund, 2002). A combination of statins (a drug that decreases the cholesterol metabolism) and PS has been reported as an interesting combination for the future in preventing coronary heart disease (Blair *et al.*, 2000; Ostlund, 2002).

The mechanism of action behind the cholesterol lowering effects of PS and phytostanols is not fully understood (Wester, 2000; Ostlund, 2002). However, currently available data indicate that PS affects the absorption via suppressing intestinal cholesterol absorption in the small bowel (Normén, 2001; Trautwein *et al.*, 2003). It has been proposed that due to the extra ethyl or methyl group in PS the hydrophobicity of PS is higher than for cholesterol (Norman & Wong, 2001). Increased hydrophobicity generates higher affinity for the mixed micelles, thereby displacing cholesterol, resulting in a decreased absorption (Lichtenstein, 2002). It has also been proposed that the absorption of cholesterol is determined by the total sterol concentration in the lumen (cholesterol + PS) (Mattson, Volpenhein & Erickson, 1977). High concentration of PS in the lumen decreases the level of cholesterol, making cholesterol precipitate as cholesterol monohydrates (Christiansen *et al.*, 2001). Both *in vitro* and *in vivo* studies have reported this displacement of cholesterol (Ikeda *et al.*, 1988b, a; Ikeda, Sugano & Tanabe, 1989). Furthermore, other possible mechanisms of action have also been proposed (Normén, 2001).

In 1995, Raisio, in Finland, launched the first phytostanol enriched spread, Benecol® (Miettinen *et al.*, 1995). Five years later Unilever launched the PS enriched margarine Becel®. Nowadays, there are many products enriched with PS or phytostanols on the commercial market and the enriched margarines have been marketed worldwide (Table 1). Enriched products coming in the future are different cooking oils, new spreads, chocolates and chocolate beverages (Moreau, 2004).

Table 1. *Countries where PS enriched products have been launched (Moreau, 2004). Benecol contain esterified phytostanols and Becel contain esterified phytosterols. Y means that the product is commercially available in that country*

Country	Year Introduced	Benecol products	Becel products	Other PS enriched products than spread
Australia	2000		Y	
Brazil	2000		Y	
Czech Republic	2000		Y	
Denmark	2000		Y	
Finland	1995	Y	Y	Cream cheese, spreads, milk, mayonnaise, cheese, pasta, yogurt, meat products, snack bars
France	2000		Y	
Germany	2000		Y	
Japan	1999	Y	Y	Cooking oil, beverages
Korea	?		Y	Beverages
South Africa	2000		Y	
Spain	2000		Y	Yogurt
Sweden	2000	Y	Y	Milk
UK	1999	Y	Y	Snack bars, mayonnaise, milk
US	1999	Y	Y	

Many studies have investigated the efficiency of PS and phytostanols for the lowering of cholesterol (Normén, 2001) and extensive reviews have recently been published (Wester, 2000; Normén, Frohlich & Trautwein, 2004). It was generally considered that the saturated stanol molecule was more efficient in the lowering action than the unsaturated sterol (Miettinen & Vanhanen, 1994). However, contradicting results were published, indicating that the two sterols were equally efficient and nowadays, fortified products both with sterols and stanols are available for the consumers (Table 1) (Normén *et al.*, 2000).

Oxidation of phytosterols

Oxidation by triplet oxygen

Oxidation in the ring structure

The oxidation of cholesterol has been extensively studied and many excellent books and comprehensive reviews have been published (Peng & Morin, 1992; Schroepfer, 2000; Guardiola *et al.*, 2002). Due to similar structures (Figure 1), much of the knowledge of the oxidation mechanisms for PS refers to studies on cholesterol (Smith, 1981). A large number of possible POP have previously been characterized and listed (Yanishlieva, Schiller & Marinova, 1980) and overviewed in Table 2.

Table 2. *Trivial- and systematic names for some common phytosterol oxidation products*

Systematic name	Trivial name
(24S)-ethylcholest-5,22-dien-3β-ol	Stigmasterol
(24S)-ethylcholest-5,22-dien-3β,7α-diol	7α-hydroxystigmasterol
(24S)-ethylcholest-5,22-dien-3β,7β-diol	7β-hydroxystigmasterol
(24S)-ethylcholest-5,22-dien-3β-ol-7-one	7-ketostigmasterol
(24S)-5α,6α-epoxy-24-ethylcholest-22-en-3β-ol	Stigmasterol-5α,6α-epoxide
(24S)-5β,6β-epoxy-24-ethylcholest-22-en-3β-ol	Stigmasterol-5β,6β-epoxide
(24S)-ethylcholest-22-en-3β,5α,6β-triol	Stigmastentriol
(24S)-ethylcholest-5,22-dien-3β,24-diol	24-hydroxystigmasterol
(24S)-ethylcholest-5,22-dien-3β,25-diol	25-hydroxystigmasterol
(24R)-methylcholest-5-en-3β-ol	Campesterol
(24R)-methylcholest-5-en-3β,7α-diol	7α-hydroxycampesterol
(24R)-methylcholest-5-en-3β,7β-diol	7β-hydroxycampesterol
(24R)-methylcholest-5-en-3β-ol-7-one	7-ketocampesterol
(24R)-5α,6α-epoxy-24-methylcholestan-3β-ol	Campesterol-5α,6α-epoxide
(24R)-5β,6β-epoxy-24-methylcholestan-3β-ol	Campesterol-5β,6β-epoxide
(24R)-methylcholestan-3β,5α,6β-triol	Campestanetriol
(24R)-methylcholest-5-en-3β,24-diol	24-hydroxycampesterol
(24R)-methylcholest-4-en-6α-ol-3-one	4-campesten-6α-ol-3-one
(24R)-methylcholest-4-en-6β-ol-3-one	4-campesten-6β-ol-3-one
(24R)-ethylcholest-5-en-3β-ol	Sitosterol
(24R)-ethylcholest-5-en-3β,7α-diol	7α-hydroxysitosterol
(24R)-ethylcholest-5-en-3β,7β-diol	7β-hydroxysitosterol
(24R)-ethylcholest-5-en-3β-ol-7-one	7-ketositosterol
(24R)-5α,6α-epoxy-24-ethylcholestan-3β-ol	Sitosterol-5α,6α-epoxide
(24R)-5β,6β-epoxy-24-ethylcholestan-3β-ol	Sitosterol-5β,6β-epoxide
(24R)-ethylcholestan-3β,5α,6β-triol	Sitostanetriol
(24R)-ethylcholest-5-en-3β,24-diol	24-hydroxysitosterol
(24R)-ethylcholest-4-en-6α-ol-3-one	4-sitosten-6α-ol-3-one
(24R)-ethylcholest-4-en-6β-ol-3-one	4-sitosten-6β-ol-3-one

The oxidation of sterols is a free radical mechanism that can be initiated by factors such as heat, light, air, water and transition metals (Smith, 1981; Dutta, Przybylski & Appelqvist, 1996). Sterols can be oxidized by many different oxygen species such as; ground-state oxygen ($^3O_2$), ozone (O_3), singlet oxygen ($^1O_2$), hydroperoxides (H_2O_2), dioxygen cation (O_2^+) and hydroxy radical ($HO^\bullet$) (Dutta, Przybylski & Appelqvist, 1996).

The most common oxygen species involved in the oxidation of sterols is triplet oxygen, $^3O_2$. Triplet oxygen has two electrons with the same spin in the antibonding 2p orbitals. The electrons are in different orbitals, which give the molecule a small electrostatic energy making this species rather unreactive (Figure 3) (Frankel, 1998).

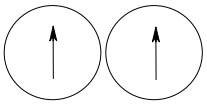

Figure 3. The outer orbitals of triplet oxygen, $^3O_2$. The electrons have the same spin giving the molecule a small electrostatic energy.

The most common oxidation mechanism for sterols is autoxidation, which is a free radical reaction that starts with the abstraction of a reactive allylic hydrogen at C_7. The abstraction of a hydrogen atom at C_4 seldom occurs because of steric hindrance and stabilization from the hydroxy group at C_3 and the tertiary C_5 (Smith, 1981).

When the hydrogen has been removed, the radical molecule reacts with the triplet oxygen to form a 7-peroxy radical (Dutta, 2004b). Hydrogen addition to the 7-peroxy radical give the more stable 7-hydroperoxide and after degradation of the 7-hydroperoxide the stable 7α-hydroxysterols, 7β-hydroxysterols and 7-ketosterols are formed (Figure 4) (Yanishlieva *et al.*, 1983; Smith, 1996).

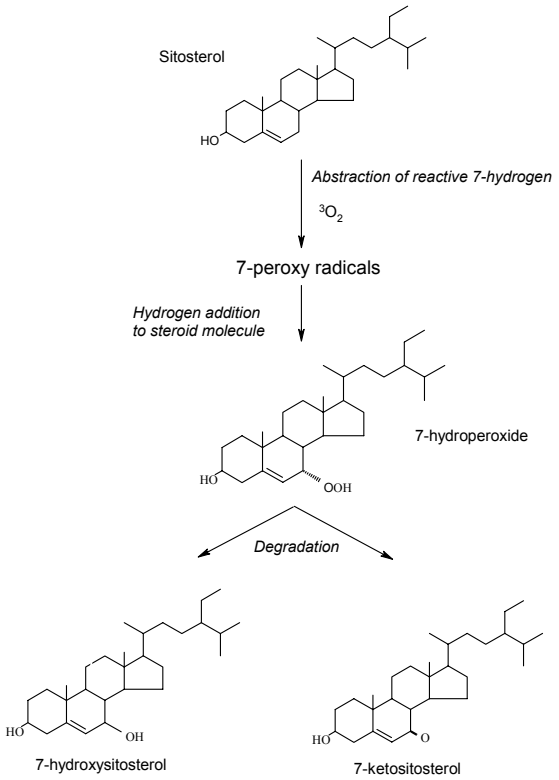

Figure 4. Reaction scheme of the formation of POP during autoxidation.

Other major oxidation products derived from the ring structure are epoxides and triols. The formation of epoxides by autoxidation occurs by a bimolecular mechanism that includes one hydroperoxy radical and one unoxidized sterol molecule and both α-epoxides and β-epoxides have been reported (Figure 5) (Bortolomeazzi *et al.*, 1999; Lampi *et al.*, 2002; Lercker & Rodriguez-Estrada, 2002; Grandgirard *et al.*, 2004b). However, autoxidation by elevated temperature does not generate any triols. The triols are formed after hydration of the epoxides, in acidic environment (Figure 5) (Smith, 1981).

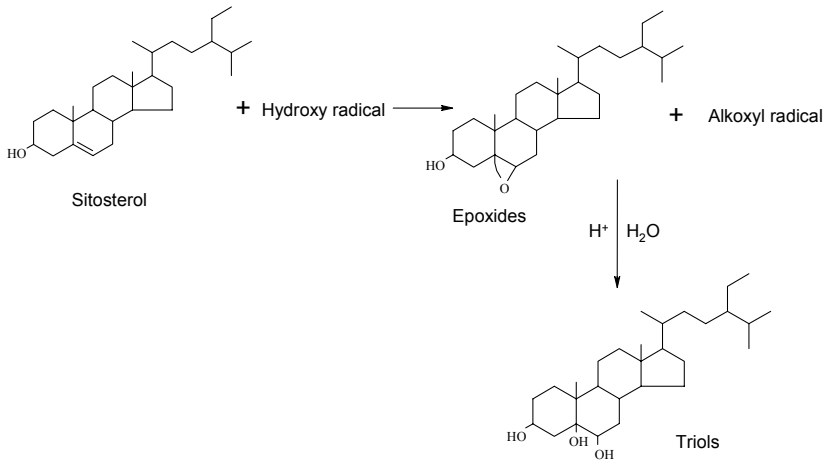

*Figure 5***.** Reaction scheme of the formation of triols and epoxides.

The primary oxidation products 7α- and 7β-hydroperoxides were reported when pure sitosterol was exposed to oxidation at 25 °C for 6 months and 150 °C during 1 h. In addition, the authors stated that other relative stable peroxides observed in the system were 6α-hydroperoxy-stigmast-4-en-3-one and 6β-hydroperoxy-stigmast-4-en-3-one (Yanishlieva, Schiller & Marinova, 1980).

As mentioned earlier, the unstable peroxides are decomposed into other more stable POP and these are the oxidation products usually analyzed. The thermal oxidation in the ring structure of sitosterol, campesterol and stigmasterol has been studied in detail (Daly, Finocchiaro & Richardson, 1983; Dutta, 1997; Dutta & Appelqvist, 1997; Lampi *et al.*, 2002). All studies reported the formation of 7α-hydroxy, 7β-hydroxy, 7-keto, 5α,6α-epoxides and 5β,6β-epoxides. Further oxidation of these POP leads to dehydration and subsequent abstraction of the hydroxy group on C_3, which generates conjugated dienes and trienes (Dutta, Przybylski & Appelqvist, 1996; Lercker & Rodriguez-Estrada, 2002).

Oxidation in the side-chain structure

Oxidation products can also be formed in the side-chain structure of the steroid molecule. However, it has been reported that the formation of oxidation products in the steroid side-chain is much less pronounced than the oxidation in the ring-

structure (Smith, 1992). The oxidation mechanisms are the same as for ring-structure oxidation and include the formation of hydroperoxides (Figure 6) that generate secondary oxidation products, which are commonly determined (Yanishlieva, Schiller & Marinova, 1980; Smith, 1992).

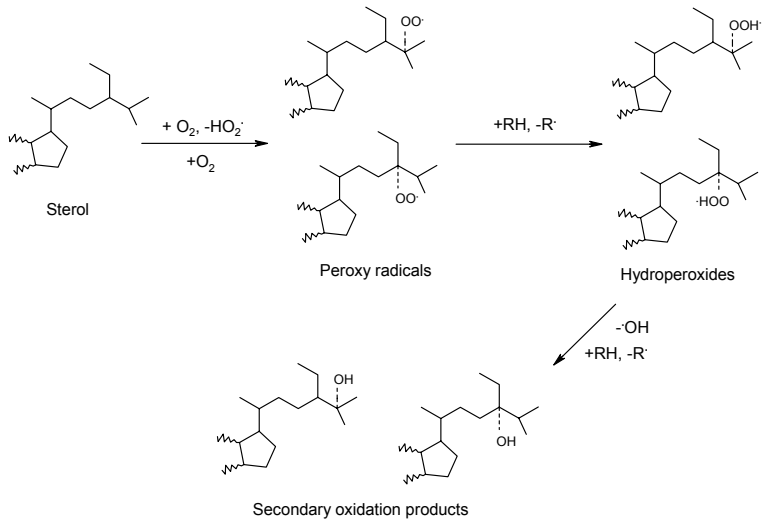

Figure 6. The reaction scheme of side-chain oxidation of phytosterols.

Due to the tertiary nature of C_{24} and C_{25} in the side chain of PS, these are the most favorable sites for the formation of hydroperoxides (Yanishlieva, Schiller & Marinova, 1980). Despite the possibility to form oxidation products in the side-chain, the research in this area is limited (Dutta, 2004b). However, the formation of 24-hydroxysitosterol and 25-hydroxysitosterol was reported after heating of sitosterol at 150 °C for 1 h or by leaving pure sitosterol in room temperature for 6 months (Yanishlieva, Schiller & Marinova, 1980). The authors also reported the formation of 24-dehydrositosterol as a product generated after the dehydration of 24-hydroxysitosterol and 25-hydroxysitosterol.

The formation of 25-hydroxystigmasterol after thermal oxidation at 180 °C during 6 h was reported as well as the presence of 25-hydroxysitosterol and 25-hydroxycampesterol in rapeseed oil (Lampi *et al.*, 2002). In addition, the generation of Δ^5-pregen-3β-ol-20-one has been observed after oxidation of stigmasterol at 180 ± 5 °C for several hours (Blekas & Boskou, 1989)

Oxidation by singlet oxygen

The oxidation of phytosterols can also be initiated by the excited state of oxygen known as singlet oxygen, $^1O_2$. This oxidation mechanism is referred to as photooxidation. In the singlet state, the two unpaired electrons in the outer orbitals have opposite spin, making the electrostatic repulsion great (Frankel, 1998).

The singlet oxygen exists in two excited states (Figure 7) and both are very reactive compared with the triplet oxygen (Figure 3).

Figure 7. The outer orbitals of singlet oxygen. The opposite spin of the electrons in the outer orbitals makes the species energy-rich and highly reactive.

The singlet oxygen is over 1500 times more reactive than ordinary triplet oxygen and an electrophilic attack can occur on either side of the double bond in the steroid molecule (Kulig & Smith, 1973; Dutta, 2004b). Singlet oxygen can be generated in several ways. The most important mechanism is when photosensitizers such as chlorophyll or riboflavin absorb light and become electronically excited. The sensitizers interact with triplet oxygen and generate the reactive singlet oxygen. There are two types of sensitizers that act with two different mechanisms. In type 1, the sensitizer (Sens) acts as a photochemically activated free radical initiator (Figure 8). The sensitizer reacts with the lipid substrate (LH) by hydrogen or electron transfer to form free radicals (Intermediate) that can react with oxygen and form hydroperoxides. Riboflavin is an example of a sensitizer that acts with this reaction mechanism (Frankel, 1998; Säynäjoki *et al.*, 2003).

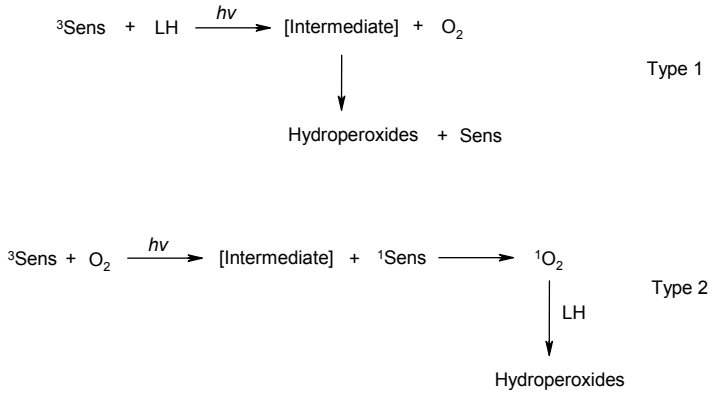

Figure 8. The reaction scheme of the photooxidation.

In type 2 the sensitizer reacts with oxygen by energy transfer (Figure 8). Reactive singlet oxygen is generated and $^1O_2$ reacts further with the lipids and forms hydroperoxides. Examples of sensitizers that act by this mechanism are chlorophyll, methylene blue and erythrosine (Frankel, 1998). Due to the high energy of the singlet oxygen the products formed from the photooxidation differ from the products generated from the oxidation with triplet oxygen. The primary hydroperoxide formed from photooxidation is 5α-hydroperoxide (Figure 9) (Yanishlieva, Schiller & Marinova, 1980; Smith, 1992; Bortolomeazzi *et al.*, 1999; Säynäjoki *et al.*, 2003).

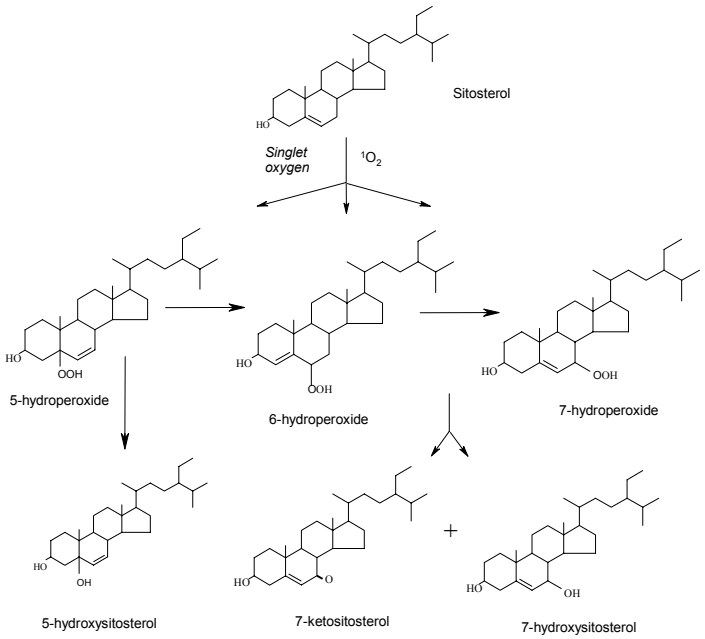

Figure 9. The formation of POP through photooxidation. Due to the highly reactive singlet oxygen, oxidation can occur on either side of the Δ^5-double bond. The primary oxidation product, hydroperoxides, generates the more stable secondary oxidation products.

Recently the formation of hydroperoxides at C_6 and C_7 was observed during photooxidation in dichloromethane in the presence of methylene blue as sensitizer and after degradation of hydroperoxides at C_5 (Säynäjoki *et al.*, 2003). As mentioned before, the hydroperoxides are energy-rich molecules and eventually generate more stable secondary oxidation products like 7-hydroxysterol and 7-ketosterol (Figure 9). However, in the area of PS the information of photooxidation is limited.

Oxidation of phytostanols

Phytostanols have similar molecular structure as PS but are saturated at C_5-C_6 (Figure 1). The saturated molecule is considered to be more stable than the counterpart (Dutta, 2004b). In a recent investigation the formation of oxidation products during heating of sitostanol at 180 °C for 3 h was reported (Soupas *et al.*, 2004). Many products were characterized with TLC, GC-FID and GC-MS and standards from cholesterol and cholestanol were used for identification of the products. In addition to epimeric hydroxides at C_5, C_6, C_7, C_{15} and C_{25}, the epoxides and 7-keto products were also observed in that study. However, further investigations in this area are required.

Occurrence of phytosterol oxidation products

The main phytosterols in food products are sitosterol, campesterol and stigmasterol. Usually, cereals are the major source of PS followed by margarines and vegetable oils (Morton *et al.*, 1995; Normén *et al.*, 2001). In cereals, PS and their conjugates are mainly located in the kernel and the levels depend on both growing location and genetic factors (Määttä *et al.*, 1999; Vlahakis & Hazebroek, 2000). Levels between 350-1200 mg/kg fresh weight have been observed and the dominating PS is sitosterol (49-64 %). The contributions from vegetables, fruits and berries are also significant (20-25%) and the main PS in these products is sitosterol (43-86% in vegetables, 72-86% in fruits and 61-93% in berries) followed by campesterol and stigmasterol (Piironen *et al.*, 2003; Piironen & Lampi, 2004).

Most studies regarding POP in food products concern vegetable oils and products prepared in oils. The first report in this area was published almost 30 years ago when 7-hydroxysitosterol was found in crude oil, neutralized oils and in soapstocks (Niewiadomski, 1975). Two of the most extensive investigations in this area are the studies of vegetable oils and food products prepared in these oils made by Dutta and Appelqvist (Dutta, 1997; Dutta & Appelqvist, 1997). The levels of POP in sunflower oil, high oleic sunflower oil and a blend of hydrogenated rapeseed oil/palm oil were reported. In addition, the amount of POP in French fries was determined. It was illustrated that the level of POP increased in the investigated vegetable oils during frying (Table 3). The authors also reported that the levels of POP increased during storage of fried potato chips prepared in the different vegetable oils. The levels were higher in the more unsaturated oils like sunflower and high-oleic sunflower oil. These results were in line with previously published results (Yanishlieva & Tasheva, 1986).

Table 3. *Levels of POP (ppm) in some vegetable oils. Before, and after 2 days of frying of French fries at 180 °C. (Dutta, 1997). The values were mean of duplicate analyses*

Frying oil	Day 0 (ppm)	Day 2 (ppm)
Rapeseed oil/Palm oil	41.0	59.4
Sunflower oil	39.9	56.6
High-oleic sunflower oil	46.7	55.9

In another study, the formation of POP in rapeseed oil was investigated, and increased levels were reported during heating of the oil at 180 °C during 0-24 h (Table 4). Oxidation products from both sitosterol and campesterol were determined and in addition to the common ring-structure products two side-chain products were quantified, 25-hydroxysitosterol and 25-hydroxycampesterol. Prior to heating, no POP were quantified in the oil. However, after 24 h at 180 °C the total amount of POP was more than 1000 μg/g oil (Table 4). The most abundant oxidation products were sitosterol-5β,6β-epoxide and campesterol-5β,6β-epoxide (Lampi *et al.*, 2002).

Table 4. *The levels of POP (ppm) in rapeseed oil during heating at 180 °C for 0-24 h, (n.d = not detected) (Lampi et al., 2002)*

Heating time (h)	Oxidation products of sitosterol (ppm)						
	7α-OH	7β-OH	5α,6α-epoxy	5β-6β epoxy	7-keto	25-OH	Total
0	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	31	52	27	38	12	2	162
12	71	105	58	92	42	4	372
24	109	154	107	172	102	2	646

Heating time (h)	Oxidation products of campesterol (ppm)						
	7α-OH	7β-OH	5α,6α-epoxy	5β-6β epoxy	7-keto	25-OH	Total
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	21	27	13	31	9	3	104
12	52	59	33	72	31	6	253
24	83	87	69	131	72	11	452

The levels of POP in a variety of crude vegetable oils and the influence of bleaching and deodorization have also been reported (Bortolomeazzi *et al.*, 2003). The bleaching of the oil decreased the level of POP but the deodorization had no influence. In another study, the level of POP in a simulated test food system was investigated (Oehrl *et al.*, 2001). Vegetable oils were fortified with PS and the formation of epoxides and epimeric 7-hydroxysterols was reported prior to heating. From the investigation, it was concluded that the accumulation of POP might be temperature-dependent because high oxidative temperature leads to oxidation products not measurable by ordinary GC.

Beside the investigations of POP in vegetable oils and products prepared in these oils, data are limited. However, some other food products have been studied. The use of 7-ketositosterol as a marker for oxidation in infant milk formulas and milk cereals was reported (Zunin, Calcagno & Evangelisti, 1998) and the investigation of POP in coffee samples and wheat flours have also been reported (Turchetto, Lercker & Bortolomeazzi, 1993; Niemelä, Wester & Lahtinen, 1996). Recently, a study was published that investigated the levels of POP in a fortified spread (Grandgirard *et al.*, 2004b). It was demonstrated that the spread contained numerous oxidation products and POP were characterized with relative retention times and GC-MS. Due to poor separation no quantitative analysis was performed but a rough estimation of the level of POP was reported as 68 μg/g spread.

Biological effects of phytosterol oxidation products

The toxic effects of COP have been extensively studied and the relation between high levels of COP and coronary heart diseases has been reported in many papers (Guardiola *et al.*, 2002). Some COP can be atherogenic, cytotoxic, mutagenic, cancerogenic and may also inhibit cholesterol biosynthesis and membrane functions (Bösinger, Luf & Brandl, 1993). Western diet contains around 300-600 mg/day of cholesterol and additional 1000-1500 mg is produced by endogenous sources, mainly from the bile (Homan & Krause, 1997; Ellsworth & Starr, 1998). Of the total cholesterol, 30-60% is absorbed in humans and only some percent of the cholesterol is oxidized (Li *et al.*, 1998; Bosner *et al.*, 1999).

Even though similar oxidation products can be generated from PS, the biological effects and safety aspects of POP are still rather unclear (Oehlr Dean & Boyd, 2004). However, POP in blood have been observed in both rats and humans (Plat *et al.*, 2001; Grandgirard *et al.*, 2004a). In addition, lymphatic absorption of POP (1.5 - 4.7%) has been reported from rat studies and it was concluded that the chain length of the sterol molecule was important since oxidation products from campesterol were absorbed to a wider extent than products from sitosterol (Grandgirard *et al.*, 1999). In another study, the levels of POP in plasma from healthy humans ranged from around 5 ng/ml for sitostanol-5α,6α-epoxide, campestanetriol and 7-ketositosterol to a much higher level for sitostanol-5β,6β-epoxide and sitostanetriol. It was suggested that the POP derived from either the food or the *in vivo* oxidation in the plasma (Grandgirard *et al.*, 2004a).

The scientific work in the area of possible toxic effects of POP is limited and contradictory. Toxic effects of some POP have been observed both *in vitro* and *in vivo* (Meyer *et al.*, 1998; Adcox *et al.*, 2001). However, lack of toxic effects of POP has also been reported (Hiroko *et al.*, 2004; Lea *et al.*, 2004). Even though POP were absorbed and accumulated in the body, no contributions from the POP to promote the development of atherosclerosis in apo E-deficient mice could be detected (Hiroko *et al.*, 2004). The absence of genotoxic effects of POP was also reported when rats were fed with a diet containing a PS concentrate with 30% POP for 90 consecutive days (Lea *et al.*, 2004).

The unoxidized PS have been investigated more thoroughly, probably because it is a component in some enriched food products. It has been reported that an increased intake (0.5 and 5 mg/kg per day per rat subcutaneous) of PS has an adverse effect on the reproductive organs of laboratory animals (Malini & Vanithakumari, 1991) and even might increase the fragility of erythrocyte membranes (Miettinen, Tilvis & Kesaniemi, 1990). The reduction of absorption of carotenoids has also been reported (Kritchevsky, 2004). However, most reviews in the area have come to the conclusion that dietary plant sterols are a safe and efficient way to lower the cholesterol levels in blood (Ling & Jones, 1995; Moghadasian, 2000; Hendriks *et al.*, 2004; Kritchevsky, 2004).

Analysis of phytosterol oxidation products

The principles for the analyses of POP are based on the same methodologies as for the analysis of COP, which include the following steps: extraction, saponification or transesterification, enrichment of oxidation products and finally qualitative or quantitative analysis by GC, GC-MS, HPLC or HPLC-MS (Dutta, 2002).

Extraction

Many different methods for the extraction of lipids have been reported in the literature including solvent extraction (Dutta, 2004b), soxhlet extraction (Christie, 2003) and supercritical fluid extraction (SFE) (Johnson & Barnett, 2003). However, the most commonly used methodology is extraction with different combinations of organic solvents. The extraction of the lipids from the sample is probably the most crucial step in the analytical methodology. Losses should be minimized and only the compounds wanted for further analyses extracted. It is very important that the extraction solvent has optimal polarity for the purpose since otherwise other non-lipid compounds could be extracted together with the lipids (Dutta, Przybylski & Appelqvist, 1996).

The most commonly used solvent system in order to separate lipids from food products are mixtures of chloroform:methanol ($CHCl_3$:MeOH) with endogenous water in the sample as ternary component. The methodology was first reported more than 40 years ago (Folch, Lees & Stanley, 1956). The combination of organic solvents has been extensively studied in the extraction of COP and also implied in POP analysis in several studies (Table 5). Cold extraction with chloroform in the analysis of coffee samples has also been reported in literature (Turchetto, Lercker & Bortolomeazzi, 1993).

Table 5. *Extraction systems used in the analysis of POP on different matrices*

Matrix	Extraction system	Reference
Commercial flours	1. water-sat. n-butanol 2. $CHCl_3$:MeOH	Finocchiaro, 1983
Potato chips & French fries	$CHCl_3$:MeOH	Lee *et al.,* 1985
Decaffeinated coffees	cold extraction with chloroform	Turchetto *et al.,* 1993
Potato chips prepared in vegetable oils	hexane:isopropanol	Dutta *et al.,* 1997
Vegetable oils and French fries	hexane:isopropanol	Dutta, 1997
Infant formulas and milk cereals	hexane:isopropanol	Zunin *et al.,* 1998

Another frequently used solvent system was described by Hara and Radin (1978). The authors used a mixture of hexane and isopropanol (H:IP) in the analysis of nervous tissue. This solvent combination was used in the analyses of infant formulas and milk cereals (Zunin, Calcagno & Evangelisti, 1998) (Table 5). The

sample was extracted with isopropanol, followed by extraction with hexane. The lipid layer was collected and the remaining sample re-extracted with additional hexane. Dutta & Appelqvist (1997) also used a two-fold extraction with H:IP (3:2, v:v) in the POP analysis of French fries and potato chips fried in different vegetable oils. However, even if different combinations of H:IP have been studied, more information is required in order to compare the effectiveness with the more extensively studied chloroform:methanol system.

Purification

Since POP is a minor component in the lipid extract, purification is vital before fractionation and separation into individual compounds (Guardiola *et al.*, 2004). After the extraction, the crude lipid extract contains triacylglycerols, phospholipids, unoxidized PS and other compounds that have to be separated in order to improve the sensitivity and efficiency of the analysis. The amount of PS in vegetable oils is often around 0.1-10% of the total lipid extract (Dutta, Przybylski & Appelqvist, 1996). Similar to the analysis of COP there are several different methodologies for the purification of PS and POP from the lipid extracts prior to determination and quantification:

- saponification
- transesterification
- chromatographic fractionation
- combination of saponification and chromatographic fractionation

In order to increase the sensitivity and effectiveness of the analytical method, the purification step should remove interfering compounds from the sample. However, it is very important to consider possible losses and extra time every purification step might bring (Guardiola *et al.*, 2004). It is also important to remember that in the analysis of POP and PS often at least three major PS are present and many different products may interfere with the analytical procedure (Dutta, Przybylski & Appelqvist, 1996).

Saponification

Saponification, also referred to as alkaline hydrolysis, is recommended in official methods for determination of unoxidized PS (AOAC, 2000). The saponification has two main purposes:

- to remove the dominant triacylglycerols and other acyl lipids from the crude lipid extract by converting them into water-soluble compounds
- to hydrolyze ester bonds in conjugated PS

By saponification the triacylglycerols are converted to water-soluble compounds that easily can be excluded by extraction with a combination of water and organic solvent (Figure 10). PS and POP remain unsaponified and dissolved in the organic phase. The saponification is vital in the determination of the total level of POP and PS since the conjugated sterols (Figure 2) are released (Dutta, Przybylski & Appelqvist, 1996).

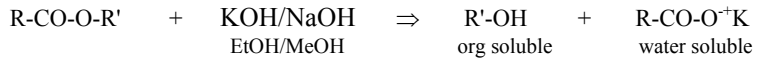

Figure 10. The reaction scheme for saponification of lipids.

Traditionally, saponification has been performed by heating the sample in methanolic or ethanolic NaOH or KOH solution for various times (Table 6) (Lee, Herian & Richardson, 1984; Herian & Lee, 1985; Lee, Herian & Higley, 1985; Yanishlieva & Tasheva, 1986; AOAC, 2000). However, in the beginning of the 1990's it was reported that heating could degrade 7-ketocholesterol and cholesterol-5α,6α-epoxide and formation of epimeric forms of 7-hydroxycholesterol and 7-ketocholesterol were reported (Tsai *et al.*, 1980; Maerker & Unruh, 1986; Smith, 1987; Park *et al.*, 1996). A recent study reported that saponification performed at 75 °C for 30 min left only 37% of the 7-ketocholesterol in the sample (Park *et al.*, 1996). Interestingly, it was also reported that the loss from saponification overnight in ambient temperature was negligible. Drawbacks reported for saponification performed at elevated temperature made many researchers to shift towards cold saponification during the 1990's (Table 6) (Tsai *et al.*, 1980; Park & Addis, 1986; Kudo *et al.*, 1989; Pie, Spahis & Seillan, 1990).

Table 6. *Summary of saponification techniques used in the analysis of POP in various food products*

Sample	Saponification procedure	Reference
Enriched spread, fat blends and PS concentrates	Ethanolic KOH (5 ml, 2 M), 18 h in ambient temp. and darkness	Louter *et al.,* 2004
Enriched spreads	Methanolic KOH (1 M), 16 h in darkness	Grandgirard *et al.,* 2004
Rapeseed oil, phytostanol enriched tripalmitin and pure sitostanol	According to Lampi *et al.,* 2002	Soupas *et al.,* 2004
Crude vegetable oils	Ethanolic KOH (5 ml, 2 M), after mixing the sample was saponified overnight	Bortolomeazzi *et al.,* 2003
Rapeseed oil	9 ml ethanol, 0.5 ml sat. KOH in water in N_2 atm., overnight in 25 °C in a shaking water bath	Lampi *et al.,* 2002
Canola-, coconut-, peanut- and soybean oil	According to Dutta *et al.,* 1997	Oehrl *et al.,* 2001
Infant formulas and milk cereals	Cold saponification overnight according to Park *et al.*, 1986	Zunin *et al.,* 1998
Potato chips prepared in different vegetable oils	95% ethanolic KOH (5 ml, 2 M), 18 h in darkness and room temp.	Dutta & Appelqvist, 1997
Vegetable oils and French fries prepared in these oils	95% ethanolic KOH (5 ml, 2 M), 18 h in darkness and room temp.	Dutta, 1997
Soybean oil and wheat flour	Methanolic KOH (20 ml, 1 M), 18 h in room temp.	Nourooz-Zadeh & Appelqvist., 1992
Different lipid medias	Refluxing with ethanolic KOH (30 ml, 10%) for 2 h	Yanishlieva *et al.,* 1986
French fries and potato chips	Refluxing 30-90 min with ethanolic KOH (1.5 M in 95% ethanol)	Lee *et al.,* 1985
Δ^5-avenasterol in trioleylglycerol	Ethanolic KOH (1 M)	Gordon & Magos, 1984

In the analysis of PS and POP in food products the use of both ethanolic and methanolic KOH has been studied in many different samples (Table 6). However, to optimize the reaction performance, the concentration of reagents and the reaction time have to be optimized and evaluated carefully for all matrices individually.

Transesterification

Another, less studied, method to remove non-wanted compounds from the PS and POP is transesterification. Replacement of saponification with transesterification eliminates the time-consuming and complicated clean-up step after the saponification (Biedermann, Konrad & Mariani, 1993). This part of the analytical procedure is recognized as an inaccuracy in the saponification methodology (Schmarr, Gross & Shibamoto, 1996). The corresponding procedure in transesterification is the separation of an organic phase from a water phase, a

separation that is free from materials acting as detergents, making it easier to perform with high accuracy (Biedermann, Konrad & Mariani, 1993). During transesterification, FAME are formed within some minutes, usually by reaction with an alcohol or alkyl-donating reagent (Figure 11) (Schmarr, Gross & Shibamoto, 1996; Duchateau, Janssen & Louter, 2004). However, for esterification of sterol esters, reaction times from 15 min to 2 h have been reported (Glass, 1971; Zubillaga & Maerker, 1988; Biedermann, Konrad & Mariani, 1993; Schmarr, Gross & Shibamoto, 1996).

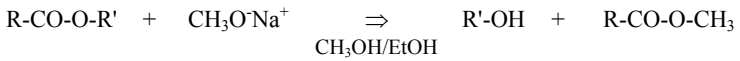

Figure 11. The reaction scheme for transesterification of lipids.

One of the advantages with transesterification is the minimized time that the sample is in contact with strong alkali (Schmarr, Gross & Shibamoto, 1996). After the transesterification, the lipid sample consists mainly of FAME, free sterols, sterol oxidation products and other minor compounds (Guardiola *et al.*, 2004). These can be separated further by chromatographic methods. Compared with the saponification procedure, investigations of transesterification in the sterol area are limited. Only a few studies are reported, mainly on cholesterol or COP (Zubillaga & Maerker, 1988; Biedermann, Konrad & Mariani, 1993; Liu, 1994; Schmarr, Gross & Shibamoto, 1996; Przygonski, Jelen & Wasowicz, 2000). So far, transesterification has not been used in the investigation of PS and POP.

Enrichment

Several chromatographic methods have been used for enrichment of sterol oxidation products (SOP = COP+POP) from crude lipid extract. For COP, column chromatography (CC) using sililic acid (Tsai *et al.*, 1980; Park & Addis, 1985; Bovenkamp, Kosmeijer-Schuil & Katan, 1988) or silica gel coated onto TLC plates (Chicoye, Powrie & Fennema, 1968; Bjorkhem, 1986) have been reported and excellent reviews in this area have recently been published (Dutta, Przybylski & Appelqvist, 1996; Ulberth & Buchgraber, 2002; Guardiola *et al.*, 2004). However, due to high effectiveness, high sensitivity and low cost, the use of SPE columns has increased during recent years. In SPE technique, separation of lipid compounds is generated through differences in the polarity between the lipids and the packing material in the cartridges, with PL being the most polar followed by SOP, SE, TAG. The majority of the published papers in the SPE area report modifications of three different analytical procedures (Ulberth & Buchgraber, 2002).

1. Si-SPE, eluted with n-hexane/diethyl ethers of increasing polarity
2. NH_2-SPE, eluted with n-hexane/ethyl acetate of increasing polarity
3. NH_2-SPE eluted with n-hexane/ethyl acetate of increasing polarity and a second separation with another cartridge.

Guardiola (Guardiola *et al.*, 1995) studied the recovery of sterols with four different elution systems on Si-SPE cartridges. Elution with hexane and diethyl ether was reported to be the most efficient and it was stated that the most efficient combination was when cold saponification was performed prior to SPE enrichment.

Many different methods have been reported in literature for separation of POP from the rest of the lipid matrix (Table 7). In all methods the total amount of POP is determined since hydrolysis of the conjugated PS are performed before SPE chromatography, as in the case of cholesterol conjugates (Addis & Park, 1996; Ulberth & Buchgraber, 2002).

*Table 7. Chromatographic methods used in the purification step prior to the determination of POP. * = POP containing fraction*

Sample	Purification procedure	Reference
Enriched spread, fat blends and PS concentrates	LC-separation on a Nucleosil 50 column (100×4 mm, id:5 µm) with a 30×40 mm guard column	Louter *et al.,* 2004
Enriched spreads	Silica SPE, 500mg Hexane:MTBE (90:10) *Hexane:MTBE (80:20)	Grandgirard *et al.,* 2004
Rapeseed oil, phytostanol enriched tripalmitin and pure sitostanol	According to Lampi *et al.*, 2002	Soupas *et al.,* 2004
Crude vegetable oils	Silica SPE Hexane:diethy ether (1:1) *Diethyl ether:methanol (1:1)	Bortolomeazzi *et al.,* 2003
Rapeseed oil	Silica SPE Hexane:diethyl ether (9:1) Hexane:diethyl ether (1:1) *Acetone	Lampi *et al.,* 2002
Canola-, coconut-, peanut- and soybean oil	According to Dutta *et al.*, 1997	Oehrl *et al.,* 2001
Infant formulas and milk cereals	According to Park *et al.*, 1986	Zunin *et al.,* 1998
Potato chips prepared in different vegetable oils	Two-fold silica SPE (500mg) Hexane:diethyl ether (75:25) Hexane:diethyl ether (60:40) *Acetone	Dutta *et al.,* 1997
Vegetable oils and French fries prepared in these oils	According to Dutta *et al.*, 1997	Dutta, 1997
Soybean oil and wheat flour	TLC developed in diethyl ether:cyclohexane (9:1)	Nourooz-Zadeh *et al.,* 1992
Different lipid medias	TLC developed in hexane: diethyl ether:acetic acid (30:70:0.7)	Yanishlieva *et al.,* 1986
French fries and potato chips	Arrestant column (3×16 cm) Argenated column (2×10 cm)	Lee *et al.,* 1985

In 1997, Dutta and Appelqvist reported a new SPE method for enrichment of POP (Table 7), introducing a double SPE step in the enrichment. The prolonged purification procedure was included in the analysis in order to fully separate the oxidized sterols from the non-wanted compounds. The completeness of the enrichment was investigated visually with HPTLC and it was reported that even if a double SPE step was performed the analysis time required was shorter than for previously reported TLC and HPLC methods (Dutta & Appelqvist, 1997).

Recently, Louter introduced straight-phase LC in the separation of POP from PS. Due to co-elution of POP and PS in GC determination the author changed from Si-SPE to LC for separation. The separation was improved and it was reported that overloading of the LC-column could cause band broadening and poor resolution of POP and PS. The method could be used for analysis of spreads (containing 20-65% water), oils, sterol esters, pure sterols and fat extracts from foods (Louter, 2004).

Determination and quantification

GC is the most frequently used analytical technique in the determination of POP. However, few studies have also reported the use of HPLC in POP analysis. The determination of some stigmasterol hydroperoxides was recently reported using photooxidation and post-column derivatization with diphenyl-1-pyrenylphosphine and fluorescence detector (Säynäjoki *et al.*, 2003). The use of HPLC with a diode array detector for the determination of 7-ketocholesterol and 7-ketositosterol as markers for oxidation of cholesterol and phytosterols in infant milk formulas and infant milk cereals have also been reported (Zunin, Calcagno & Evangelisti, 1998).

Due to numerous numbers of possible POP, high efficient capillary columns in combination with a flame ionization detector (FID) provide a powerful and highly specific analytical system. In order to increase the volatility, thermo stability and generate better peak shapes, the samples are silylated (turned into trimethylsilyl ether derivatives). Many different methods for silylation have been reported in the analysis of PS and POP and the most frequently reported are the use Tri-Sil reagent and BSTFA in TMCA (Lampi *et al.*, 2002; Grandgirard *et al.*, 2004b; Louter, 2004). Silylation of PS was recently reviewed and the use of MSHFBA (N-methyl-N-(trimethylsilyl)-heptafluorobutyrmide), BSTFA/TMCS (N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethyl-chlorosilane), Tri-Sil reagent (HMDS and TMCS) and BSTFA/TBME (N,O-bis(trimethylsilyl)trifluoroacetamide in tert-butylmethyl ether were reported (Lampi, Piironen & Toivo, 2004). The reagents have different reaction times for complete silylation of the products so careful evaluation of the silylation step is vital for every new application. It is also crucial that traces of water are removed prior to silylation, otherwise the water will compete with the sterol for the silyl donors and the silylation of the sample might be incomplete (Guardiola *et al.*, 2004).

For the separation on GC, non-polar and mid polar capillary GC columns are frequently used (Table 8). Generally, non-polar columns are more thermo-stabile compared with mid-polar columns.

Table 8. *Capillary GC columns used in the analysis of PS and POP*

Matrix	Analyte	GC-column (m×mm×μm)	Reference
Gingseng seed oil	PS	DB-5 (60×0.32×0.25)	Beveridge *et al.,* 2000
Rice bran oil	PS	SPB-5 (30×0.25×0.1)	Xu *et al.,* 1999
Veg oils and cereal lipids	PS	BP5 (25×0.33×0.5)	Dutta *et al.,* 1996
Sesame seed	PS	BP5 (19×0.33id)	Kamal Eldin *et al.*, 1992
Enriched spread Fat blends PS concentrates	POP	CP-Sil-8CB (30×0.32×0.12)	Louter *et al.,* 2004
Enriched spreads	POP	DB5-MS & DB1-MS (30×0.25×0.25)	Grandgirard *et al.,* 2004
Crude vegetable oils	POP	SPB5 (30×0.25×0.25)	Bortolomeazzi *et al.,* 2003
Rapeseed oil	POP	RTX-5W (60×0.32×0.25)	Lampi *et al.,* 2002
Canola-, coconut-, peanut- and soybean oil	POP	RTX-50 (30×0.32×0.25)	Oehrl *et al.,* 2001
Potato chips prepared in different vegetable oils	POP	CP-Sil-5-CB (30×0.25×0.25)	Dutta *et al.,* 1997

As a consequence of large number of POP often present in samples, co-elution and bad base-line separation are commonly reported in literature (Dutta, 2002; Lampi *et al.*, 2002; Grandgirard *et al.*, 2004b). This was reported when a mixture of COP and POP was analyzed on a non-polar capillary column. The analytical time on the GC was more than 60 min and co-eluted POP were; unoxidized brassicasterol/7α-hydroxycampesterol, 7α-hydroxystigmasterol/unoxidized campesterol, 7α-hydroxysitosterol/unoxidized stigmasterol, 7β-hydroxystigmasterol/unoxidized sitosterol, 7β-hydroxysitosterol /stigmasterol-5β,6β-epoxide, sitosterol-5β,6β-epoxide/campestanetriol and sitosterol-5α,6α-epoxide/stigmastentriol (Dutta, 2002).

Recently, the separation of several POP on two different capillary GC columns (DB1-MS and DB5-MS) of same dimensions (30m×0.25mm×0.25μm) was reported. Relative retention times and elution patterns for many POP and COP on both the GC columns, along with co-elution and poor base line separation for some oxidation products on both the GC columns, were reported. The authors also observed that minor changes in the temperature program improved the separation of the co-eluted peaks. However, the improvement was followed by co-elution of other products. The authors suggested that by combining data from the two

systems a more efficient separation could be obtained with an additional number of identified peaks (Grandgirard *et al.*, 2004b).

The identification of the POP is often performed with relative retention times (RRT) and it is common to include mass spectrometry (MS) (electron impact) in the identification. Fragmentation pattern and mass spectra have been reported for many POP (Dutta, 1997; Dutta & Appelqvist, 1997; Lampi *et al.*, 2002), and recently reviewed (Dutta, 2002). Aringer & Nordström (1981) published an extensive work including both GC and GC-MS data for 165 different dioxygenated C_{27}, C_{28} and C_{29} steroids. The main interest for the authors was the specificity of the cholesterol metabolizing liver enzyme systems *in vitro*. The report included data on different epimers of dihydroxy oxysterols, including 6β-hydroxy-, 7α- and 7β-hydroxy-, 15-hydroxy, 25-hydroxy-, 26-hydroxy- derivates of C_{28} and C_{29} steroids. Also, 3β-hydroxycholest-5-en-7-one of both C_{28} and C_{29} sterols, both epimers of 6-hydroxycholest-4-en-3-one of C29 steroid, 7α-hydroxycholest-4-en-3-one of both C28 and C29 sterols, and 26-hydroxycholest-4-en-3-one of C_{29} sterol were characterized. The authors identified the 5α-cholestane-3,12-dione of C_{29} sterol, 7α-hydroxy-5β-cholestan-3-one of both C_{28} and C_{29} sterols, as well as 5β-cholestst an-3,12- and 3,15-dione of C_{29} sterol. Both epimers of 5,6-epoxycholestan-3β-ol of both C_{28} and C_{29} sterol were also characterized.

Quantification of POP is often performed with internal standard (IS) technique and 5α-cholestane is frequently used (Dutta & Appelqvist, 1997; Oehrl *et al.*, 2001; Grandgirard *et al.*, 2004b; Louter, 2004). However, 5α-cholestane is less polar than POP and therefore cannot be added prior to purification. This is a drawback, since it is preferable to add the IS as soon as possible in the analytical procedure. Lately, the use of COP and cholestanol as IS has been reported (Lampi *et al.*, 2002; Bortolomeazzi *et al.*, 2003) and also the use of the corresponding multideuterated COP (Dzeletovic *et al.*, 1995). However, the use of such technique is both time consuming and expensive.

Objectives

Nowadays, many consumers want food products that not only have a good taste but also have a positive effect on health. In addition, it is important that the products are safe and quality assured. During recent decades the lipid sources used by consumers have changed radically. From the use of animal fat with high content of cholesterol and saturated lipids, it is nowadays more common to include fats of vegetable origin in the preparation of various food products.

With an increased consumption of vegetable oils, food products prepared in these oils and PS enriched food products, the intake of PS and POP increases steadily. However, so far no standard analytical method for the determination of POP in food products has been published.

The overall aim of this thesis was to study the formation of POP during heat-facilitated oxidation and also to evaluate and optimize an analytical method for determination of POP in food products.

The specific objectives of this thesis were:

- to study the formation of POP during thermal oxidation.
- to separate POP generated through autoxidation using preparative TLC technique. Attention was given to medium polar oxidation products generated both from the ring-structure and from the side-chain structure of the steroid molecule.
- to characterize the separated POP using various analytical techniques.
- to evaluate and optimize an analytical method including transesterification and amino SPE-technique for purification and concentration of POP.
- to investigate and optimize the separation of a mixture of POP on different polarity capillary GC columns, individually and in combination.
- to study the levels and formation of POP in edible vegetable oils during heating at 180 °C for 0-2 h.
- to study the content of POP in a PS ester enriched commercially available spread.

Material and methods

This section gives a short description of the materials and methods used in the present study. Further details of the analytical work are presented in **Papers I-IV**.

In general, the standard mixture of sitosterol and campesterol (60:40) was purchased from Research Plus Inc. (Bayonne, NJ) and the standard of stigmasterol was purchased from Sigma-Aldrich (Stockholm, Sweden). Standard samples of COP were purchased from Steraloids Inc. (Newport, RI). All other solvents and chemicals were, unless otherwise stated in the papers, purchased from VWR AB, Sweden. The capillary GC columns were purchased from J&W Scientific (Folsom, CA). Before GC-analysis the samples were silylated using Tri-Sil reagent (Pierce, Rockford, IL, USA). When extraction of samples was performed a previously described method was used (Hara & Radin, 1978) and the fatty acid composition and total sterol composition were also investigated with previously described methods (Dutta *et al.*, 1994). Samples of olive oil, peanut oil, maize oil and a PS ester enriched spread were purchased at a local supermarket in Uppsala.

Formation and characterization of POP (Papers I and II)

The thermal oxidation of pure samples of stigmasterol and a mixture of sitosterol and campesterol was performed in an air-ventilated oven for 72 h at 120 °C. After the oxidation, the POP were separated using preparative TLC chromatography. The zones that contained POP were collected by scraping the plates and extracted with 3×5 ml chloroform:methanol (2:1, v:v) for further purification. For efficient separation of the POP two different TLC systems were used. The first system contained ether:cyclohexane (9:1, v:v) and the second chloroform:ether:methanol (95:5:2, v:v:v). The second system was refrigerated (5 °C) in order to increase the separation efficiency.

The separated oxidation products were characterized by their TLC mobility and as TMS-ether derivatives using GC (CE Instruments, Milan, Italy) connected to a MS (Finnigan, Manchester, England). The products were separated on a DB5-MS capillary column (30m×0.25mm×0.5µm). The carrier gas was helium (80 kPa) and the injection mode splitless. The injector temperature was 250 °C and the oven was programmed from 60 °C to 280 °C (50 °C/min) and from 280 °C to 300 °C (1 °C/min). The electron energy was 70 eV, the ion source 200 °C and full-scan mass spectra were recorded.

In addition, three of the oxidation products from stigmasterol were characterized on a 400 MHz NMR instrument (Bruker, DRX, Germany). The samples were dissolved in $CDCl_3$ and recorded at 30 °C using TMS as a reference. Analyses performed were ^1H NMR spectroscopy, COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy), HMBC (heteronuclear multiple bond correlation) and HSQCDEPT (heteronuclear single quantum coherence

distortionless enhancement by polarization transfer) using the Bruker standard pulse program.

Method optimization Papers III and IV

The analytical method optimized in the present work included transesterification, SPE-enrichment and GC-separation. The efficiency and accuracy of the optimized method was investigated by spiking unoxidized maize oil with various amounts of the previously separated POP (five spiked samples at each level). The optimized methodology was used for the investigation of POP in vegetable oils and an enriched spread.

The transesterification procedure used in this study was based on a method previously described by Schmarr, Gross and Shibamoto (1996) with some modifications. In brief, 0.5 g of unoxidized maize oil was spiked with a known amount of POP and 5 ml 10% sodium methylate in dry methanol, diluted with MTBE (4:6, v:v), was added. The sample was mixed by vortex for 30 sec and after 20 min the sample was mixed by vortex another 30 sec. After an additional 20 min, 1.5 ml water and 4 ml dichloromethane were added and the sample was mixed by vortex again. The tube was centrifuged at 3000 rpm for 3 minutes and the upper aqueous layer discarded. The remaining organic phase was neutralized with 2 ml 1% citric acid, mixed by vortex, centrifuged at 3000 rpm for 3 min and again the upper layer was discarded. Finally, the organic phase was evaporated under nitrogen, some anhydrous sodium sulphate was added with 5 ml hexane and the sample was centrifuged at 3000 rpm for 3 min prior to SPE-enrichment.

The SPE-enrichment was also based on the method previously described by Schmarr, Gross and Shibamoto (1996). However, a two-fold SPE-enrichment step was optimized and described. In brief, the POP-containing hexane phase from the previous described transesterification was applied onto a pre-solved amino SPE-cartridge. The unwanted esters were eluted with 2×5 ml hexane:MTBE (2:1, v:v) and finally the POP were eluted in 5 ml acetone. The acetone was dried under nitrogen and the sterol products dissolved in 5 ml hexane and the same procedure as previously described was performed with a new SPE-cartridge. Before GC-separation the mixture of POP was derivatized to trimethylsilyl-ethers, as described in **Paper I**.

The GC separation was optimized using the previously characterized POP (29 compounds, Table 2). The investigated systems were a DB5-MS column, a DB35-MS column, having similar dimensions (25m×0.2mm×0.33μm), and a combination of these two columns joined together by a press-fit connector (NTK Kemi, Sweden). The two columns were connected in the order of decreasing polarity, i.e., DB35-MS was connected to the injector side and the DB5-MS column was connected to the detector side.

The GC equipment was a CP-3800 (Varian AB, Stockholm, Sweden) fitted with an on-column injector and Star Chromatography Workstation software (Version 5.52). The detector temperature was set at 320 °C and the injector temperature was programmed at 65 °C for 0.5 min and raised to 290 °C (180 °C/min). The samples were injected by an auto-sampler, CP8400 (Varian AB, Stockholm, Sweden). The initial oven temperature was 65 °C (0.5 min), programmed at a rate of 90 °C/min to 290 °C (10 min) and then with 0.3 °C/min to 305 °C (17 min). The carrier gas (He) was programmed at a constant flow of 0.8 ml/min for both the individual and for the two combined columns.

Determination of POP in food products

Approximately 400 ml of the commercial available edible vegetable oils (olive oil, maize oil and peanut oil) were individually oxidized at 180 ± 5 °C for 0 - 2 h in an 800 ml glass bottle with additional stirring. In addition, the lipids from a PS ester enriched spread were extracted as previously described by Hara and Radin (1978). The collected samples were stored at room temperature in darkness for subsequent analysis.

The fatty acid composition and the sterol composition of the products were determined as described elsewhere (Dutta *et al.*, 1994) and the POP content as described in previous sections. Mean values from duplicate analyses were reported from both the investigations of vegetable oils and the enriched spread. No factors were included in the calculations of the POP levels in the products.

Results and discussion

Formation and characterization of POP (Papers I & II)

In order to increase the formation of POP the unoxidized sample of PS was dissolved in chloroform and a thin film of sterols were generated through evaporation in a rotary evaporator. This procedure increased the contact between air and the PS making the oxidation more efficient. Different durations of the oxidation were investigated and 72 h was reported to generate considerably higher amounts of POP both from the ring structure and the side chain structure. However, even if the PS was oxidized as a thin film the amount of POP was low and only a few mg of each product were separated and purified. Recently, a similar study was published where POP were prepared using thermal oxidation (Grandgirard *et al.*, 2004b). However, in that study a two-fold oxidation was used, which increased the amount of generated POP.

The common POP have previously been separated and characterized in many studies (Aringer & Nordström, 1981; Daly, Finocchiaro & Richardson, 1983; Nourooz-Zadeh & Appelqvist, 1992; Dutta, 1997; Dutta & Appelqvist, 1997; Bortolomeazzi *et al.*, 1999). However, in the present study focus was on products that originated from the side-chain structure and other medium polar oxidation products. The POP were purified and separated with preparative TLC, a technique that is rapid and "easy to use". The mixture of POP was applied as a thin band over the TLC plate using an automatic TLC-applicator (CAMAG Linomat IV, Muttenz, Switzerland). The application of a thin and narrow band is crucial in the separation of numerous POP. Two different TLC systems were used in this study and for satisfactorily separation of the POP both systems were required. The mobility of the POP was compared to the solvent front and calculated as $R_f = R_{substance}/R_{front}$ (Table 9).

Table 9. *TLC mobility on two different solvent systems for medium polar POP separated and characterized in the present thesis. System 1 contained ether:cyclohexane (9:1, v:v) and system 2 was refrigerated and contained chloroform:ether:methanol (95:5:1, v:v:v).* $R_f = R_{substance}/R_{front}$

	TLC (R_f)	
Oxidation product	System 1	System 2
24-hydroxycampesterol	0.32	0.13
25-hydroxycampesterol	0.39	0.17
4-campesten-6α-ol-3-one	0.33	0.18
4-campesten-6β-ol-3-one	0.40	0.23
24-hydroxysitosterol	0.32	0.13
25-hydroxysitosterol	0.39	0.17
4-sitosten-6α-ol-3-one	0.33	0.18
4-sitosten-6β-ol-3-one	0.40	0.23
24-hydroxystigmasterol	0.48	0.16
25-hydroxystigmasterol	0.38	0.13
24-ethyl-5,22-choladien-3β-ol-24-one	0.48	0.21

Tentative identification of the POP was performed by comparison with commercial available COP. The oxidation product from sitosterol and campesterol had similar mobility in the TLC systems described above. In order to separate those two PS a sophisticated RP-HPLC technique can be included prior to the oxidation (Holen, 1985; Grandgirard *et al.*, 2004b). However, this procedure is time-consuming in order to accumulate required amounts of the individual PS.

For the characterization of the separated POP, GC-MS was used. Each oxidation product generates a characteristic full-scan mass spectrum ("fingerprint") and this information is crucial for identification of various oxidation products in samples. In the present study, full-scan mass spectra for the separated medium polar compounds as their TMS-ether derivatives were recorded and some important fragments are listed in Table 10 (**Papers I and II**).

Table 10. *Some characteristic mass fragments for the medium polar POP separated in this thesis. MS spectra for the POP were recorded as their TMS-ether derivatives*

Oxidation product	M^+ (m/z)	Other significant fragments (m/z)
24-hydroxycampesterol	560	470, 455, 380, 345, 159, 215, 129
25-hydroxycampesterol	560	545, 470, 455, 215, 131
4-campesten-6α-ol-3-one	486	471, 430, 359, 127
4-campesten-6β-ol-3-one	486	471, 430, 359, 127
24-hydroxysitosterol	574	559, 484, 469, 445, 394, 229, 129
25-hydroxysitosterol	574	559, 484, 131
4-sitosten-6α-ol-3-one	500	485, 444, 169, 131
4-sitosten-6β-ol-3-one	500	485, 444, 169, 131
24-hydroxystigmasterol	572	557, 529, 439, 349, 157
25-hydroxystigmasterol	572	557, 482, 392, 131
24-ethyl-5,22-choladien-3β-ol-24-one	456	366, 345

The most favorable positions for an oxidative attack in the side-chain of a steroid molecule are the tertiary carbons (C_{24} and C_{25} for PS) (Yanishlieva & Marinova, 1980; Dutta, 1997). These carbons can stabilize the formed radicals, making them extra susceptible to oxidation. Consequently, some of the reported POP in this study were 24-hydroxy and 25-hydroxy products from all three PS investigated. However, the levels of these POP was low after thermal oxidation under the conditions used in this study.

The fragmentation pattern was similar for the generated 25-hydroxysterols from sitosterol, campesterol and stigmasterol. The base peak was identified at m/z 131 and derived from the well-known α-fragmentation of C_{24}-C_{25} (Aringer & Nordström, 1981; Park & Addis, 1992; Pizzoferrato, Nicoli & Lintas, 1993). This fragment has previously been reported as exclusive for 25-hydroxy compounds.

Other important identified fragments from all 25-hydroxysterols were the loss of one methyl group (M^+-15) and the loss of a TMSO group (M^+-90). The loss of two TMSO groups was also reported (M^+-180) but this fragment was low in abundance for all 25-hydroxysterols.

The purified 24-hydroxysterols were characterized for all three investigated PS. The characteristic fragmentation of a dihydroxy steroid molecule (M^+-15, M^+-90, and M^+-180) was recorded for all three products and some other significant fragments from the side-chain structure were also identified (Table 10). The characteristic fragment at m/z 129 is typical of molecules having a Δ^5-steroid structure (Budzikiewicz, 1972).

A third oxidation product that was separated and characterized from stigmasterol was 24-ethyl-5,22-choladien-3β-ol-24-one. This oxidation product is probably formed during prolonged oxidation of 24-hydroxystigmasterol. Full-scan mass spectra indicated the molecular ion at m/z 456, the loss of the side-chain at m/z 345, and the loss of a TMSO group at m/z 366 (Table 10).

Medium polar oxidation products separated and characterized from the ring structure of sitosterol and campesterol were 4-campesten-6α-ol-3-one, 4-campesten-6β-ol-3-one, 4-sitosten-6α-ol-3-one and 4-sitosten-6β-ol-3-one. These products have been reported as oxidation products from PS, but full-scan mass spectra as their TMS-ether derivatives have not been published previously (Yanishlieva, Schiller & Marinova, 1980; Aringer & Nordström, 1981). The α- and β-isomer had different mobility on the TLC-systems and could be separated (Table 9). The molecular ions for these compounds generated from sitosterol and campesterol were reported at m/z 500 and m/z 486, respectively (Table 10). Other important fragments were the loss of a methyl group (M^+-15) and the loss of a fragment of m/z 56 (M^+-56). These fragments were characteristic for these isomers and derived from the loss of one methyl group and a fragment from ring A (Brooks, 1972; Björkhem, Gustafsson & Sjövall, 1973; Harvey, Middleditch & Vouros, 1973).

In addition to the GC-MS analyses for the oxidation products of stigmasterol, NMR analyses were performed. Various NMR techniques were used for structure characterization and confirmation for the medium polar products. ^{1}H NMR and ^{13}C NMR data in $CDCl_3$ for 24-hydroxystigmasterol, 25-hydroxystigmaterol and 24-ethyl-5,22-choladien-3β-ol-24-one are presented in Table 11.

Table 11. *^{1}H NMR and $^{13}CNMR$ data in $CDCl_3$ for 24-hydroxystigmasterol, 25-hydroxystigmasterol and 24-ethyl-5,22-choladien-3β-ol-24-one. a = Numbering of carbons in the molecule (Figure 1), b = 24-ethyl-5,22-choladien-3β-ol-24-one*

	24-hydroxy		25-hydroxy		24-keto compound[b]	
Pos[a]	^{13}C-shift	^{1}H-shift	^{13}C-shift	^{1}H-shift	^{13}C-shift	^{1}H-shift
3	72.0	3.52 (*m*)	72.0	3.52 (*m*)	71.8	3.52 (*m*)
6	121.7	5.35 (*m*)	121.8	5.35 (*m*)	121.8	5.35 (*m*)
17	56.3	1.22 (*m*)	56.1	1.23 (*m*)	55.4	1.25 (*m*)
18	12.5	0.71 (*s*)	12.4	0.71 (*s*)	12.4	0.72 (*s*)
19	19.6	1.01 (*s*)	19.7	1.01 (*s*)	19.7	1.01 (*s*)
20	40.6	2.14 (*overl.)*	40.9	2.12 (*m*)	40.4	2.28 (*m*)
21	21.5	1.05 (*d*)	21.5	1.06 (*d*)	19.6	1.10 (*d*)
22	136.0	5.41 (*dd*)	142.3	5.36 (*dd*)	152.6	6.68 (*dd*)
23	131.3	5.29 (*d*)	127.6	5.07 (*dd*)	127.9	6.01 (*d*)
24	77.0	-	57.7	1.76 (*m*)	201.4	-
25	36.6	1.70 (*m*)	72.3	-	33.6	2.55 (*q*)
26	17.2	0.88 (*d*)	27.1	1.11 (*s*)	8.6	1.09 (*t*)
27	20.0	0.85 (*d*)	27.3	1.17 (*s*)		
28	31.7	1.54 (*overl.*)	27.7	1.60/1.10 (*m*)		
29	8.0	0.85 (*t*)	13.0	0.84 (*t*)		

In general, all three oxidation products gave similar chemical shifts (^{1}H and ^{13}C) for C_3, C_6, C_{17}, C_{18} and C_{19}, indicating that the ring-structure was intact and that the performed autoxidation only had affected the side chain. NMR-analysis also concluded that the double bond in the side chain was positioned between C_{22}-C_{23} and was of trans conformation ($J_{H22,H23}$ 15 Hz) (Table 11).

In the ^{1}H-NMR spectrum of 25-hydroxystigmasterol two singlets corresponding to methyl groups were identified (C_{26}-C_{27}). For unoxidized stigmasterol these singlets would be doublets since C_{25} would bear an extra hydrogen atom. However, the two singlets had a slightly downfield shift, indicating that C_{25} was substituted with a hydroxy group. In HMBC both methyl groups coupled to C_{25} at 72.3 ppm (typically hydroxy substituted). COSY and TOCSY, with different delay times, gave the complete spin-system system (H_{20}-H_{24}→H_{28}-H_{29}) (Table 11).

In the ^{1}H-NMR spectrum of 24-hydroxystigmasterol, two olefinic protons (5.29 (*d*) and 5.41 ppm (*dd*)) that coupled to each other were identified (C22-C23). The doublet at 5.29 ppm (C23) indicated a quaternary carbon adjacent to C23. COSY and TOCSY gave the intact spin system (H_{20}-H_{24}), and in HMBC cross peaks from methyl protons at 0.88 and 0.85 ppm to a carbon atom at 77.0 ppm also provided strong evidence of a hydroxylated C_{24} (Table 11).

In the ^{1}H-NMR spectrum for 3β-hydroxy-5,22-dien-24-ethyl-24-cholanone two methyl signals were missing. A pair of olefinic protons was identified more downfield, i.e., 6.68 ppm (*dd*) and 6.01 ppm (*d*), indicating a carbonyl function close to the olefinic protons (Table 11). COSY and TOCSY analyses indicated the

intact spin system (H_{20}-H_{24}) and it was concluded that C_{25}-C_{27} had been removed from this molecule. In HMBC, cross peaks from H_{22}, H_{23} and H_{25}, H_{26} indicated that the ketofunction (201.4 ppm) was in C_{24} position.

Method optimization Papers III and IV

Transesterification was mainly chosen for optimization due to the shorter analysis time compared with the more commonly used saponification technique. The transesterification methodology has previously been described in the analysis of COP in sausages and PS in edible vegetable oils and fats (Biedermann, Konrad & Mariani, 1993; Schmarr, Gross & Shibamoto, 1996). However, it has so far not been used for the determination of POP. In the present study, the volume of organic solvent per sample was optimized so the analyses were accurately performed in a 15 ml glass tube. In addition, the transesterification time was reduced to 40 min at room temperature (from 60 min; Schmarr, Gross & Shibamoto, 1996) and the completeness of the transesterification was visually checked with TLC. In order to improve the efficiency of the reaction, mixing was performed 3 times during the reaction time (0 min, 20 min and 40 min). Prior SPE-enrichment the addition of a small amount of anhydrous Na_2SO_4 effectively removed small amount of remaining moisture **(Paper IV)**.

For the elution of FAME from the amino cartridges various proportions of hexane:isopropanol were investigated and visual evaluation by TLC indicated that the most efficient combination was hexane:MTBE (2:1, v:v). The elution was performed using approximately 0.8-1.0 ml/min. This is often used in the SPE-technique and it is important that the elution speed is not too high because losses of POP can occur. Different elution volumes of acetone were investigated and it was observed that 5 ml was suitable in order to quantitatively elute the POP. TLC-analysis of the acetone fraction eluted from the first SPE-cartridge indicated remaining traces of FAME. Therefore, a two-fold SPE-step was developed and considered as an efficient technique to separate non-desirable lipids from the POP **(Paper IV)**.

The separation of a mixture of the purified POP using a single capillary column indicated co-eluting peaks and poor base line separation **(Paper III)**. The separation using the non-polar DB5-MS column generated the following co-eluted compounds: unoxidized stigmasterol/7α-hydroxysitosterol, 7β-hydroxy-stigmasterol/7β-hydroxycampesterol/unoxidized sitosterol, campesterol-5β,6β-epoxide/4-campesten-6β-ol-3-one, 4-campesten-6β-ol-3-one/campesterol-5α,6α-epoxide, campesterol-5α,6α-epoxide/stigmasterol-5β,6β-epoxide, stigmasterol-5β,6β-epoxide/7β-hydroxysitosterol, campestanetriol/sitosterol-5β,6β-epoxide, sitosterol-5β,6β-epoxide/4-campesten-6β-ol-3-one, stigmastentriol /sitosterol-5α,6α-epoxide, 25-hydroxystigmasterol/24-hydroxystigmasterol and sitostanetriol /7-ketocampesterol. In contrast, the more polar column, DB35-MS, generated these co-eluted peaks: unoxidized campesterol/7β-hydroxycampesterol, 7β-hydroxystigmasterol/unoxidized stigmasterol, unoxidized sitosterol/7β-hydroxysitosterol, campestanetriol/stigmasterol-5β,6β-epoxide, stigmasterol-

5β,6β-epoxide/campesterol-5α,6α-epoxide, 4-campesten-6β-ol-3-one/25-hydroxy-stigmasterol/24-hydroxystigmasterol and sitostanetriol/sitosterol-5α,6α-epoxide.

Due to the co-elution of the numerous POP just mentioned, two capillary columns of different polarity were combined with a pressfit connector and the separation efficiency of a mixture of 29 POP was investigated **(Paper III)**. The more polar column, DB35-MS, was connected to the injector side and the less polar, DB5-MS, was connected to the detector side. Compared with analyses performed with the individual columns, improved and adequate base-line separation was observed for many of the POP when the combined columns were used (Figure 12). For identification of the peaks in Figure 12, see Table 12.

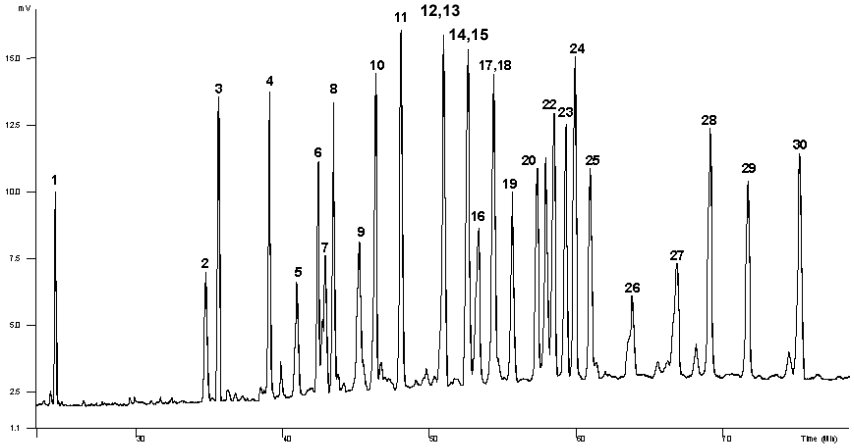

Figure 12. GC-chromatogram of the separation of a mixture of 29 POP on a combination of capillary GC columns of different polarity, DB35-MS and DB5-MS (25m×0.2mm×0.33μm). For peak identification, see Table 12.

In the system with the combined columns 7α-hydroxycampesterol (No. 2) had the shortest retention time (34.7 min) and 7-ketositosterol (No. 30) the longest (75.3 min). Three pairs of POP co-eluted; 24-hydroxysitosterol/campesterol-5β,6β-epoxide (Nos. 12 and 13), stigmasterol-5β,6β-epoxide/campesterol-5α,6α-epoxide (Nos. 14 and 15) and stigmasterol-5α,6α-epoxide/campestanetriol (Nos. 17 and 18). Almost base line separation was reported for 25-hydroxystigmasterol/24-hydroxystigmasterol (R_s=1.4) (Nos. 20 and 21) and 24-hydroxystigmasterol /sitosterol-5α,6α-epoxide (R_s=1.3) (Nos. 21 and 22) (Figure 12).

Table 12. *Identification of the separated POP in Fig 12*

Oxidation product	Peak no.	Oxidation product	Peak no.
5α-cholestane (IS)	1	4-campesten-6β-ol-3-one	16
7α-hydroxycampesterol	2	Stigmasterol-5α-6α-epoxide	17
7α-hydroxystigmasterol	3	Campestanetriol	18
7α-hydroxysitosterol	4	Stigmastenetriol	19
Unoxidized campesterol	5	Sitosterol-5β,6β epoxide	20
Unoxidized stigmasterol	6	25-hydroxystigmasterol	21
7β-hydroxycampesterol	7	24-hydroxystigmasterol	22
7β-hydroxystigmasterol	8	Sitosterol-5α,6α-epoxide	23
24-hydroxycampesterol	9	4-sitosten-6β-ol-3-one	24
Unoxidized sitosterol	10	Sitostanetriol	25
7β-hydroxysitosterol	11	4-campesten-6α-ol-3-one	26
24-hydroxysitosterol	12	7-ketocampesterol	27
Campesterol-5β,6β-epoxide	13	7-ketostigmasterol	28
Stigmasterol-5β,6β-epoxide	14	4-sitosten-6α-ol-3-one	29
Campesterol-5α,6α-epoxide	15	7-ketositosterol	30

This is the first time in the area of POP analysis that a combination of two capillary columns has been investigated. The introduction of the combined columns improved the separation of POP but further optimization of both column material and GC-settings are necessary in order to improve the separation for additional POP. Since optimization of the separation of POP has been an issue in POP analysis for many years the use of capillary columns in a combination can be one step in further development to achieve more efficient analytical methods.

Method evaluation Paper IV

The method optimized in this study was evaluated by using authentic samples of POP separated and characterized as previously described in this section. This is the first time a method for the analysis of POP has been evaluated with oxidation products originated from phytosterols. Due to structural similarities, and the fact that COP is commercially available, the most common choice is to use COP as standard samples for method development.

The recovery for the whole analytical procedure was determined at three different levels (0.5-10 μg) for numerous POP using 5α-cholestane as internal standard. The POP were added before the transesterification and higher quality of the recovery data were reported for oxidation products generated from stigmasterol than for the other investigated POP. At the lowest spiking level (1 μg) the recoveries ranged from 93% to 112% for 7-ketostigmasterol and 24-hydroxystigmasterol, respectively. Similar results were determined at the two higher levels (5-10 μg).

However, for the oxidation products of sitosterol and campesterol a wider range of the recoveries were reported (84-121%). Due to difficulties with the matrix or other analytical problems, recoveries both below and above 100% are commonly reported. It is very important to consider the whole analytical chain in order to identify the source of the problems (IUPAC, 2002). Data reported in the present study were in line with other reports and it is obvious that the determination of POP and COP is a delicate task (Lampi *et al.*, 2002; Ubhayasekera, Verleyen & Dutta, 2004).

The precision of the method was determined as the repeatability (McCluskey & Devery, 1993) with five analyses of each oxidation product, at three different levels. The coefficient of variance (CV) for most of the products used in this study, for all levels investigated, was less than, or just above 15%. This was consistent with previously reported data on COP (Lampi *et al.*, 2002; Ubhayasekera, Verleyen & Dutta, 2004). Another parameter that was evaluated for some POP was the linearity. The linearity was determined over the range 0.5 μg to 10 μg in 0.5 g unoxidized maize oil and the injected amount on the GC was between 1-20 ng. The correlation coefficient (r^2) for all evaluated POP were above 0.95 ($r^2 = 1$ represents a perfect fit to a set of data points). The linearity was of higher quality for the oxidation products generated from stigmasterol. It is worthy to note that extremely pure authentic samples of POP are necessary for studying method validation parameters.

The final parameter evaluated was the detection limits of the method. It was determined using the lowest spiking levels of authentic samples of POP in 0.5 g of unoxidized maize oil (**Paper IV**). However, the detector response of the relevant peaks indicated that levels of POP below the spiking levels could be quantified. It was estimated that the lowest amount of detectable POP was less than 0.1 μg/g oil, using the described method procedure (**Paper IV**). However, it has to be carefully considered that every matrix should be evaluated individually since the background noise is different in all matrixes.

From the evaluated parameters it was concluded that the methodology was accurate and reliable. Good recovery and precision were obtained and the linearity at low concentrations for the investigated POP were of good quality. However, for complete validation and the possibility to use the method in a routine analysis, additional parameters should be evaluated. A more precise limit of detection should be calculated and the robustness of the technique investigated by using extremely pure standards of POP.

Determination of POP in food products Paper IV

The optimized method was used to study the formation of POP during heating of three commercial available edible vegetable oils of different fatty acid (FA) profile (Table 13). The three oils investigated were maize oil, olive oil and peanut oil.

Table 13. *Fatty acid profile (%) and sterol composition (mg/100 g oil) of the edible vegetable oils included in the study. The FA that were below 1% for all oils were excluded in the table. Reported values are mean of duplicates*

	Fatty acid composition (%)								Sterol composition (mg/100 g)		
Vegetable oil	16:0	18:0	18:1	18:2	18:3	20:0	22:0	24:0	Campe	Stigma	Sito
Maize oil	11.1	1.9	29.9	54.7	0.8	0.4	0.2	0.2	42	37	179
Olive oil	11.3	3.2	76.8	5.9	0.7	0.4	0.1	0.0	18	6	78
Peanut oil	11.5	3.3	43.9	34.3	0.2	1.4	2.7	1.3	36	31	143

Maize oil was the oil with highest amount of unoxidized PS (258 mg/100 g oil) followed by peanut (210 mg/100 g oil) and olive oil (103 mg/100 g oil) (Table 13). The dominating phytosterol in all oils were sitosterol. During oxidation of the vegetable oils (heating at 180 °C for 0-2 h), major POP formed were epimeric 7-hydroxysterols, epoxides, triols and 7-ketosterols. The formation of POP during 1 h of heat-facilitated oxidation of maize oil is illustrated in Figure 13.

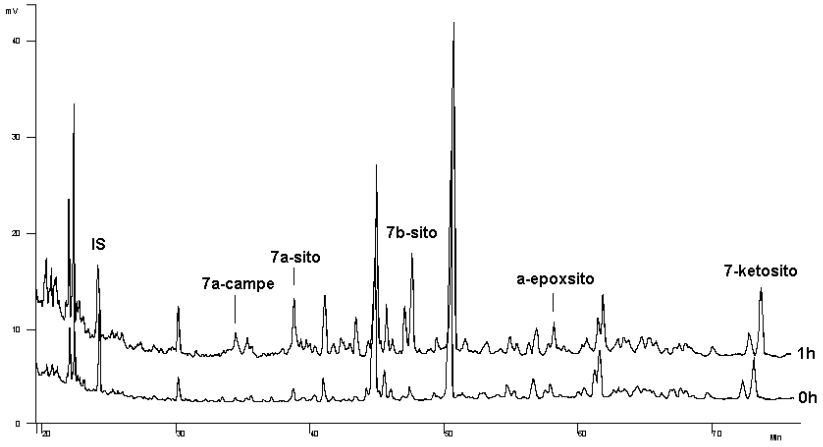

Figure 13. GC-chromatograms of POP from unoxidized maize oil (lower chromatogram) and maize oil oxidized at 180 °C during 1 h (upper chromatogram). Increased formation during heating is illustrated for some common POP. (a = α, b = β).

During heating of maize oil the total level of POP increased from 4.3 μg/g oil to 12.2 μg/g oil. The same trend was reported from olive oil (from 7.7 μg/g oil to 17.6 μg/g oil) and this was in line with previously published results (Dutta, 1997). The level of POP in peanut oil was almost unchanged during the heating (7.1 to 6.8 μg/g oil). However, the small decrease in amount of POP might have been

caused by other simultaneous reactions in the oil during heating, such as isomerization and decomposition of the sterols or the presence of compounds with antioxidant properties (Osada *et al.*, 1993). Extended oxidation time could be used in order to identify higher levels of POP and observe clearer trends in the formation of POP during heating.

A sample of a PS ester enriched spread was also included in the study. The dominating FA were palmitic acid (16:0, 14%), oleic acid (18:1, 28%), linoleic acid (18:2, 44%) and linolenic acid (18:3, 6%). The spread contained sitosterol (44%), campesterol (29%) and stigmasterol (27%) and the total amount of determined POP was almost 35 μg/g lipid. Major oxidation products identified were epimeric 7-hydroxysterol, 24-hydroxysterols, epoxides, triols and 7-ketosterols. The investigated spread contained 35% lipids making the POP content in the spread 12.3 μg/g. The identification of POP in a similar spread was recently reported. The authors roughly estimated the POP content in the spread to 68 μg/g (Grandgirard *et al.*, 2004b). The differences in the estimation of POP in a PS ester enriched spread might partly be due to the limited number of analyses performed and also the fact that different analytical methods were used. In addition, the same sample of the food products was not analyzed and variations in POP levels in different spreads may exist.

Due to increased interest from consumers for food products with combinations of good taste and a positive health effect, the PS enriched products have to be more deeply investigated. The two studies just mentioned are, as far as we know, the only investigations in this area that have focused on the oxidation products in the spreads and there is still much information needed in order to report a more detailed characterization of the products.

Conclusions

One of the main aims of the present study was to investigate the formation of medium polar POP during thermal oxidation. Oxidation products generated from the ring-structure have previously been observed in many studies and recently reviewed (Dutta, 2002). However, data from oxidation products generated from the side-chain of the steroid molecules are limited (Yanishlieva, Schiller & Marinova, 1980; Lampi *et al.*, 2002; Dutta, 2004b). In the present work it was demonstrated that oxidation in the side-chain of the steroid molecule occurs at the tertiary carbons, C_{24} and C_{25}. Oxidation products generated from these carbons were separated from stigmasterol, campesterol and sitosterol. For stigmasterol it was also observed that the hydroxy group on C_{24} was further oxidized to the corresponding ketogroup. The separation of the POP was performed on preparative TLC, a simple but efficient technique. The oxidation products were characterized in detail using numerous analytical techniques such as analytical TLC, GC-FID, GC-MS and NMR.

A rearrangement of the double bond from Δ^5 to Δ^4 in the steroid ring-structure during oxidation has previously been reported (Yanishlieva, Schiller & Marinova, 1980). In the present study, four oxidation products generated from this rearrangement were separated from sitosterol and campesterol; 4-campesten-6α-ol-3-one, 4-campesten-6β-ol-3-one, 4-sitosten-6α-ol-3-one and 4-sitosten-6β-ol-3-one. The products were characterized by TLC and GC and full-scan mass spectra, as their TMS-ether derivatives, were recorded by GC-MS.

Another aim of this study was to evaluate and optimize an analytical method for the determination of POP in food products. The method included transesterification followed by enrichment with amino-SPE and quantification of the POP by GC. This procedure has never previously been used in the analysis of POP and was chosen mainly due to the shorter analysis time compared with the more commonly used saponification technique (Dutta & Appelqvist, 1997; Lampi *et al.*, 2002). In order to separate the FAME and unoxidized PS from the POP, a two-fold SPE step was developed and found to be efficient. The optimized method was evaluated with authentic samples of POP. Good recovery, linearity, repeatability and low detection limit were reported.

The separation of POP by GC was improved using a combination of capillary GC-columns with different polarity. This is the first time in this area a combination of capillary columns has been investigated and improved separation for a mixture of the 29 characterized POP was observed. However, due to co-elution of some POP further improvement of GC-parameters, column polarities and column dimensions are necessary.

Further, the formation of POP during heat-facilitated oxidation (180 °C for 0-2 h) of three vegetable oils (maize oil, peanut oil and olive oil) was studied. The levels of POP increased during heating of olive oil and maize oil. However, the levels of POP during heating of peanut oil were almost unchanged. These results were basically in line with previously reported data, where vegetable oils have been oxidized for longer times (Dutta, 1997; Lampi *et al.*, 2002). Numerous POP were also identified in a PS ester enriched spread and the level of POP was calculated to 12 μg/g spread. This level was considerably lower than a recently roughly estimated level of 68 μg/g spread (Grandgirard *et al.*, 2004b). This discrepancy is probably due to the use of different samples and analytical techniques and justifies further studies to obtain a harmonized and standardized method for the analyses of POP.

The per head consumption of potato chips and French fries was recently reported to be around 1 kg/year and 4 kg/year, respectively (Svensson *et al.*, 2003). A daily intake of POP from these food products was estimated to 1-5 μg/day/person using data obtained in the present and previous studies (Dutta, 1997; Dutta & Appelqvist, 1997). However, the intake of POP when consuming the recommended level of PS enriched spread was much higher and estimated to be more than 300 μg/day/person (1700 μg/day/person using data from Grandgirard *et al.*, 2004b).

As mentioned earlier, many studies have reported COP to be mutagenic, cytotoxic or atherogenic (Osada, 2002), while data concerning biological effects of POP are limited (Oehlr Dean & Boyd, 2004). Studies on mutagenic properties on COP have reported that the epoxides and triols are the most active (Osada, 2002) and the present study indicates that corresponding products are also formed from PS. If these oxidation products have the same biological effects as the corresponding COP, it is a matter of health concern.

Hitherto data on POP occurrence in PS enriched spreads, and in food products prepared from heated vegetable oils, suggest exposures of POP in the range of some micrograms up to almost 2000 μg/person and day, see above. These levels are 10-100 times higher compared with the estimated average daily dietary exposure of some already recognized food mutagens, e.g. acrylamide, polyaromatic hydrocarbons, heterocyclic amines and nitrosamines are ~50-100, 1-2, <1 μg/day, respectively (Jägerstad & Skog, 2004). The high exposure of POP, and the fact that POP, like COP, might have other biologically effects, warrant further studies.

Future prospects

As mentioned in the introduction, Beneke (1862) discovered the first phytosterol more than a century ago. In his wildest dreams he could probably never imagine that the compound he found had health promoting properties and would be one of the first compounds to gain declaration as a "functional food" ingredient. However, still many questions concerning phytosterols remain to be answered.

During the years with this project many ideas of future prospects in the area of PS and POP have appeared. This is an attempt to summarize some of these ideas and hopefully inspire other scientists to start working in this area.

- The oxidation of PS deserves more attention. It is crucial to study both primary and secondary oxidation products in order to gain deeper knowledge of the oxidation mechanisms.

- The use of optimized and reliable analytical methods for determination of POP is vital. It is also important that the methodologies are evaluated using pure and individual oxidation products generated from PS.

- A harmonized and, eventually, an official method for the determination of POP needs to be described and accepted worldwide. When this kind of information has been published, a database with POP-levels in food products can be coordinated.

- The possibility to use HPLC in the determination of POP in various food products should be studied. One of the advantages with the use of HPLC would be the exclusion of the time-consuming silylation step.

- The levels of POP in commercial available edible vegetable oils and other food products should be determined. In addition, the possibility to reduce the formation of POP during cooking and storage should be investigated.

- The levels of POP in commercial available food products enriched with PS esters needs to be determined. The number of products launched on the market will increase during coming years and it is urgent to investigate the levels of POP both in fresh and stored products.

- The daily intake of POP for consumers eating food with a "normal" base and for those who use the enriched food products should be determined.

- The metabolism of PS and POP in humans needs to be evaluated in detail especially when the intake of these compounds is increasing.

- Investigations of possible long-term biological effects of individual POP should be performed. Some studies have been done in this area but the results so far have been contradictory and further investigations are required.

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