Sterols and Oxysterols

Occurrence and Analysis in By-product Feed Fats and Animal Tissues

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Cover photograph: Ball and stick representation of the cholesterol molecule in gold showing the electron distribution as a blue wire mesh (Provided by: Wimal Ubhayasekera)

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Sterols and Oxysterols. Occurrence and Analysis in By-product Feed Fats and Animal Tissues

Abstract

The ingredients of animal feed formulation, especially the fatty sources, play a significant role in the production of high-quality food of animal origin. Industrial fat by-products are a major source of feeding fats in Europe. The main objectives of the project were firstly to assess feed fats and oils for their composition and content of sterols (cholesterol and phytosterols) and oxysterols, and secondly to assess the levels of cholesterol and oxycholesterols of chicken and rabbit tissues after feeding with especially formulated feeds containing *trans* fatty acids and oxidized lipids. The lack of standardized analytical procedure prompted the evaluation of an in-house purification method by comparing it with a number of commonly used methods for the analysis of oxysterols. The saponification and transesterification steps showed rather comparable results. A two-dimensional capillary GC column with different stationary phases (a 35% phenyl column coupled to an apolar 5% phenyl column) was used for better resolution of a large number of oxysterols. This new system improved the separation efficiency and reduced the analytical time for a wide range of oxysterols. The satisfactory purification method and the reliable separation of oxysterols facilitated the qualitative and quantitative assessment of sterols and oxysterols in samples of by-products from chemical and physical refining. A large variation in the levels of sterols and oxysterols was observed in the fat by-products from chemical and physical refining processes for edible fats and oils. The observed variations in the contents and composition of sterols and oxysterols were mainly due to the origins, production facility and different processing conditions of the by-product samples. The highly oxidized lipid and *trans* fatty acid feeds significantly increased the contents of cholesterol and oxycholesterols in edible parts, e.g. the muscles and livers of chickens and rabbits ($0.01 < p \leq 0.05$). Hence, the consumption of products from animals fed with higher levels of *trans* fatty acids and oxidized lipid feeds may contribute to higher ingestion of cholesterol and oxycholesterols by humans.

Keywords: Animal tissues, chicken, cholesterol, fat by-products, feed fats, oxycholesterols, oxyphytosterols, phytosterols, rabbit, two-dimensional capillary GC

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Dedication

To My Loving Late Mother & Father,

ප්‍රනාමය

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Contents

List of Publications

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I Ubhayasekera, S.J.K.A., Verleyen, T., Dutta, P.C. (2004). Evaluation of GC and GC-MS methods for the analysis of cholesterol oxidation products. *Food Chem,* 84, 149-157.

II Ubhayasekera, S.J.K.A. and Dutta, P.C. (2009). Resolution of complex mixtures of oxysterols by two-dimensional capillary column gas chromatography. *Analytical Method* (*Submitted*)

III Ubhayasekera, S.J.K.A. and Dutta, P.C. (2009). Sterols and oxidized sterols in feed ingredients obtained from chemical and physical refining processes of fats and oils. *J Am Oil Chem Soc,* 86, 595-604.

IV Ubhayasekera, S.J.K.A., Tres, A., Codony, R., Dutta, P.C. (2009). Effect of feed fat by-products with *trans* fatty acids and heated oil on cholesterol and oxycholesterols in chicken. *J Am Oil Chem Soc* (*Accepted*)

V Ubhayasekera, S.J.K.A., Tres, A., Codony, R., Dutta, P.C. (2009). Effect of dietary fat co-products and by-products high in *trans* fatty acids and oxidized lipids on cholesterol and cholesterol oxides in rabbit meat, liver and plasma. *Food Chem* (*Submitted*)

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The contribution of the author to the papers included in this thesis was as follows:

I Planning of research project with the co-authors, sample preparation, analysis, evaluation of the results and writing of the manuscript

II Planning of research project with the supervisors, sample preparation, analysis, evaluation of the results and writing of the manuscript

III Planning and execution of analytical work, evaluation of the results and writing of the manuscript

IV Planning analytical work performed on the tissue samples from the animal experiments along with the co-authors, analysis, data processing, interpretation of results and writing of the manuscript

V Planning analytical work performed on the tissue samples from the animal experiments along with the co-authors, analysis, data processing, interpretation of results and writing of the manuscript

Abbreviations

AOCHE	Acid oil from chemical refining
AOPHY	Acid oil from physical refining
COPs	Cholesterol oxidation products (oxycholesterols)
DB-1MS	100% dimethyl polysiloxane
DB-5MS	5% phenyl- and 95% dimethyl polysiloxane
DB-17MS	50% phenyl- and 50% dimethyl polysiloxane
DB-35MS	35% phenyl- and 65% dimethyl polysiloxane
FAME	Fatty acid methyl ester
GC	Gas chromatography
GC-FID	Gas chromatography - flam ionization detector
GC-MS	Gas chromatography - mass spectroscopy detector
HDL-	High density lipoprotein
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LDL-	Low density lipoprotein
OXL	Oxidized (heated) lipids (oils)
POPs	Phytosterol oxidation products (Oxyphytosterols)
PUFA	Polyunsaturated fatty acids
RRT	Relative retention time
RT	Retention time
SFA	Saturated fatty acids
SIM	Selective ion monitoring
SOPs	Sterol oxidation products (Oxysterols)
SPE	Solid phase extraction
TFA	*Trans* fatty acids
TLC	Thin layer chromatography
TMS	Trimethylsilyl
UFA	Unsaturated fatty acids

1 Background

The present study is part of a multinational EU research project named "Feeding Fats Safety" (FOOD-CT2004-007020). It is essential to control each step in the food chain to prevent public health risks and guarantee consumer safety. The main objectives of our part of the project were firstly to assess feed fats and oils for their composition and content of sterols (cholesterol and phytosterols) and their oxidation products (oxysterols) and secondly to assess the levels of cholesterol and oxycholesterols of chicken and rabbit tissues after feeding with especially formulated feeds containing *trans* fatty acids and oxidized lipids.

Feeding fats and oils, which originate from either animal or plant sources or both and vary in their fatty acid composition, play an important role in human health. The main constituent of fats and oils is triacylglycerols, whereas sterols and tocopherols are the minor components. Cholesterol is the major animal sterol, while over 200 phytosterols have been identified in the products of phyto origin; the most abundant phytosterols are campesterol, stigmasterol, and sitosterol (Dutta *et al.*, 2006).

Cholesterol is vital for normal physiological functions in animals and is also associated with atherosclerosis, the most common cause of death in the world. Bioactive lipids, especially lipoproteins, cholesterol, oxysterols, etc., are accumulated in the plaque formed in the arteries causing ultimately atherosclerosis, and are positively related to high blood cholesterol levels. The quality of dietary fats and increased oxidative stress *in vivo* also contribute in this process (Staprans *et al.*, 2005). In contrast, phytosterols are known to possess cholesterol-lowering properties in humans at a considerably higher dose compared with the normal daily intake (Hovenkamp *et al.*, 2008).

Cholesterol and phytosterols can be oxidized forming a large number of oxysterols (SOPs) in fats and oils, food lipids and animal tissues. Extensive studies have been conducted on the biological functions of oxycholesterols (COPs), which are considered as a health risk. However, studies on oxyphytosterols (POPs) in fats and oils and in foods are limited (Hovenkamp *et al.*, 2008). At present there is no standard method to analyze COPs, POPs or SOPs. Therefore, it is essential that SOPs should be carefully analyzed using validated and reliable methods.

The first part of the studies presented in this thesis was to develop a suitable purification procedure for COPs. The next part was to synthesize authentic samples for POPs due to the commercial unavailability of these compounds. The subsequent task was to set up an experiment with the two-dimensional capillary column GC technique in order to improve the resolution for a large number of SOPs. The last sets of experiments concerned the assessment of sterols and SOPs in selected by-product feeding fats obtained from edible oil refineries and distributors of feed fats in Europe. The final phase concerned studying the effects of low, medium and high levels of *trans fatty* acids and oxidized lipids in the feeds on the levels of cholesterol and COPs in chicken and rabbit.

2 Feeding Fats

Feed fats provide several essential nutrients, such as polyunsaturated fatty acids, and behave as a solvent for several important nutrients, such as lipid-soluble vitamins. Several lipid oxidation products in feed fats may deposit or form in muscles and other animal tissues, decreasing the quality of animal products and causing health risks to the consumer. Ultimately the lipid composition, stability and nutritional quality of meat depend directly on the composition and quality of the fats added to the feed. In addition, feed fats also may supply minor components such as persistent pollutant, which can bio-accumulate giving undesirable effects on animals and consumers (Nuchi *et al.*, 2009). For this reason, the maximum level of contaminants (dioxins and dioxin-like compounds, 0.1-1.5 ng WHO-TEQ/kg weight/day) in feeds and feedstuffs is regulated by the European Union (EU) (Regulations CE 466/2001). For normal growth of animals certain percentage of fats and oils in feeds, is essential. In addition to fats and oils fat by- and co-products, recycled fats and oils and other fatty materials from the food chain are also used as feed fat ingredients. Only some of the fat by- and co-products are forbidden for use for feed formulation (e.g. recycled cooking oils and oils from exhausted bleaching earth) (Nuchi *et al.*, 2009). The regulatory measures do not cover all these components, partly due to a lack of information. There is confusion in the market about the classification of these fats as feedstock, allowing the use of all kinds of feedstock of unknown or low quality and occasionally contaminated recycled fat materials. This lack of knowledge hinders the improvement and proper use of several by- and co-products from oil refining, in that it obstructs the development of legislation on this particular subject (Nuchi *et al.*, 2009).

Since 1950, the animal feed industry has been using vegetable fats in feed formulation. Acid oil by-products from both chemical and physical refining

processes are an example of such raw materials for feeds. The quantities and the types of acid oils available for feeds are directly related to the usage of refined oils. The compositions of these by-products vary according to the refining method used. Chemically refined fats/oils contain fewer free fatty acids than those prepared by physical refining. Moreover, these oil fractions contain sterols and tocopherols as well (Balazs, 1987). The presence of unwanted compounds in feedstock may result according to the nature, preparation and handling procedures of the sample (Gasperini *et al.*, 2007).

2.1 Chemistry of Fats and Oils

Fats and oils are triacylglycerols (TAGs) consisting of fatty acids esterified to a glycerol molecule and belong to the group of compounds known as lipids. A chemical structure is shown in Figure 2.1. Fats and oils provide essential nutrients like fatty acids and lipid-soluble vitamins for the normal growth and physiological functions of organisms (Williams, 2000).

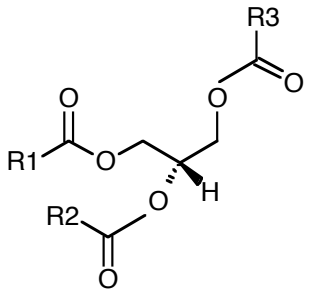


Figure 2.1. Chemical structure of acylglycerol (R1, R2, R3= acyl groups).

Saturated Fatty Acids

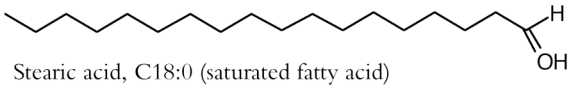
The chemical, physical and biological functions of a TAG are dependent on the fatty acid molecules, which it contains. Fatty acids can be of different chain lengths and those without double bonds are saturated fatty acids (SFA). They are the most common compounds in fats and oils (Figure 2.2), consisting of straight chains and an even number of carbons. SFAs are believed to cause hypercholesterolemia and elevated LDL cholesterol levels are major factors for atherosclerosis. C12:0, C14:0, and C16:0 concomitantly elevate anti-atherogenic HDL-cholesterol (Parodi, 2009).

Unsaturated Fatty Acids

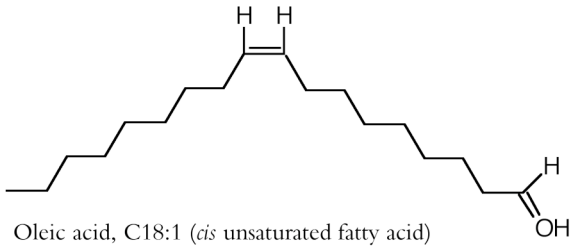
Unsaturated fatty acids (UFAs), which dominate lipids, contain one allyl group (monounsaturated fatty acids; MUFAs), or two or three allyl groups (polyunsaturated fatty acids; PUFAs) in their acyl residues (Figure 2.2) (Wood *et al.*, 2004).

Trans Fatty Acid

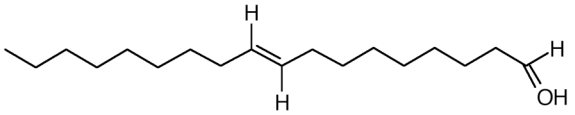
Trans fatty acids (TFAs) are unsaturated fatty acids which have at least one double bond in the *trans* configuration and which form during the hydrogenation of vegetable oils (Figure 2.2). Small amounts of *trans* fatty acids are found in ruminants, otherwise fatty acids are mainly found in plant and animal lipids. The TFA isomers present in industrial sources are C16:1t, C18:2t, C18:3t and C18:1 (elaidic acid), the last of which is the most common (Gebauer *et al.*, 2007).



Stearic acid, C18:0 (saturated fatty acid)



Oleic acid, C18:1 (*cis* unsaturated fatty acid)



Elaidic acid, C18:1 (*trans* unsaturated fatty acid)

Figure 2.2. The chemical structures of some common fatty acids.

2.2 Sterols

Sterols (cholesterol and phytosterols/plant sterols), a large group of naturally occurring compounds having a 1,2-cyclopentanophenthren skeleton, are a minor component of the lipids in plants and animals (Figure 2.3). Sterols are biosynthetically derived from squalene and belong to the group of triterpenes that are important structural compounds of cell and organelle membranes. Sterols regulate membrane fluidity and permeability, as well as membrane-associated metabolic processes in eukaryotic cell membranes (Piironen & Lampi, 2004).

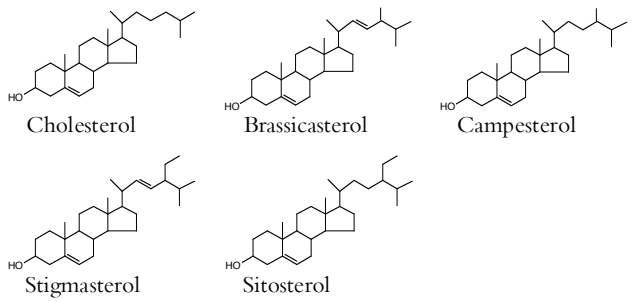


Figure 2.3. The chemical structures of some common sterols.

Cholesterol is a ubiquitous compound of all animal tissues. The analogues of cholesterol in the plant kingdom are named phytosterols (plant sterols). The major differences of phytosterols concern side chain substitution with or without double bonds (Figure 2.3). The principle plant sterols are β-sitosterol, campesterol, and stigmasterol. Brassicasterol is also present in substantial amounts in plants from the plant family *Cruciferae* (Dutta *et al.*, 2006).

Phytosterols 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). 4,4-dimethyl and 4-monomethyl phytosterols are intermediates in the biosynthetic pathways that lead to the end products 4-desmethyl phytosterols. However, these intermediates are commonly found at low levels in most plant tissues. Saturated phytosterols are called phytostanols and occur in trace levels in many plant species, but in high levels in tissues of a few cereal species. Cholestanol is the saturated counterpart of cholesterol in fats and oils of animal origin.

2.3 Refining Processes for Fats and Oils

Crude oil refining is carried out by chemical (Figure 2.4) and physical processes (Figure 2.5), which include degumming, neutralization, bleaching, and deodorization. The amount of sterols is reduced in the neutralization and deodorization/distillation steps; e.g. up to 21% of the sterols are removed from crude oil to produce soap stock in chemical refining (Figure 2.4). The unsaponifiable fraction in soap stock contains a considerable amount of sterols (~70%) (Dutta *et al.*, 2006; Verleyen *et al.*, 2002).

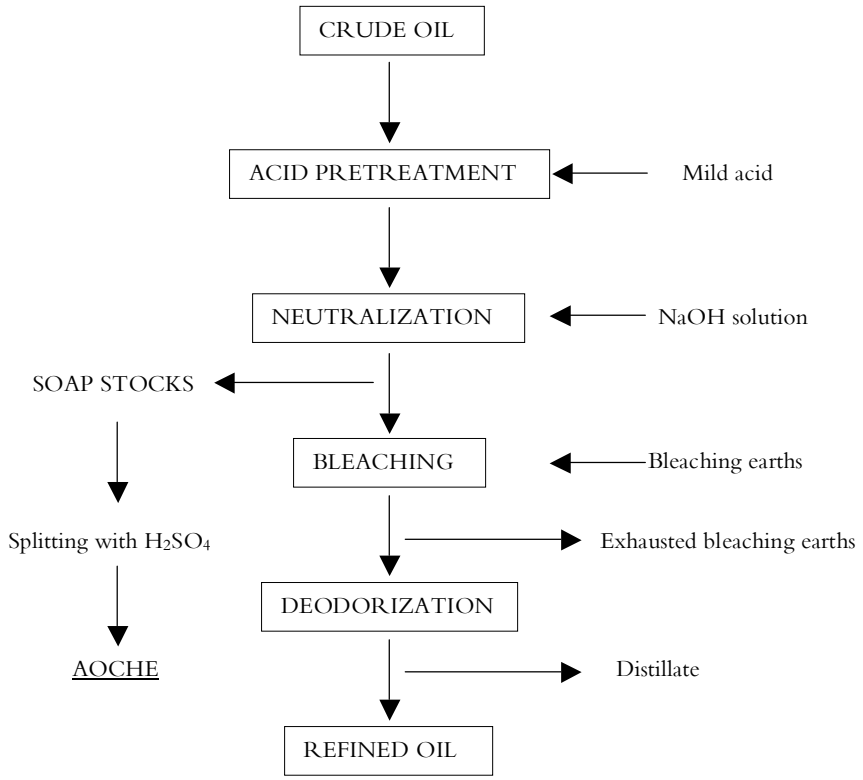


Figure 2.4. Schematic diagram of the steps in the chemical refining process. AOCHE, the acid oil fraction collected after refining as a by-product.

A considerable amount (2-20%) of sterols is present in the distillate fraction resulting from physical refining (Figure 2.5). There are only a few reports on the levels of sterols in the by-product fractions collected from chemical and physical refining processes; e.g. Table 2.1 (Dumont & Narine, 2007; Durant *et al.*, 2006; El-Mallah *et al.*, 2006; Verleyen *et al.*, 2002; Verleyen *et al.*, 2001; Dowd, 1998; Dowd, 1996).

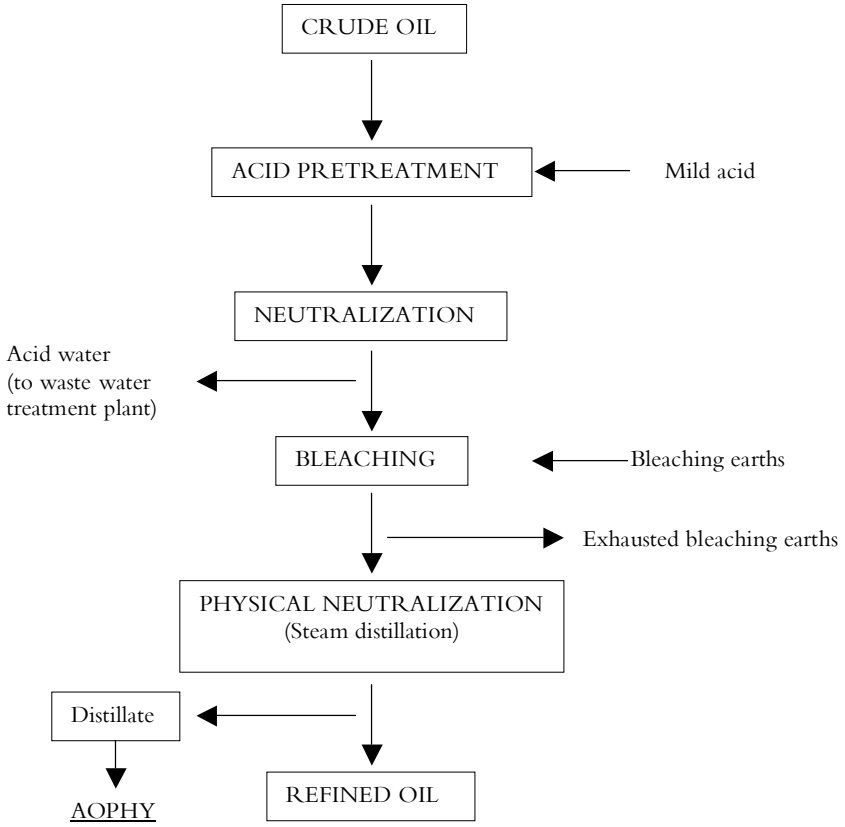


Figure 2.5. Schematic diagram of the steps in the physical refining process; AOPHY, the acid oil fraction collected after refining as a by-product.

Table 2.1. Total sterol content (mg/100g) of soybean oil by-product fractions*

Samples	Chemical refining (soap stock) (AOCHE)	Physical refining (distillate) (AOPHY)
A	590	1360
B	250	2020
C	400	4160
D	890	1020

A, B, C and D are from different factories; *, table adapted from (El-Mallah *et al.*, 2006).

Hydrogenation Process and *Trans* Fatty Acids

Hydrogenation is used to modify fats and oils in order to solidify and change their stability and functionality. Saturated and *trans* fatty acids are

formed from unsaturated fatty acids during the hydrogenation process. In practice the fats and oils are mixed with a suitable catalyst (e.g. nickel), and heated to the desired temperature (140–225°C) under the desired flow and pressure of hydrogen. In addition to saturation and isomerization of fatty acids, the contents of sterols and tocopherols decrease considerably during the hydrogenation process (Dutta *et al.*, 2006).

2.4 Lipid and Sterol Oxidation

The mechanisms of lipid and sterol oxidation follow common pathways, e.g. autoxidation, photoxidation and enzymatic oxidation (Dutta *et al.*, 2006). Autoxidation in food lipids is the dominant oxidation process, initiated by either triplet oxygen or free radicals, which generate a series of autocatalytic free radical reactions (Figure 2.6). The autoxidation reactions lead to a breakdown of the lipids and to the formation of a wide array of oxidation products. Autoxidation is a free radical ($R^{\bullet}$) chain reaction, which takes place at unsaturated lipids (RH) in fats and oils. The oxidation of unsaturated lipids includes three processes: initiation, propagation and termination. The reaction of a peroxy radical with another suitable substrate like unsaturated fatty acids or sterol molecules yields a substrate hydroperoxide and a lipid radical, thus converting the number of lipid radicals in the reaction sequence (Frankel, 2007).

Initiation: $RH \rightarrow R^{\bullet} + H^{\bullet}$

Propagation: $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

$ROO^{\bullet} + RH \rightarrow ROOH + L^{\bullet}$

Termination: $ROO^{\bullet} + H^{\bullet} \rightarrow ROOH$

$R^{\bullet} + H^{\bullet} \rightarrow RH$

Figure 2.6. Lipid autoxidation pathway.

Similar to fatty acids, sterols are susceptible to oxidation when exposed to heat, light, and radiation, forming cholesterol oxidation products (oxycholesterols, COPs) and phytosterol oxidation products (oxyphytosterols, POPs) (Table 2.2).

Table 2.2. Nomenclature and abbreviations of some common sterols and oxysterols

Trivial name	Abbreviation	Systematic name
Cholesterol		Cholest-5-en-3β-ol
7α-hydroxycholesterol	7α-HC	Cholest-5-en-3β,7α-diol
7β-hydroxycholesterol	7β-HC	Cholest-5-en-3β,7β-diol
7-ketocholesterol	7-KC	3β-hydroxycholest-5-en-7-one
α-epoxycholesterol	α-EC	5,6α-epoxy-5α-cholestan-3β-ol
β-epoxycholesterol	β-EC	5,6β-epoxy-5β-cholestan-3β-ol
Cholestanetriol	CT	5α-cholestan-3β,5,6β-triol
25-hydroxycholesterol	25-HC	Cholest-5-en-3β,25-diol
Sitosterol		(24R)-ethylcholest-5-en-3β-ol
7α-hydroxysitosterol	7α-HSito	(24R)-ethylcholest-5-en-3β,5α-diol
7β-hydroxysitosterol	7β-HSito	(24R)-ethylcholest-5-en-3β,5β-diol
7-ketositosterol	7-KSito	(24R)-ethylcholest-5-en-3β-ol-7-one
α-epoxysitosterol	α-ESito	(24R)-5α,6α-epoxy-24-ethylcholestan-3β-ol
β-epoxysitosterol	β-ESito	(24R)-5β,6β-epoxy-24-ethylcholestan-3β-ol
Sitostanetriol	SitoT	(24R)-ethylcholestan-3β,5α,6β-triol
Stigmasterol		(24S)-ethylcholest-5,22-dien-3β-ol
7α-hydroxystigmasterol	7α-HStig	(24S)-ethylcholest-5,22-dien-3β,7α-diol
7β-hydroxystigmasterol	7β-HStig	(24S)-ethylcholest-5,22-dien-3β,7β-diol
7-ketostigmasterol	7-KStig	(24S)-ethylcholest-5,22-dien-3β-ol-7-one
α-epoxystigmasterol	α-EStig	(24S)-5α,6α-epoxy-24-ethylcholest-22-en-3β-ol
β-epoxystigmasterol	β-EStig	(24S)-5β,6β-epoxy-24-ethylcholest-22-en-3β-ol
Stigmastentriol	StigT	(24S)-ethylcholest-22-en-3b,5α,6β-triol
Campesterol		(24R)-methylcholest-5-en-3β-ol
7α-hydroxycampesterol	7α-HCam	24R)-methylcholest-5-en-3β,7α-diol
7β-hydroxycampesterol	7β-HCam	(24R)-methylcholest-5-en-3β,7β-diol
7-ketocampesterol	7-KCam	(24R)-methylcholest-5-en-3β-ol-7-one
α-epoxycampesterol	α-ECam	(24R)-5α,6α-epoxy-24-methylcholestan-3β-ol
β-epoxycampesterol	β-ECam	(24R)-5β,6β-epoxy-24-methylcholestan-3β-ol
Campestanetriol	CamT	(24R)-methylcholestan-3b,5α,6β-triol
Brassicasterol		(24S)-methylcholest-5,22-dien-3β-ol
7α-hydroxybrassicasterol	7α-HB	(24S)-methylcholest-5,22-dien-3β,7α-diol
7β-hydroxybrassicasterol	7β-HB	(24S)-methylcholest-5,22-dien-3β,7β-diol
7-ketobrassicasterol	7-KB	(24S)-methylcholest-5,22-dien-3β-ol-7-one
α-epoxybrassicasterol	α-EB	(24S)-5α,6α-epoxy-24-methylcholest-22 en-3β-ol
β-epoxybrassicasterol	β-EB	(24S)-5β,6β-epoxy-24-methylcholest-22-en-3β- ol
Brassicastanetriol	BT	(24S)-methylcholest-22-en-3β,5α,6β-triol

These compounds are known collectively as sterol oxidation products (oxysterols, SOPs). Oxysterols are a group of compounds which are similar to sterols and which contain an additional functional group in each of them, such as hydroxy, ketone or epoxide in the sterol nucleus and/or on the side chain of the molecule. In addition to autoxidation, SOPs can be formed by photoxidation and by enzymatic pathways where some of the oxysterols are exclusively formed following the latter pathways (Diczfalusy, 2004; Lercker & Rodriguez-Estrada, 2002).

Sterol autoxidation usually starts at C-7 by the abstraction of a hydrogen atom following the addition of an oxygen atom forming primary oxysterols, isomers of 7-hydroperoxysterols (Figure 2.7). These 7-hydroperoxysterols can further convert into 7α-hydroxysterols and 7β-hydroxysterols. In addition, 7-ketosterols can be formed by the dehydration of isomeric 7-hydroxysterols (Figure 2.7). The side chain oxidation occurs at C-20, C-24, C-25, C-26 and C-27 with free radical attacks at these positions resulting in the production of relevant hydroperoxides, which can be further converted into 20α-hydroxysterols, 24-hydroxysterols, 25-hydroxysterols, 26-hydroxysterols, 27-hydroxysterols (Diczfalusy, 2004; Lercker & Rodriguez-Estrada, 2002).

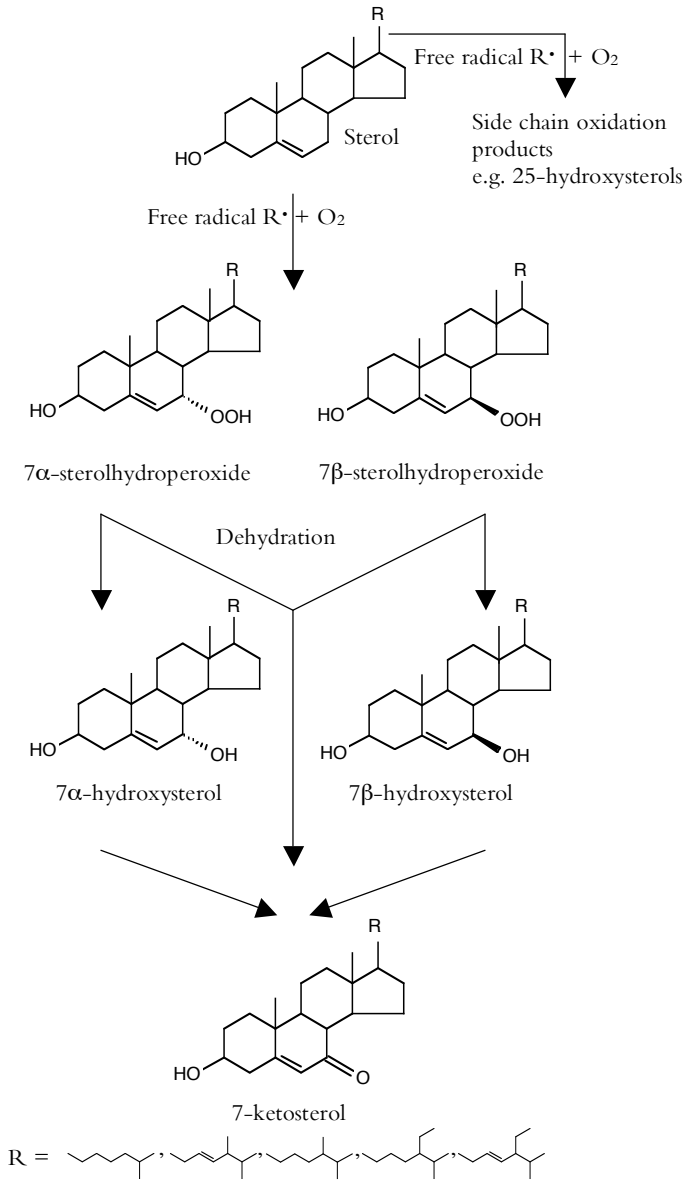


Figure 2. 7. Autoxidation of sterols.

The formation of isomeric epoxysterols occurs due to interaction between sterol molecules and hydroxy radicals (Figure 2.8) and these epoxy compounds can be further hydrolyzed in an acidic medium converting them into steroltriols.

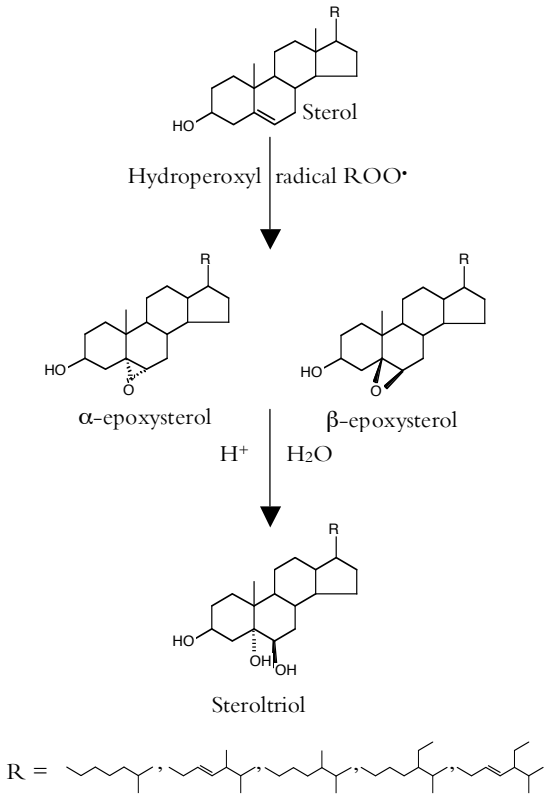


Figure 2.8. Formation of sterol epoxides and steroltriols.

2.5 Cholesterol and Phytosterols in Foods

Cholesterol is the most studied sterol and its content in different food and feed items has been extensively analyzed. The various reports published have shown the amounts of cholesterol, as shown in Table 2.3.

Phytosterols are sterols synthesized only in plants, which mammals obtain *via* dietary sources such as vegetable oils, nuts, seeds and grains. Sitosterol is the main dietary phytosterol (56-79%), while campesterol and stigmasterol contribute approximately 18% and 9% of the total, respectively.

Table 2.3. Cholesterol in some common food

Sample	Cholesterol (mg/100g)	Reference
Egg	400	(Piironen *et al.*, 2002)
Beef- shoulder/chuck	55	
Cattle liver	300	
Pork-chop	47	
Pig liver	60	
Chicken-leg/thigh	84	
Chicken liver	318	(Qureshi *et al.*, 1980)
Rabbit-leg	53	(Zotte, 2002)
Rabbit liver	271	(de Wolf *et al.*, 2003)
Shrimp	142	(Piironen *et al.*, 2002)
Butter	200	
Chicken fat	58	(Hui, 1996)
Lard	95	
Tallow	109	
Fish oil	700	

Most of the vegetable oils contain 100-500 mg/100 g of total phytosterols. The variation of the levels can have many causes, viz. the origin, variety, and growth conditions of the plant, and the oil processing technique and duration. Wheat and corn germ oils contain higher amounts (1700-2600 and 1070 mg/100 g respectively), whereas palm and coconut oils contain lower amounts (approx. 70 mg/100 g) (Piironen & Lampi, 2004). Therefore, over the recent years a number of phytosterols-containing "functional foods" have become available in the market both due to the positive research findings as well as the profit. At present there exist spreadable fats, salad dressings, yoghurts, milk, cheese, plant oils, bakery products, and orange juice fortified with phytosterols (Ryan *et al.*, 2009).

2.6 Occurrence of Oxysterols (SOPs) in Foods

Oxycholesterols

COPs can form in all types of foods and food ingredients that contain cholesterol. The amount of COPs varies according to the food, and whether it is fresh or processed, since the formation of COPs is influenced by several factors (see Section 2.4 Lipid and Sterol Oxidation). COPs have been extensively analyzed in several products, and isomeric 7-hydroxycholesterol and 7-ketocholesterol have been reported to be the most common. The total content of COPs differs from product to product; e.g. milk powder has been reported to contain 0.025 mg/100 g, fresh egg to contain trace amounts, processed egg 22-113 mg/100 g, and raw beef, pork and chicken 1.4×10^{-3}, 0.13×10^{-3} and 0.2×10^{-3} mg/100 g, respectively, while cooked beef, pork and chicken samples have been found to contain 5.96×10^{-3}, 6.79×10^{-3} and 1.46×10^{-3} mg/100 g, respectively (Kerry *et al.*, 2002). The main source of the COPs in meat is heat processing, mainly over-heating. Fresh meat has been reported to contain zero or trace amounts of COPs and cooked meat 18-190 mg/100 g (Paniangvait *et al.*, 1995).

Oxyphytosterols

Various amounts of POPs have been quantified in foods of phyto origin such as French fries, potato chips, wheat flour, functional foods, margarines, and crude and refined vegetable oils, as recently reviewed by Ryan *et al.*, (2009) in Table 2.4.

Table 2.4. Oxyphytosterols (POPs) in some common foods*

Sample	Total POPs (mg/100g lipid)	Major POPs
Crude vegetable oil		
Sunflower	0.3-1	7-hydroxysterols from campe- and stigmasterol and 7-ketositosterol
Corn	0.5-7	
Olive	0.4-6	
Heated oil (180°C), 0-24 hours		
Rapeseed	27-110	7-hydroxysterols, 7-ketosterol, epoxysterols and 25-hydroxysterols from campe- and sitosterol
Heated oils (150°C), 0-1 hour		
Sunflower	5-24	7-hydroxysterols, 7-ketosterol and epoxysterols from sitosterol
Olive	0-4	
Heated oils (200°C), 0-1 hour		
Sunflower	5-82	7-hydroxysterols, 7-ketosterol and epoxysterols from sitosterol
Olive	0-37	
Oils exposed to sunlight, 0–30 days		
Sunflower	1-35	7-hydroxysterols, 7-ketosterol and epoxysterols from sitosterol
Soybean	0.1-33	
Olive	Trace-29	
Rapeseed	0.2-108	
Corn oil refined	4	7β-hydroxyphytosterols and α-epoxyphytosterols
Rapeseed oil refined	10	7β-hydroxyphytosterols and α-epoxyphytosterols
Bakery products	0.2-0.3**	7-ketosterols from campe-, brassica-, stigma- and sitosterol
Infant foods, stored for 0-9 months at 25°C	0.06-0.07**	7-ketosterol, α-epoxysterol and sterol triol from sitosterol
Potato chips fried in oil, stored 0-25weeks		
Palm	0.5-0.9	7-hydroxysterols and 7-ketosterols from campe- and sitosterol
Sunflower	4.6-4.7	
High oleic sunflower	3.5-5.8	
French fries fried (200°C×15 min) in oil		
Rapeseed/palm	3.2	7-hydroxysterols, epoxysterols, steroltriols and 7-ketosterols from campe- and sitosterol
Sunflower	3.7	
High oleic sunflower	5.4	
Phytosterol esters enriched margarine	1.2	7-hydroxysterols, epoxysterols, steroltriols, 7-ketosterols and 24-hydroxysterols from campe-, stigma- and sitosterol

*, adapted from Ryan *et al.*, (2009); **, refers to mg/100g sample

2.7 Determination of Sterols and Oxysterols

The procedure for the determination of sterols and SOPs is similar to that for the determination of cholesterol and COPs (Dutta *et al.*, 2006). The investigation of SOPs is challenging for numerous reasons. Some of the SOPs are epimers having similar molecular weights. The SOPs are present in trace levels (ppm or ppb) and are to be found in complex mixtures in the unsaponifiable fraction of lipids. Therefore, the formation and destruction of some of the SOPs may occur during work-up steps. The analytical procedure has to be designed in such a way that it will guarantee an efficient recovery of sterols and SOPs from the food or from biological samples, and minimize the generation of artefacts during the sample clean-up and work-up steps (Guardiola *et al.*, 2004). Most common analytical protocols used for sterol and SOP analysis follow more or less the same route and an overview is shown in Table 2.5.

Extraction

Sterols and SOPs are lipid-soluble compounds present in minor quantities in food and biological samples in a complex mixture. These compounds are co-extracted with other fat-soluble substances in food and tissue matrices, such as acylglycerols, esterified and free sterols and SOPs, free fatty acids and phospholipids, etc. The extraction of total lipids can be achieved using organic solvents and there are several lipid extraction methods, which minimize losses. Sterols/SOPs need extensive work-up and cleaning procedures before quantification due to their higher polarity compared with triacylglycerols. It is important to choose a solvent system, which is capable of disrupting the associative forces binding the sterols/SOPs and lipids to non-lipid material in food and biological matrices. Different methods for the extraction of sterols and SOPs together with lipids are widely used, e.g.:

(i). Chloroform/methanol (Folch *et al.*, 1957)
(ii). n-hexane/2-propanol (Hara & Radin, 1978)
(iii). Dichloromethane/methanol (Maxwell, 1986)

There are other solvent mixtures that can also be used for the extraction of lipids for the analysis of sterols and SOPs (Guardiola *et al.*, 2004). Accelerated solvent extraction (ASE) is well known for high efficiency in lipid extraction, but there are not many available reports on sterol or oxysterol analysis using this method (Ulberth & Buchgraber, 2002).

Purification and Enrichment

Crude lipid extracts contain 0.1-3% sterols, of which less than 0.1% are SOPs. Therefore, sterols and SOPs need to be purified in order to analyze them with less interference. The most common purification methods are saponification and transesterification (Dutta *et al.*, 2006; Lampi *et al.*, 2004).

Table 2.5. Procedures generally applicable for the analysis of sterols and SOPs in foods and tissues lipids*

Work-up steps	Principle
Extraction	Isolation of lipids from matrices
Saponification	Hydrolysis of acyllipids and sterol esters converting them to water-soluble fatty acid salts and the unsaponifiable fraction in organic solvent. Recovery of the unsaponifiable fraction containing sterols, SOPs and lipid soluble vitamins, etc The reaction is shown below: R-CO-OR´ + KOH/NaOH → R´-OH (sterol or SOPs) + R-CO-OK^+/Na^+
Transesterification	Acylglycerols are converted to FAME and sterol- and oxysterol esters are converted to free sterols and SOPs by the reaction with alkyl-donating reagent. The lipid fraction contains FAME, free sterols and SOPs, lipid soluble vitamins, etc. The reaction is shown below: R-CO-O-R´ + $CH_3O^-Na^+$ → R´-OH + R-CO-O-CH_3 (FAME, sterol or SOPs methyl ester)
Enrichment/ Purification	Isolation of sterols and oxysterols can be accomplished by various methods of preparative chromatography e.g. prep-TLC, prep HPLC, SPE, etc.
Derivatization	Polar hydroxyl groups are converted into corresponding volatile trimethylsilyl ethers as shown in the reaction below $(CH_3)_3$Si-Cl + OH-R (free sterols/SOPs) → $(CH_3)_3$Si-O-R (TMS ether of sterols or SOPs)
Determination	TMS-ether derivatives of sterols and oxysterols are separated, identified and quantified by GC and GC-MS, or by HPLC and HPLC-MS

*Reviewed by Guardiola *et al.*, (2004).

Cold saponification with alcoholic KOH, performed overnight in darkness, and hot saponification at 60-100°C have also been used for the determination of oxysterols in lipids (Table 2.5). Hot saponification leads to artefact formation by degrading unstable SOPs, e.g. 7-ketocholesterol (Park & Addis, 1986). Apart from the disadvantages of possible artefact formation, saponification also has some practical drawbacks. Saponified triacylglycerols form a soap solution giving bad separation of the evolved emulsions, leading to a loss of compounds of interest (Schmarr *et al.*, 1996). Nevertheless,

direct saponification has also shown excellent results for COPs in food samples (Dionisi *et al.*, 1998). Transesterification has become an alternative method to saponification due to the shorter analytical time and less artefact formation of the former (Schmarr *et al.*, 1996).

Generally, enrichment is performed for SOPs, due to the trace amounts of SOPs compared with sterols in various matrices, in order to increase sensitivity and reduce interferences (Guardiola *et al.*, 2004). Several preparative chromatographic methods have been used, e.g. preparative TLC, preparative HPLC, LC, SPE, etc. Among these, SPE is a popular method due to its high effectiveness and low cost. Silica and aminopropyl phases have shown the best efficiency and reproducibility for SOPs (Ulberth & Buchgraber, 2002). Since SPE exploits differences in the polarity of interfering compounds and analytes, adequate separation is achieved by stepwise elution with increasing solvent polarity and thereby enrichment of SOPs can be successfully accomplished (Guardiola *et al.*, 2004). The SOPs-containing fraction is often eluted with acetone or a mixture of acetone and methanol (60:40, v/v). When SOPs/POPs are enriched, 2-fold SPE is necessary to remove completely unoxidized phytosterols (Dutta & Appelqvist, 1997).

Separation and Identification

The separation of SOPs is rather complicated, because one is dealing with a mixture of oxysterols generated from cholesterol, brassicasterol, campesterol, stigmasterol, and sitosterol. Capillary GC is generally the method of choice because of its excellent resolving power with sensitive flame ionization detection in the *nano* gram (ng) range. Coupling the mass spectrometer as a detector for GC-MS adds more value to the analysis (Dutta *et al.*, 2006; Guardiola *et al.*, 2004; Dutta & Appelqvist, 1996). To overcome the thermal degradation and volatilization of SOPs, trimethylsilyl (TMS) ether derivatization has often been employed. For TMS etherification, various reagents used are; *N*, *O*-*bis*-(trimethylsilyl)trifluoro acetamide (BSTFA), trimethylchlorosilane (TMCS) and hexamethyl-disilazane (HMDS) dissolved in pyridine. The TMS ether derivatives are then directly injected into the GC system. It is worth mentioning that the reaction medium must be free from water for a complete derivatization (Guardiola *et al.*, 2004).

Fused silica capillary columns with apolar stationary phases (100% dimethyl polysiloxane or 5% diphenyl- and 95% dimethyl polysiloxane) and

with an inner diameter of 0.20-0.32 mm and a length of 25-60 m have been used in the analysis of COPs and POPs by GC. The elution order of most common COPs and POPs is as follows: 7α-hydroxysterols, unoxidized sterols, 7β-hydroxysterols, β-epoxysterols, α-epoxysterols, steroltriols, 25-hydroxysterols and 7-ketosterols (Grandgirard *et al.*, 2004b; Guardiola *et al.*, 2004; Bortolomeazzi *et al.*, 2003; Dutta, 2002). It is important that SOPs should be separated completely from the unoxidized sterols even when being analyzed by using high-efficient GC capillary columns, because of co-eluting and overlapping of unoxidized sterols and oxysterols (Dutta, 2002). The 2-fold SPE system is quite efficient in removing almost all the unoxidized sterols (Dutta & Appelqvist, 1997) and recently a single step SPE method was developed for POPs (Azadmard-Damirchi & Dutta, 2009). Some recently published methodologies for the analysis of oxysterols are shown in Table 2.6.

Further improvement in separation has been attempted by using two-dimensional GC capillary columns (Johnsson & Dutta, 2005). The columns were a DB-5MS (a non polar column, 5% diphenyl- and 95% dimethyl polysiloxane) and a DB-35MS (a mid-polar column, 35% diphenyl- and 65% dimethyl polysiloxane) with similar dimensions (25 m × 0.2 mm × 0.33 μm). The major drawbacks of this system were a longer analytical time (more than 75 min) and the co-elution of three pairs of POPs. A comparatively enhanced baseline separation was achieved with some co-eluting peaks. They were 24-hydroxysitosterol/β-epoxycampesterol, β-epoxystigmasterol/α-epoxycampesterol, and α-epoxystigmasterol/campestanetriol.

Table 2.6. Some published methodologies for the analysis of SOPs (COPs +POPs) in different matrices

Matrix	Lipid extraction	Purification	Enrichment	Capillary GC columns	Analytes	Reference
Heated vegetable oils		Transesterification (10% MeOH) a with MTBE for 20 min at RT)	2-fold NH_2-SPE (500 mg)	Combine DB-35MS and DB-5MS (25 m×0.2 mm×0.33 μm)	POPs	(Johnsson & Dutta, 2006)
Indian sweets	hexane: *2*-propanol (3:2, v/v)	Cold saponification (RT, overnight, with 1M KOH in EtOH)	Si-SPE (500 mg)	DB-5MS (30 m×0.25 mm×0.5 μm)	COPs	(Ubhayasekera *et al.*, 2006)
Spread enriched with phytosterols	chloroform: MeOH (2:1,v/v)	Cold saponification (RT, 16h, dark) with 1M KOH in MeOH	Si-SPE (500 mg)	DB-5MS (30 m×0.25 mm×0.25 μm) DB-1MS (30 m×0.25 mm×0.25 μm)	SOPs	(Grandgirard *et al.*, 2004b)
Standard mixture of COPs and POPs				DB-5MS (30m×0.25mm×0.25μm) DB-17MS (30m×0.25mm×0.15μm)	SOPs	(Apprich & Ulberth, 2004)
Crude, bleached and deodorized vegetable oil		Cold saponification (RT, 18h, with 2N KOH in EtOH)	Si-SPE (500 mg)	DB-5MS (30 m×0.25 mm×0.25 μm)	POPs	(Bortolomeazzi *et al.*, 2003)
Rapeseed oil		Cold saponification (at 25°C, overnight with saturated KOH)	Si-SPE (500 mg)	DB-5MS (60 m×0.32 mm×0.1 μm)	POPs	(Lampi *et al.*, 2002)
Potato chips	hexane: *2*-propanol (3:2, v/v)	Cold saponification (RT, overnight, with 1M KOH in EtOH)	2-fold Si-SPE (500 mg)	DB-5MS (25 m×0.25 mm×0.25 μm)	POPs	(Dutta & Appelqvist, 1997)
Poultry meat	chloroform: MeOH (2:1,v/v)	Si-SPE (10 g)		DB-5MS (30 m×0.25 mm×0.25 μm)	COPs	(Regueiro & Maraschiello, 1997)

2.8 Why are Sterols and Oxysterols Important?

Despite their similar chemical structures, cholesterol and phytosterols differ distinctly from each other in their biological activities or functions, including their intestinal absorption and metabolic fate. Since humans do not synthesize phytosterols, dietary consumption is the only source of ingestion. The normal diet includes all kinds of sterols from both animal and plant sources, and usually contains similar amounts of cholesterol and phytosterols (200–300 mg/day). However, the body seems to allow cholesterol to enter the body to a great extent (50%), with an almost negligible amount of phytosterols being absorbed (5%). The deposition of cholesterol in arteries and the aorta may result in atherosclerotic plaque from increased cholesterol consumption (Normen & Frohlich, 2004). In contrast, phytosterol intakes in high amounts, e.g. 1.5–2 g/day, display hypocholesterolaemic properties (Jones & AbuMweis, 2009).

Extensive evidence of the deleterious biological effect of various COPs has placed the focus on both exogenous and endogenous COPs. COPs in diet are efficiently absorbed into the blood circulation system and transported by the low-density lipoprotein (LDL) to the liver (Diczfalusy, 2004). Many COPs exhibit atherogenic properties and have an ability to modulate the cholesterol metabolism by influencing several specific enzymes in it, e.g. HMG-CoA reductase. The cytotoxic, mutagenic and probably carcinogenic effects described for some oxycholesterols have been tested *in vitro* (Valenzuela *et al.*, 2003; Osada, 2002; Staprans *et al.*, 1998). The cytotoxic properties of many COPs, for example 7-keto, 7-hydroxy, and 25-hydroxy cholesterol, together with cholesterol epoxides and cholesteroltriol, are widely documented and their formation in membranes occurs during lipid peroxidation. COPs are toxic towards different types of vascular cells, smooth muscle cells, fibroblasts and macrophages (Meynier *et al.*, 2005).

In comparison to the published reports on COPs, POPs have been poorly studied. The dietary intake of POPs from phytosterol-enriched foods is estimated to be 1.7 mg/day or less (Jones & AbuMweis, 2009), whereas approximately 1% of the cholesterol consumed in the Western diet is oxidized, corresponding, for example, to 2–3 mg of total COPs/day (Paniangvait *et al.*, 1995). Similar to phytosterols, only a few percentages of POPs are absorbed by animals (Grandgirard, 2002). However, none of the

reports shows the risk levels of COPs or POPs for human ingestion. To cause cytotoxic effects on the human hepatoma cell line in the same intensity, the amount of POPs needed is higher than that of COPs (Ryan *et al.*, 2005). Hovenkamp *et al.* (2008) have summarized the potential biological effects of POPs, however, more detailed investigations are required to evaluate the biological effects of POPs.

2.9 Effect of Feeding Fats on Cholesterol and Oxycholesterols in Animals

Oxidized Fats and Oils

In thermally abused, heated oils, PUFAs oxidize forming different primary and secondary oxidation products, which accelerate cholesterol oxidation *in vivo* and *in vitro* (Staprans *et al.*, 2005; Garcia-Cruset *et al.*, 2002). Oxidized oil contains fewer amounts of antioxidants than fresh oil. A study on the effect of heated sunflower oil consumption on α-tocopherol and the oxidative stability of chicken tissues has suggested that the chronic ingestion of oxidized lipid may compromise free-radical scavenging activity *in vivo* by depleting α-tocopherol in the gastrointestinal tract, or possibly in plasma and other tissues (Sheehy *et al.*, 1994). Pigs fed with meals prepared using fresh and oxidized dietary fats have been reported to give pork containing 20.5 and 23.5 μg/g COPs, respectively (Monahan *et al.*, 1992).

Hypercholesterolemia has been observed in animals fed with feeds containing an increasing amount of lipids and COPs. A study on hamsters showed that the animals had elevated cholesterol levels when fed with either a normolipidic or a hyperlipidic diet with or without a mixture of COPs (1.4 mg/day) (Meynier *et al.*, 2005). The total cholesterol level in chickens fed with an oxidized poultry fat diet was found to be higher (78 mg/100 g of the sample) than that in chickens fed with a fresh poultry fat diet (52 mg/100 g of the sample) in a study where *in vivo* lipid oxidation also correlated with the oxidized lipid diet (Racanicci *et al.*, 2008).

The production of β-ring and side chain oxycholesterols has been evaluated in chicken meat after feeding experiments with different fat sources as ingredients. Lard, sunflower and olive oil were used as dietary fat in a study where, after six weeks of feeding, raw chicken meat contained 3.7, 53, and 12 μg/100 g total COPs and 70, 56, and 51 mg/100 g of cholesterol respectively (Maraschiello *et al.*, 1998). It has been reported that

the fatty acid composition of meat can be modified by the alteration of the fat composition of the feed. To produce n-3 fatty acid rich meat, linseed oil or fish oil (good n-3 fatty acid sources) is frequently added to feeds. However, when included in feed, linseed oil is reported to increase meat oxidation (Wood *et al.*, 2004). Different dietary fatty acids may influence lipid oxidation *in vivo* and the rate is determined by the amount of antioxidants in the medium. The effect of dietary fat containing α-tocopherol on the concentration of COPs in chicken muscle has been studied. Chickens were fed for 35 days with 3 different feeds using palm, soybean and linseed oils as the dietary fat sources. Three different α-tocopherol levels (20, 40, and 200 mg/kg) were used for each fat source, totally formulating 9 different feeds. The content of COPs in raw muscle was highest and lowest in chickens fed with linseed oil and palm oil, respectively. However, the effect of the dietary fats also depended on the amount of α-tocopherol in the diet (Eder *et al.*, 2005).

Chickens fed with animal fats and vegetable oil have been reported to show variable amounts of cholesterol in fresh meat; e.g. 56 and 36 mg/100 g of the sample, respectively (Bonoli *et al.*, 2007). However, the meat of chickens fed with beef tallow, oxidized sunflower oil and linseed oil shows no significant difference in the amount of cholesterol found in fresh chicken meat (96–100 mg/100 g). There is no evidence of a correlation between the amount of cholesterol and the degree of unsaturation of the oil fed. However, the contents of total COPs in different samples of fresh chicken meat have been found to be different depending on the feeding oil; i.e. 126, 190, 142, and 143 μg/100 g of the sample from beef tallow, sunflower oil, oxidized sunflower oil and linseed oil, respectively (Grau *et al.*, 2001). The substitution of rapeseed oil for lard in feed has been reported to decrease the concentration of cholesterol in chicken meat by 13% (Skrivan *et al.*, 2000).

Studies on the effects of oxidized lipids in rabbits are limited. One of the published studies shows that the polymerization of oil by heating lowers the plasma cholesterol level in rabbit (Hur *et al.*, 2005).

Trans Fatty Acids

It has been well documented that TFAs generate more cholesterol than SFAs when present in the human diet (Morin, 2005). In one study, two groups with 50 males in each were supplied with diets containing TFA and SFA separately for 5 weeks. Blood samples were analyzed, resulting in 3.36 and 3.21 mmol/L LDL-cholesterol and 1.16 and 1.30 mmol/L HDL-

cholesterol in males fed with a TFA and an SFA diet, respectively (Judd *et al.*, 2002). Very little is known about the effects of TFA in feed fats on fat deposition in animals and their subsequent effects on human health in quantitative terms (Faulconnier *et al.*, 2006). Recently, it was shown that female broilers fed with a TFA-containing diet had similar body fat and protein contents and did not differ significantly from the control group, except for the body fatty acid composition. The authors showed that 50% of the ingested TFA was incorporated into the body fat and that it could negatively affect the nutritional value of chicken meat (Javadi *et al.*, 2008).

Studies on the effect of dietary *cis* fatty acids (CFA), TFA and SFA on plasma lipids and the lipoprotein metabolism in rabbits and rats revealed that rabbits fed with a TFA diet possess plasma cholesteryl ester transfer protein (CEPT) activity while rats are devoid of that activity. With the presence of cholesterol (0.2%, w/w) in the above-mentioned feeds, the plasma LDL-cholesterol content was significantly higher in rabbits fed with the TFA diet compared to those fed with the CFA diet. However, the studies on rats showed that the content of total cholesterol in plasma was 20% lower in TFA-fed rats compared to rats fed with the CFA diet. The differences in the effects of dietary TFA in these animal models emphasize the significant role of CEPT activity in determining the distribution of plasma cholesterol in response to dietary *trans* fatty acids (Gatto *et al.*, 2001). It has also been reported that dietary TFAs increase plasma CEPT activity in humans (Mozaffarian *et al.*, 2009).

TFAs influence the fatty acid metabolism of adipocytes, resulting in reduced triglyceride uptake, reduced esterification of newly synthesized cholesterol and increased production of free fatty acids. TFAs appear to affect the lipid metabolism through several pathways and to increase the cellular accumulation and secretion of cholesterol by hepocytes *in vitro* (Combe *et al.*, 2007; Gebauer *et al.*, 2007; Mozaffarian *et al.*, 2006).

3 Objectives of the Present Study

Fats and oils are high-energy feed ingredients and essential for the normal growth of animals. By-products from the food chain are used in feed formulation. The origin and quality of these by-products can vary, and, therefore, it is essential to characterize the composition and quality of and the contaminants in these by-products and their effect as feed fats on the quality and safety of animal food products. Sterols and oxysterols are lipid-soluble compounds that accumulate in bio-systems and can constitute health risks. Therefore, the present thesis has focused on sterols and oxysterols in feed fats and tissues of chickens and rabbits fed with the feed fat by-products. Prior to that, it was necessary to conduct a substantial amount of analytical method control and separation of SOPs by two-dimensional capillary GC. The main objectives have been:

- To evaluate the analysis methods for COPs by comparing it with commonly used enrichment methods.
- To investigate and to optimize the separation of a mixture of SOPs in two-dimensional capillary GC column systems using standard samples of cholesterol, COPs, phytosterols, and authentic samples of POPs.
- To assess the sterols and SOPs in the by-product feed fats obtained from chemical and physical refining processes.
- To investigate the effect of *trans* fatty acids (TFA) and oxidized lipids (OXL) in feeding fats on cholesterol and COPs in rabbit meat, liver and plasma.
- To investigate the effect of *trans* fatty acids (TFA) and oxidized lipids (OXL) in feeding fats on cholesterol and COPs in chicken meat, liver and plasma.

4 Materials and Methods

This section provides a summary of the materials, work plan and methodologies used in the present study. A brief description of the materials is shown below. Details of the materials and analytical procedure are stated in the attached Papers (I-V).

4.1 Materials

Synthesis of Oxyphytosterols (POPs)

Due to the unavailability of the standard samples of POPs for purchasing, some of the common POPs were synthesized in the laboratory. These were 7α- and 7β-hydroxy-, 7-keto-, α-epoxy-, β-epoxy- and triols from a mixture of brassica-, campe-, stigma- and sitosterol. The synthesis of these compounds was performed following published methods (Dutta, 2002; Li *et al.*, 1999). Details on the chemical synthesis, GC analysis, purity and identification by GC-MS, are presented in Paper II.

Feed Fats

By-product samples from chemical refining process (AOCHE, 25 samples) and physical refining process (AOPHY, 16 samples) have been assessed for their composition and content of sterols and their oxidation products. Details are presented in Paper III.

Tissue Samples from Chickens and Rabbits

The feeding trials with chickens and rabbits using three levels of TFA and OXL feeds, were conducted at the Department of Animal Science, Polytechnic University of Valencia, Barcelona, and Veterinary School,

University Autonoma, Barcelona, Spain. For each trial (TFA and OXL), chickens (7 days old, n=96) and rabbits (28 days old, n=144) were randomly distributed into 3 different dietary treatments (low, medium and high) with 8 replicates per treatment where 4 chickens and 6 rabbits constituted per each replicate. Chickens were 47 days old and rabbits were 63 days old when they were slaughtered. The protocols for animal trials and slaughtering conditions were according to EU guidelines. The experimental oils containing *trans* fatty acids (TFA) had 0.14% and 10.01% total TFA at low and high levels, respectively. The oxidized oil (OXL) samples were prepared from a mixture of sunflower oil and olive oil (70:30, v/v). The fresh oil mixture was a low OXL sample and had a *p*-anisidine value 2.74. The high OXL sample was from the same mixture after industrial frying and had a *p*-anisidine value 67.43. The medium level samples were prepared by mixing equal portions of the low and high TFA and OXL samples, respectively. After the feeding trials, the animals were sacrificed. Homogenized samples of meat and liver from chickens and rabbits were vacuum-packed and frozen at -20°C. The plasma samples of the same animals were collected in 4.5 mL plastic tubes and frozen at -20°C. The frozen tissue samples were sent from the Nutrition and Food Science Department, University of Barcelona, Spain by DHL courier air transport in dry ice box. After arrival at the Department of Food Science, SLU, Uppsala, the samples were stored at -20°C until further analysis. Details on the animal experiments are described in Paper IV and V.

It should be mentioned that the high levels of TFA and OXL in the feeding trials were selected based on the maximum values found in the 125 feed fats samples from different categories during the first phase of the EU projects (unpublished). The high TFA and OXL levels do not reflect feed formulations commonly used in Europe. Furthermore, it should be made clear that the feeding trials with chickens and rabbits have no direct link to the work presented in Paper III, other than to show that feed fat ingredients from the food chain can contain variable amounts of sterols and oxysterols.

The analytical procedures described in Paper III, IV and V is shown in Figure 4.1.

4.2 Work Plan

1. Optimization of COPs purification methods (Paper I).
2. Synthesis of oxyphytosterols (n=28) from a mixture of unoxidized brassicasterol, campesterol, stigmasterol, and sitosterol (Paper II-III).
3. Separation of a mixture of sterols and a large number of authentic samples of oxysterols (n=37) in two sets of two-dimensional capillary GC column systems (Paper II-III).
4. Determination of sterols and SOPs in by-product samples of feed fats and oils collected from chemical and physical refining processes (n=82) (Paper III).
5. Optimization of a lipid extraction method for meat and liver samples (Papers IV-V).
6. Optimization of the cold saponification time for determination of COPs in plasma. Determining the most suitable solvent system for COPs enrichment by solid phase extraction (SPE) (Paper IV-V).
7. Assessment of cholesterol and COPs in tissue samples from chickens and rabbits fed with different levels of TFA and OXL (n=144) (Paper IV-V).

4.3 Methods for Analysis of SOPs

The few existing purification methods for SOPs were compared to find out the most suitable one. To calculate the recovery percentage, linearity response and relative response factors, various amounts (1, 5, 10, 15, and 20 μg) of standard samples of COPs were mixed with a fixed amount of 5α-cholestane and 19-hydroxycholesterol (19-HC) as the internal standards. The response factor for each oxycholesterol was calculated using the area ratio versus the weight ratio of each compound by constructing a multilevel calibration curve (Lee *et al.*, 1984). The efficiency and accuracy of the methods were investigated by spiking COPs with different amounts of crude tallow (5,10 and 20 μg) (Beaten, Overmere, Belgium). Three different saponification methods and the transesterification method were compared and the detailed methodology is described in Paper I.

Two different multidimensional capillary GC column systems with different selectivity were used to optimize the separation of the SOPs. The first system consisted of DB-5MS and DB-17MS columns with similar dimensions (10 m × 0.18 mm × 0.18 μm), for the second system a DB-5MS column (15 m × 0.18 mm × 0.18 μm) and a DB-35MS column (10 m × 0.2

mm × 0.33 μm) were coupled. The sample preparation for the SOPs and the GC analysis are described in detail in Paper II.

Method optimization experiments were carried out prior to the extraction of lipids from the experimental tissue samples of meat and liver of chickens and rabbits. This was necessary to identify the minimum sample amount, the optimal volume of the solvent and the number of extractions that would be required for the complete lipid extraction from different tissues. A slightly modified version of the method of lipid extraction developed by Hara and Radin (1978) was used. Then the completeness of the lipid extraction was checked by TLC. Known amounts of pure 7-ketocholesterol were spiked to conduct recovery tests. The recovery percentages for COPs were verified by GC and GC–MS.

Further, a method optimization experiment was conducted to achieve completeness of saponification, the recovery of some common standard samples of COPs throughout the work-up steps and enrichment by SPE, prior to the analysis of the COPs in plasma samples from chickens and rabbits. A plasma sample in triplicate was spiked with known amounts of a standard sample of cholesterol and COPs with 19-HC as an internal standard. Three different saponification durations and two different SPE methods were exploited, as shown in Table 4.1. The method described by Appelqvist *et al.,* (1995), with slight modifications, was tested for the saponification of COPs in plasma. COPs were enriched by saponification using 1 mL of plasma with 5 mL of 10% KOH in 95% EtOH in a hot or cold condition with different time intervals (see Table 4.1 below). The reaction was stopped by the addition of 5 mL of saturated NaCl solution. Then the unsaponifiables were extracted twice with 2 mL of hexane.

Table 4.1. Different saponification methods and time intervals used for optimization to determine the oxycholesterols in plasma of chickens and rabbits

Experiment	1	2	3	4	5	6
Sample (mL)	1	1	1	1	1	1
Saponification time	45 min	2 hrs	18 hrs	45 min	2 hrs	18 hrs
Condition*	Hot (55°C)	Cold	Cold	Hot (55°C)	Cold	Cold
SPE method**	1	1	1	2	2	2

* Cold, at room temperature in the dark; **SPE methods 1 and 2 are described under SPE1 and SPE2, respectively.

The pooled hexane phases were washed with a 5 mL of a 5% NaOH solution, followed by 5 mL of a saturated NaCl solution. The hexane fraction was dried under nitrogen and dissolved in 500 μL of hexane:diethyl ether (75:25). Further enrichment of the COPs fraction was performed by solid phase extraction (SPE). Enrichment of COPs by SPE was regularly checked on a silica gel 60 F_{254} pre-coated high performance thin-layer chromatography (TLC) plate (10 x 10 cm, 0.1 mm thick) in the solvent system diethyl ether:cyclohexane (90:10, v/v). After elution, the TLC plate was sprayed with a reagent containing 1 g each of phosphomolybdic acid and caesium sulphate dissolved in 5.4% sulphuric acid to develop colour. The plate was dried in an air oven at 120°C for 15 minutes. The COPs were determined by GC as their trimethylsilyl (TMS) ether derivatives.

SPE 1

The unsaponifiable fraction was dissolved in 500 μL of hexane:diethyl ether (75:25, v/v) and applied to a silica cartridge (500 mg/6 mL; International Sorbent Technology Ltd., Mid Glamorgan, UK.) pre-equilibrated with 3 mL of hexane. Then the cartridge containing COPs was washed with 3 mL of hexane:diethyl ether (75:25, v/v) and 6 mL of hexane:diethyl ether (60:40, v/v). The COPs were eluted with 4 mL of acetone:methanol (60:40, v/v) solvent mixture. This fraction was dried under nitrogen and derivatized to TMS-ether prior to GC analyses.

SPE 2

The unsaponifiable fraction was dissolved in 500 μL of hexane:diethyl ether (75:25, v/v) and applied to a silica cartridge (500 mg/6 mL; International Sorbent Technology Ltd., Mid Glamorgan, UK.) pre-equilibrated with 3 mL of hexane and 3 mL of hexane:diethyl ether (75:25, v/v). The COPs-containing cartridge was washed with 3 mL of hexane:diethyl ether (75:25, v/v) and 18 mL of hexane:diethyl ether:acetic acid (65:35:0.2, v/v). Finally the COPs were eluted with 3 mL of methanol. This fraction was dried under nitrogen and derivatized to TMS-ether prior to GC analyses.

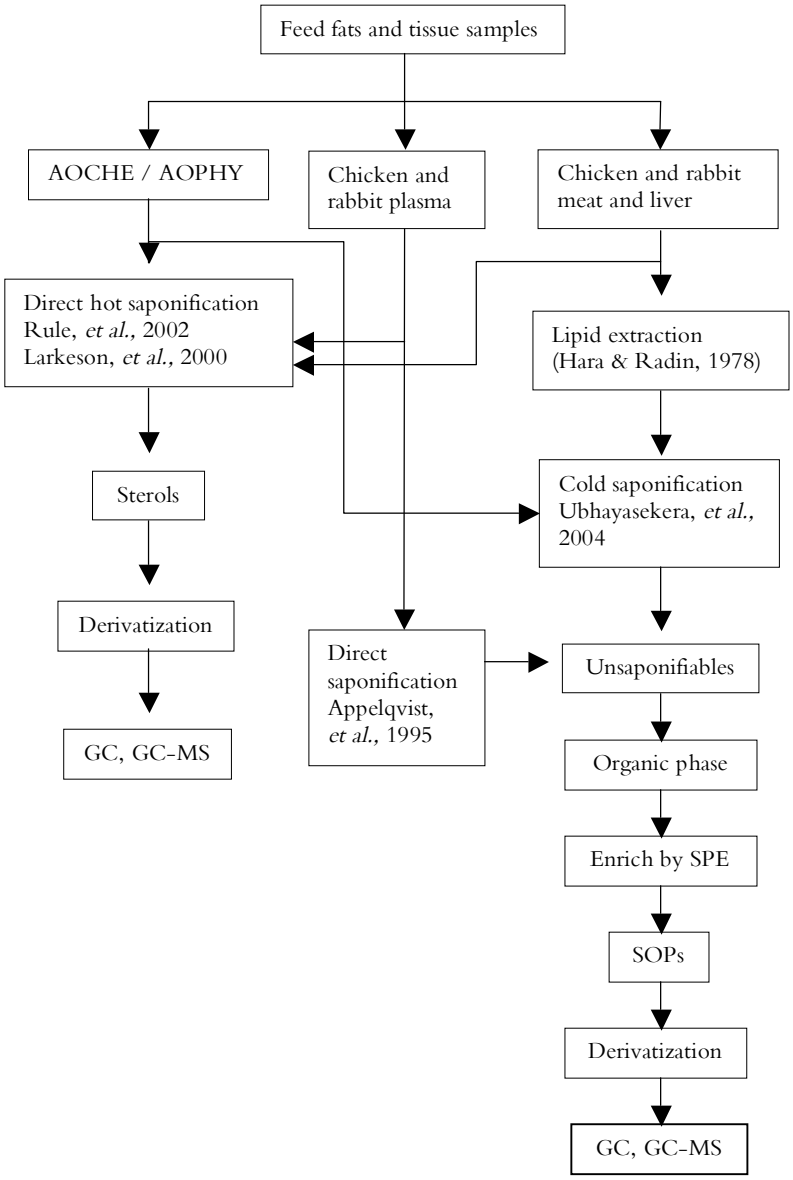


Figure 4.1. Flow chart showing a summary of the analytical procedures for the determination of sterols and SOPs in feed fats (AOCHE and AOPHY) (Paper III) and tissue (meat, liver and plasma) samples from chickens and rabbits (Paper IV and V).

5 Results and Discussion

The predominant oxysterols (SOPs), which were analyzed in this study were C-7 oxygenated sterols, epimeric epoxysterols and steroltriols. These SOPs were quantified and identified in the feed fat by-products of edible fat and oil refining processes and in the tissue samples from chickens and rabbits. The separation, quantification and identification were conducted by GC and GC-MS.

5.1 Paper I

Three different saponification methods and the transesterification method were used for the purification of oxysterols as the first step in the determination of the recovery percentages of COPs. In saponification, the bulk lipids were separated from the unsaponifiables, leaving a small fraction that was less than 1% of the total lipids. On the other hand, in transesterification most of the lipid components were converted into FAME, keeping the total amount of lipids almost constant. The results for the recovery percentages at the spiking levels of 5, 10, and 20 μg for each standard COPs determination by the three different saponification methods and the transesterification method are shown in Figure 5.1. The COPs recovery percentages were higher in cold saponification with 95% EtOH (57-88%) at the 5 μg spiked level compared to the other methods and spiking levels (Figure 5.1-A). In transesterification, the recovery of COPs varied from 16-52% at the 5 μg spiking level.

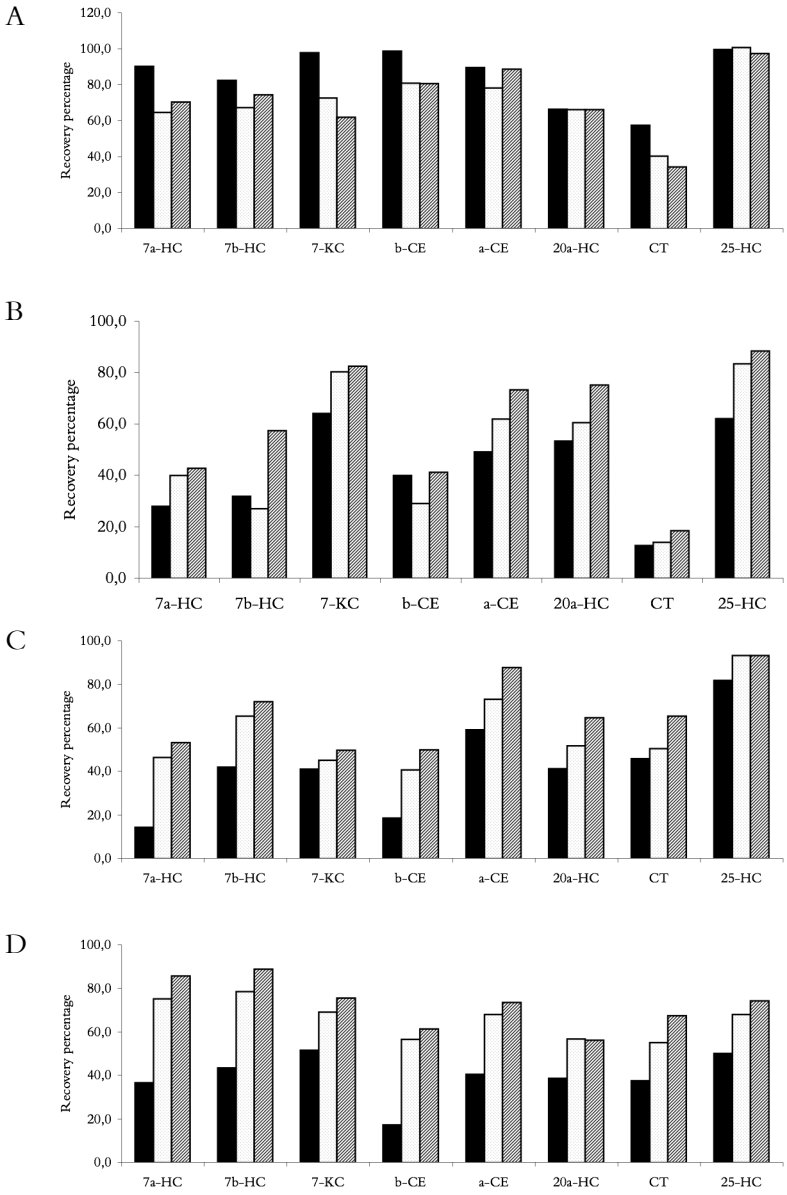


Figure 5.1. Recovery percentages for standard COPs spiked with 5, 10, and 20 μg (black, white, and gray bars, respectively) for four different purification methods; A, cold saponification with 1M KOH in 95% EtOH; B, cold saponification with 1M KOH in 95% MeOH; C, hot saponification with 1M KOH in 95% EtOH; D, transesterification. 7a-HC, 7α-hydroxycholesterol; 7b-HC, 7β- hydroxycholesterol; 7-KC, 7-ketocholesterol; b-CE, β-epoxycholesterol; a-CE, α-epoxycholesterol; CT, cholesteroltriol; 25-HC, 25-hydroxycholesterol.

The cold saponification with 95% EtOH gave the highest COPs recovery only for β-CE (76%) and α-CE (78%). The highest recoveries of 20α-HC (60%) and 7-KC (81%) were obtained after saponification both with EtOH and MeOH, compared to the other methods at the 10 μg spiking level for COPs (Figure 5.1). Moreover, the recovery of 25-HC is the highest in hot saponification with 95% EtOH at the 10 μg spiking level. The highest recovery of 7α-HC (75%) and 7β-HC (78%) was achieved in transesterification at the 10 μg COPs spiking level. Transesterification at the 20 μg spiking level produced the highest recovery for most of the COPs compared to other purification methods (57-89%). The recovery percentages obtained for β-CE (78%), α-CE (88%) and 25-HC (97%) are the highest in cold saponification with 95% EtOH at the 20 μg spiking level for COPs. CT shows the lowest recovery percentages in cold saponification with 95% MeOH: 13, 14, and 18% at the 5, 10, and 20 μg COPs spiking levels, respectively (Figure 5.1). CT is the most polar oxysterol among the other common COPs and can be soluble in the water-MeOH interface. Thus, it caused very low recovery percentages compared with the other COPs. The recovery percentages for 25-HC were similar in both cold and hot saponification with 95% EtOH at all levels of spiking, confirming the lack of artefact formation or further degradation of these compounds during hot saponification. However, most of the other COPs showed low recovery in hot saponification in comparison to cold saponification, at all levels of spiking.

A considerable loss was demonstrated for 7-KC at all spiking levels for COPs. In hot saponification, oxycholesterols may degrade further forming artefacts during heating, especially 7-KC (Park & Addis, 1986). Among the three methods of saponification, cold saponification with 95% EtOH demonstrated the highest recoveries for all the spiking levels. It is worth mentioning that a direct comparison between cold saponification with 95% EtOH and transesterification is not possible owing to the fundamental differences in the principles of the methods. The results demonstrated in cold saponification with 95% EtOH confirm that it is more suitable for purification of trace amounts of COPs than transesterification. This may be due to the bulk amount of lipids together with trace amounts of COPs, making enrichment difficult in the transesterification method (Figure 5.1 A and D).

5.2 Paper II

Phytosterols are structurally similar to cholesterol, but have an additional bond and an alkyl side chain. The analytical methods for POPs or SOPs are based on those developed for COPs. The complicated nature of the complex mixture of SOPs causes poor resolution of the individual oxysterols in different GC columns, as described in Section 2.7. The synthesized oxysterols (7-hydroxy isomers, epoxy isomers, triols and 7-keto sterols from brassica-, campe-, stigma-, and sitosterol) were separated by GC and confirmed by GC-MS. The full scan mass spectra of the synthesized POPs from brassicasterol are shown in Figure 5.2. Similar POPs synthesized from campesterol, stigmasterol and sitosterol were also confirmed by their mass spectra in comparison to the published data (Dutta, 2002).

Paper II describes an improved capillary GC separation for individual SOPs in comparison to the recently published results for a wide range of SOPs (Table 2.2). The idea was to separate a mixture of common oxysterols, i.e. oxycholesterols, oxybrassicasterols, oxycampesterols, oxystigmasterols, and oxysitosterols, together with their unoxidized sterols (more than 35 sterols and SOPs). To increase the separation efficiency for complex mixtures of oxysterols and reduce the GC analytical time, two different multidimensional capillary column systems were exploited: system 1, DB-17MS/DB-5MS (10 m × 0.18 mm × 0.18 μm) and system 2, DB-35MS (10 m × 0.2 mm × 0.33 μm)/DB-5MS (15 m × 0.18 mm × 0.18 μm). The elution order of SOPs in all the trials was 7α-HS, 7β-HS, β-ES, α-ES, ST, 25-HS, and 7-KS (Figure 5.3). Co-elutions or poor baseline separations were observed for α-CE/CT, 7α-HStig/brassicasterol, β-BE/sitosterol, α-BE/BT, StigT/α-SitoE, and SitoT/7-KB in system 1 (Figure 5.3-A). System 2 gave a better resolution with an efficient separation than system 1 (Figure 5.3-B).

A

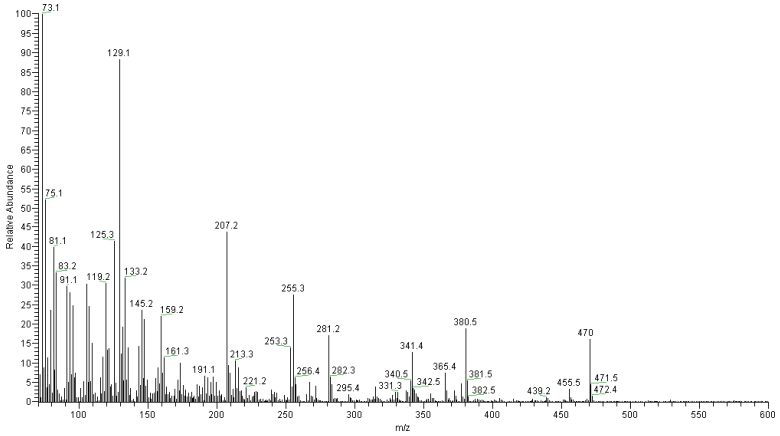


Figure 5.2A. Full scan mass spectrum of trimethylsilyl (TMS) ether derivatives of brassicasterol; m/z=470.

B

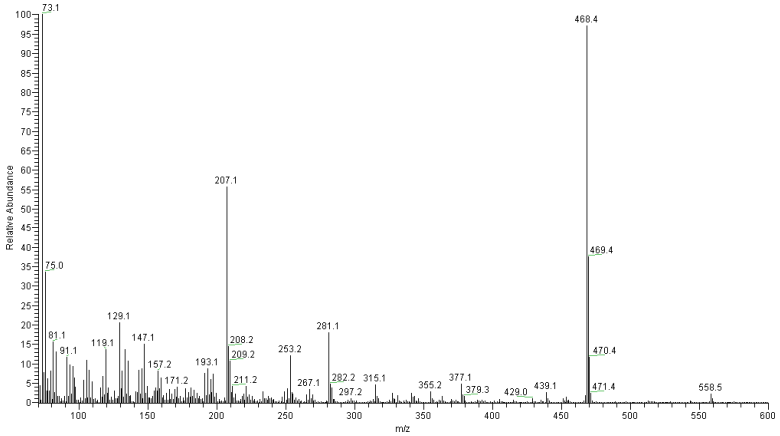


Figure 5.2B. Full scan mass spectrum of TMS ether derivatives of 7α-hydroxybrassicasterol; m/z=558.

C

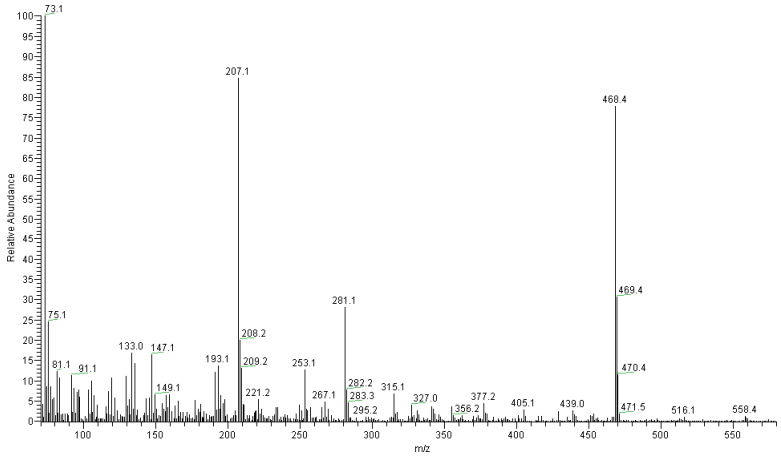


Figure 5.2C. Full scan mass spectrum of TMS ether derivatives of 7β-hydroxybrassicasterol; m/z=558.

D

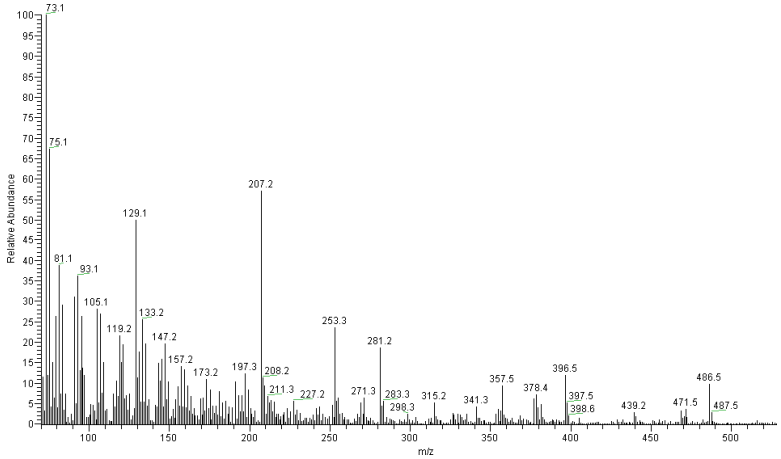


Figure 5.2D. Full scan mass spectrum of TMS ether derivatives of β-brassicasterolepoxide; m/z=486.

E

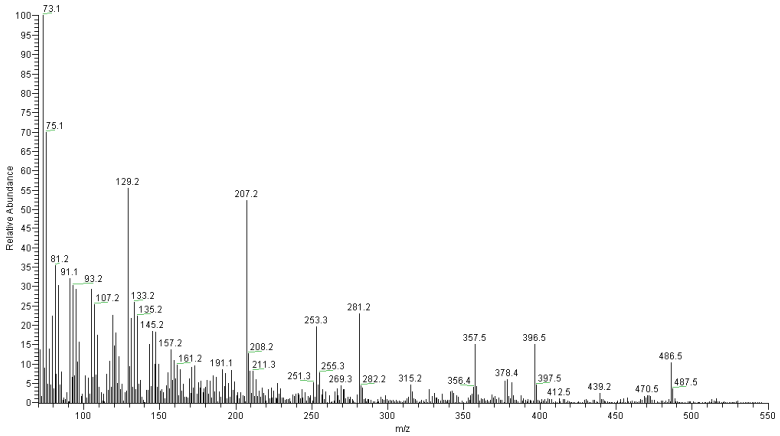


Figure 5.2E. Full scan mass spectrum of TMS ether derivatives of α-brassicasterolepoxide; m/z=486.

F

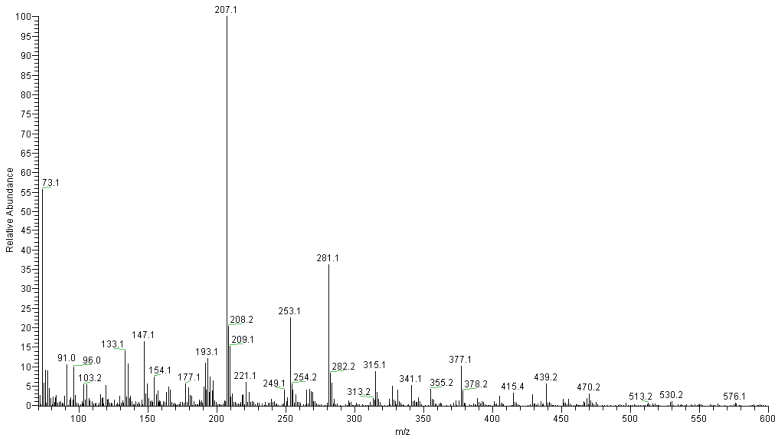


Figure 5.2F. Full scan mass spectrum of TMS ether derivatives of brassicastanetriol; m/z=576.

G

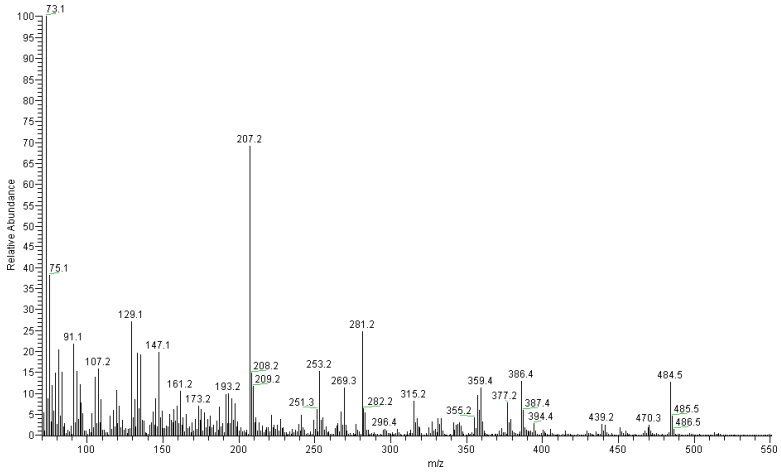
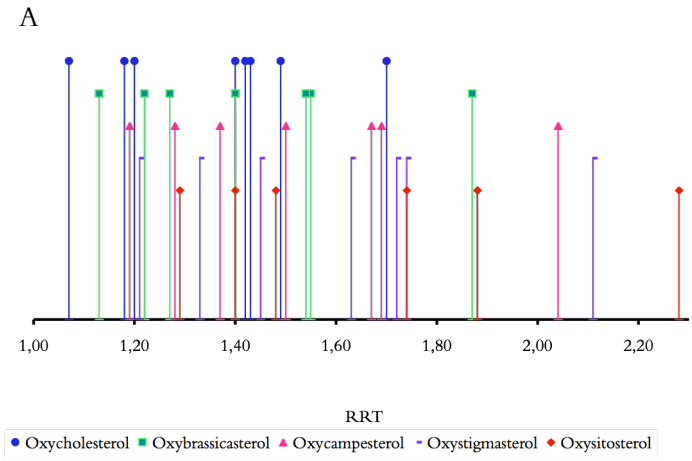


Figure 5.2G. Full scan mass spectrum of TMS ether derivatives of 7-ketobrassicasterol; m/z=484.



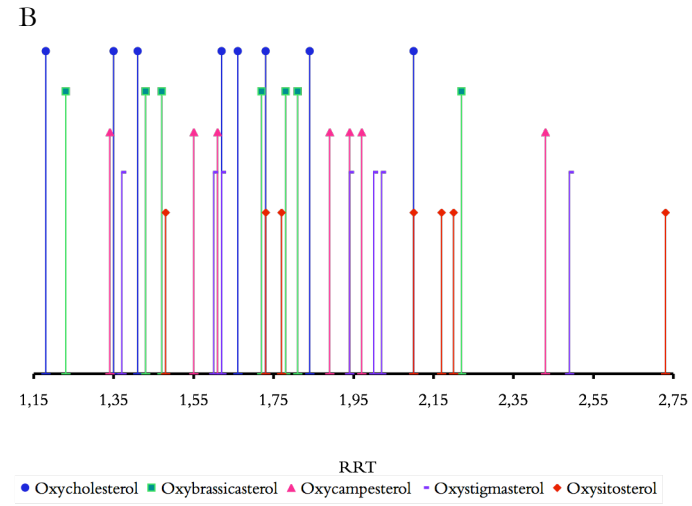


Figure 5.3. A, Schematic diagram of the elution order of sterols and SOPs and the relative retention time (RRT) for cholesterol, brassicasterol, campesterol, stigmasterol and sitosterol in the two-dimensional capillary GC systems (DB-17MS/DB-5MS); B, Schematic diagram of the elution order of sterols and SOPs and the RRT for cholesterol, brassicasterol, campesterol, stigmasterol and sitosterol in the two-dimensional capillary GC system (DB-35MS/DB-5MS). The chromatographic conditions are given in the methodology section. The elution order of oxycholesterols: 7α-HC, cholesterol, 7β-HC, β-CE, α-CE, CT, and 7-KC; oxybrassicasterols: 7α-HB, brassicasterol, 7β-HB, β-BE, α-BE, BT, and 7-KB; oxycampesterols: 7α-HCam, campesterol, 7β-HCam, β-CamE, α-CamE, CamT, and 7-KCam; oxystigmasterols: 7α-HStig, stigmasterol, 7β-HStig, β-StigE, α-StigE, StigT, and 7-KStig; oxysitosterols: 7α-HSito, sitosterol, 7β-HSito, β-SitoE, α-SitoE, SitoT, and 7-KSito.

It is remarkable that the separation of individual mixtures of unoxidized sterols, and oxysterols in GC system 2 (Figure 5.4) confirm the separation of POPs with better resolution, except for α-CamE and β-StigE compared with system 1 (Figure 5.4). The retention times for the individual sterols and SOPs separated by system 2 of the 2-dimensional capillary column are shown in Table 5.1.

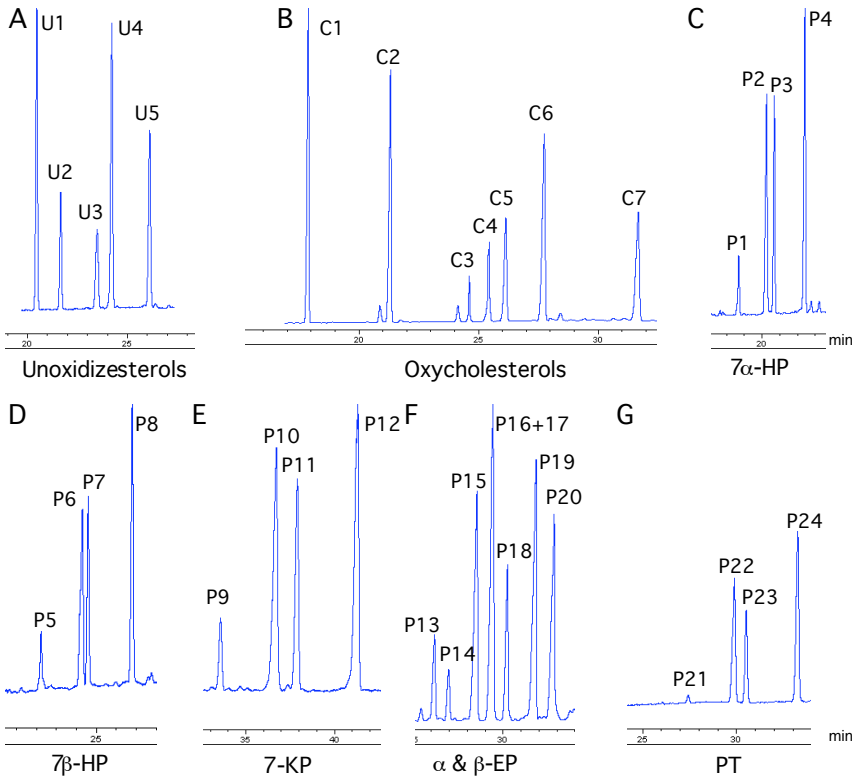


Figure 5.4. GC chromatograms of individual separation of sterols and oxysterols: A, unoxidized sterols (U1=cholesterol, U2=brassicasterol, U3=campesterol, U4=stigmasterol, U5=sitosterol); B, Oxycholesterols (C1=7α-HC, C2=7β-HC, C3=β-CE, C4=α-CE, C5=CT, C6=25-HC, C7=7KC); C, 7α-hydroxyphytosterols (P1=7α-HB, P2=7α-HCam, P3=7α-HStig, P4=7α-HSito); D, 7β-hydroxyphytosterols (P5=7β-HB, P6=7β-HCam, P7=7β-HStig, P8=7β-HSito); E, 7-ketophytosterol (P9=7KB, P10=7KCam, P11=7KStig, P12=7KSito); F, α & β-epoxyphytosterols (P13=β-BE, P14=α-BE, P15=β-CamE, P16=αCamE, P17=β-StigE, P18=α-StigE, P19=β-SitoE, P20=α-SitoE); G, Triols; for peak identification and abbreviation see Table 5.1. (P21=BT, P22=CamT, P23=StigT, P24=SitoT). The chromatographic conditions are given in the methodology section in Paper II.

Table 5.1. Retention times (RT) for sterols and SOPs separated on a two-dimensional capillary GC column (DB-35MS + DB-5MS)[a]

Sterols and SOPs[b]	Peak name	RT (min)	Sterols and SOPs[b]	Peak name	RT (min)
Cholesterol	U1	20.44	7β-HStig	P7	24.56
Brassicasterol	U2	21.64	7β-HSito	P8	26.77
Campesterol	U3	23.48	7-KB	P9	33.56
Stigmasterol	U4	24.21	7-KCam	P10	36.68
Sitosterol	U5	26.12	7-KStig	P11	37.87
7α-HC	C1	17.86	7-KSito	P12	41.21
7β-HC	C2	21.34	β-BE	P13	26.06
β-CE	C3	24.56	α-BE	P14	26.95
α-CE	C4	25.20	β-CamE	P15	28.51
CT	C5	26.16	α-CamE[c]	P16	29.40
25-HC	C6	27.76	β-StigE[c]	P17	29.40
7-KC	C7	31.70	α-StigE	P18	30.22
7α-HB	P1	18.66	β-SitoE	P19	31.82
7α-HCam	P2	20.23	α-SitoE	P20	32.83
7α-HStig	P3	20.67	BT	P21	27.40
7α-HSito	P4	22.40	CamT	P22	29.86
7β-HB	P5	22.21	StigT	P23	30.49
7β-HCam	P6	24.28	SitoT	P24	33.24

[a], The chromatographic conditions are given in the methodology section in Paper II; [b], abbreviations are shown in Table 2.2; [c], co-eluted peaks (P16/P17).

When a mixture of unoxidized sterols and SOPs was introduced into system 2, 7α-HCam/cholesterol, 7β-HB/7α-HSito, stigmasterol/7β-HCam, sitosterol/CT, 7β-HSito/α-BE, α-CamE/β-StigE, and StigT/7-KC demonstrated either co-elution or poor resolution (for the complete chromatogram see Paper II).

It is necessary to remove unoxidized sterols by 2-fold SPE before the determination of SOPs by GC to reduce the interferences or co-eluting peaks. Oxybrassicasterols are only present in the family *Cruciferae* (rapeseed oil). The results of this study clearly indicate that two-dimensional capillary GC column with different selectivity are more suitable than a single capillary GC column for the analysis of SOPs. The number of studies on the analysis of SOPs is limited.

Table 5.2. Some recent literature data on the separation of SOPs (COPs+POPs) by capillary GC column

SOPs	Column	Co-eluting or poor baseline separation	Total run time	Reference
Oxybrassica, oxycampe, oxystigma, oxysitosterols	DB-5MS (50m×0.2mm × 0.33μm)	Brassicasterol/7α-HCam, 7α-HStig/7β-HC/Campesterol, 7α-HSito/Stigmasterol, 7β-HSitg/Sitosterol, 7β-Hsito/β-EStig/25-HC, β-ESito/CamT, α-ECam/7β-HSito, CamT/α-ESito, α-ESito/7-KCam	60 min	(Dutta, 2002)
Oxycampe, oxystigma, oxysitosterols	DB-5MS (25m × 0.2mm×0.33μm)	Sigmasterol/7α-HSito, 7β-Stig/7β-HCam, α-ECam/β-Stig,β-EStig/7β-HSito, CamT/β-ESito, StigT/α-ESito, SitoT/7-KCam	30 min	(Johnsson & Dutta, 2005)
Oxycampe, oxystigma, oxysitosterols	DB-35MS (25m×0.2mm×0.33μm)	Campesterol/7β-HCam, 7β-HStig/Stigmasterol, Sitosterol/7β-HSito, CamT/β-EStig, β-EStig/α-ECam, Sito/α-ESito	40 min	″
Oxycampe, oxystigma, oxysitosterols	DB-35MS + DB-5MS (25m×0.2mm×0.33μm)	β-Stig/α-EStig, α-EStig/CamT, Stigmasterol/7β-HCam	75 min	″
Oxychole, oxycampe, oxystigma, oxysitosterols	DB-5MS (30m×0.25mm×0.25μm)	Brassicasterol/7α-HCam, β-CE/7β-HB/7α-Hsito, 7β-HStig/Sitosterol/TC, BT/α-ECam, α-EStig/7-KC, 7-KB/α-ESito/CamT, 7α-Htig/7β-HC, 25-HC/β-EStig, SitoT/7-Kcam, no separation for 7β-HCam	22 min	(Grandgirard *et al.*, 2004b)
Oxychole, oxycampe, oxystigma, oxysitosterols	DB-1MS (30m×0.25mm × 0.25μm)	β-CE/7β-HB, 7β-HCam/Sitosterol, BT/7β-HSito, Choleterol/7α-HB, 7α-HStig/campesterol/7β-HC, Stigmasterol/α-CE/β-EB/7α-HSito/α-EB, 7βHStig/CT	32 min	″
Oxychole, oxycampe, oxystigma, oxysitosterols	DB-5MS (30m×0.25mm×0.25μm)	α-EC/7α-Sito, CT/β-ECam, α-ESito/CamT, 7-KStig/SitoT	28 min	(Apprich & Ulberth, 2004)
Oxychole, oxycampe, oxystigma, oxysitosterols	DB-17MS (30m×0.25mm×0.15μm)	7α-HSitg/7β-HC, α-ECam/β-EStig, α-CE/CT	21 min	″
Oxychole, oxybrassica, oxycampe, oxystigma, oxysitosterols	DB-35MS + DB-5MS (25m×0.2mm×0.33μm)	Brasicasterol/7α-HStig, β-CE/β-BE/Sitosterol, α-CE/CT, α-EB/BT, StigT/β-ESito, SitoT/7-KB	44 min	(Johnsson & Dutta, 2005)

The only other reported study of SOPs was carried out using a single GC capillary column separation (Apprich & Ulberth, 2004; Grandgirard *et al.*, 2004b). The single column analysis of SOPs using a DB-5MS column (30 m × 0.25 mm × 0.25 μm) showed co-elution of α-CE/7α-HSito, CT/β-CamE, CamT/α-SitoE, and 7-KStig/SitoT, while 7α-HStig/7β-HC, α-CE/TC, and α-CamE/β-StigE were co-eluted by a DB-17MS column (30 m × 0.25 mm × 0.15 μm), with the analytical times of 21 and 28 minutes, respectively (Apprich & Ulberth, 2004).

It has been reported that a mixture of POPs from campe-, stigma- and sitosterols was separated with multidimensional capillary GC columns (Johnsson & Dutta, 2005). A non-polar DB-5MS and a mid-polar DB-35MS column with similar dimensions (25 m × 0.2 mm × 0.33 μm) were used to separate POPs with enhanced baseline separation. However, three pairs of POPs were co-eluted: 24-hydroxystigmasterol/β-CamE, β-StigE/α-CamE and α-StigE/CamT. In addition, the analytical time required was more than 70 minutes. The main obstacles reported in the earlier studies are oxysterol co-elution peaks, poor baseline separation, and longer analytical times when using GC (Johnsson & Dutta, 2005; Apprich & Ulberth, 2004; Grandgirard *et al.*, 2004a; Dutta, 2002). In this study, the present author has made an effort to overcome the above difficulties and has shown that a two-dimensional GC capillary column system with different selectivity is more suitable than a single capillary column system in the analysis of SOPs. Figure 5.4 and Table 5.2 (published data) show that the column combinations of system 2 are capable of separating complex mixtures of unoxidized sterols and SOPs.

5.3 Paper III

Acid oil fractions are the major by-products from vegetable oil refining. They have a low commercial value and are increasingly being used in animal feed formulations (Dumont & Narine, 2007; Gasperini *et al.*, 2007). However, there is a lack of information on the levels of sterols and SOPs in these valuable by-products. An attempt was made to provide information on the sterols and SOPs in these by-products in Paper III.

Prior to the analysis of the total sterols by GC and GC-MS, analytical thin layer chromatography (TLC) was conducted (Dutta & Appelqvist, 1997) for

some of the samples to observe the lipid pattern and to estimate roughly the amounts of internal standard to be used during the GC analysis.

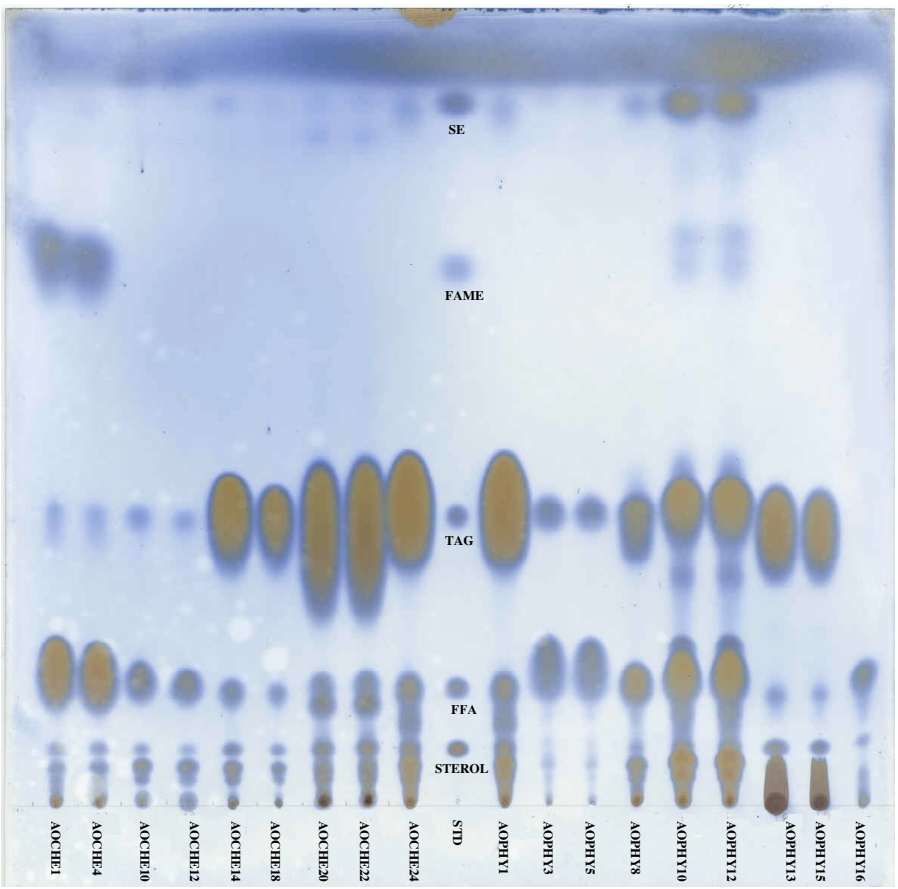


Figure 5.5. A thin layer chromatogram showing the distribution of lipids in the samples of by-product feeding fats from chemical (AOCHE) and physical (AOPHY) refining processes. The samples, along with a standard TLC-reference were spotted on a TLC plate and developed in the solvent system hexane: diethyl ether: acetic acid (85:15:1, v/v/v). FFA, free fatty acids; TAG, triacylglycerols; FAME, fatty acid methyl esters; SE, sterolesters.

The by-product samples were collected from chemical and physical refining processes in Europe and contain different amounts of total sterols and SOPs. Twenty-five by-product samples from chemical refining (AOCHE) were analyzed for total sterols and SOPs. The levels of total

sterols in these samples are shown in Figure 5.6A. Among the 25 samples, 7 samples contained brassicasterol (0.01-0.35 g/100 g) and 2 samples of animal origin contained only cholesterol (0.32 and 1.47 g/100 g).

The content of SOPs in the AOCHE samples showed wide variation ranging from 0.02-17 mg/100 g from campe-, stigma-, and sitosterol (Figure 5.6B). The most common SOPs in all the AOCHE samples were 7-hydroxysterols, and 7-ketosterols. In samples, which contained cholesterol, C7 oxysterols formed as prominent thermal oxidation products of cholesterol. In addition to that, some samples contained varied amounts of oxybrassicasterols having the highest value for 7-ketobrassicasterol (trace amounts to 6.5 mg/100 g).

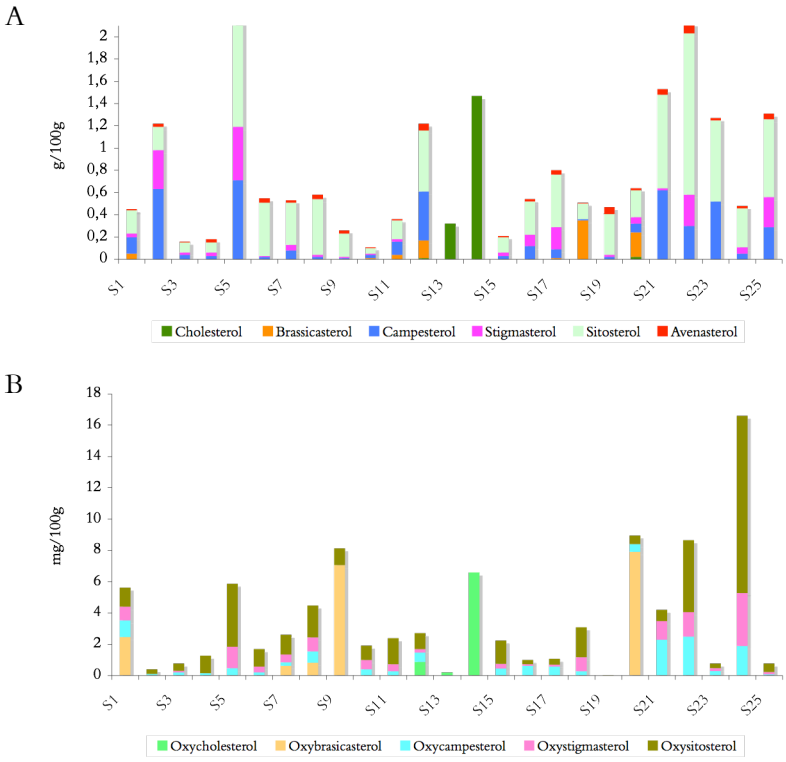


Figure 5.6. Content of sterols and SOPs in feed fat by-products collected from chemical refining process (AOCHE). A, Content of sterols in the AOCHE; B, Content of SOPs in the AOCHE.

Sixteen by-product samples from physical refining (AOPHY) were analyzed for total sterols and SOPs. The levels of total sterols in these samples are shown in Figure 5.7A. Most of these by-product samples were of plant origin, while 2 samples originated from a mixture of vegetable oils and animal fats, and one sample contained cholesterol as only sterol. There were 3 samples, which contained brassicasterol together with other sterols.

The content of SOPs in the AOPHY samples varied from 0.01 to 1.5 mg/100 g (Figure 5.7B). In general, a high amount of oxidation products are shown in samples, which contain brassicasterol as one of the individual sterols (1.4 mg/100 g).

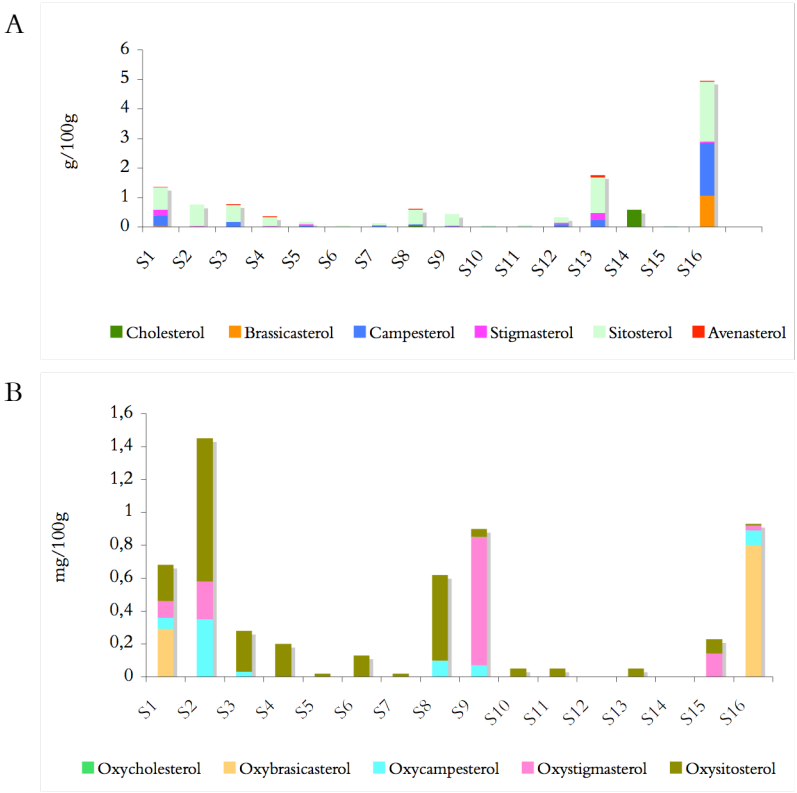


Figure 5.7. Content of sterols and SOPs in feed fat by-products collected from physical refining process (AOPHY). A, Content of sterols in the AOPHY; B, Content of SOPs in the AOPHY.

To the best of our knowledge, this is the first comprehensive work on the content of SOPs in soapstock and distillate fractions from edible oil refining processes. There are no available previous reports, which can be compared with our findings in this study. The AOPHY samples contained relatively lower levels of SOPs due to the high temperature applied during vacuum distillation, which obviously accelerated the breakdown of SOPs, transforming them into other unidentified degradation products. None of the AOPHY samples of mixed and animal origin contained quantifiable amount of COPs. This may be because COPs are more likely to degrade and breakdown at high temperatures than other oxysterols.

During the refining of edible fats and oils, the content of total sterols decreases due to the degradation and formation of products through isomerization, dehydration, polymerization, and due to the formation of hydrocarbons or sterenes and SOPs. These qualitative and quantitative changes in the sterols can be traced in the refined oil and in by-products like soapstock and distillate fractions collected after chemical and physical processes.

Moreover, the high amounts of natural antioxidants in AOPHY distillates probably prevented further formation of SOPs. Some sterols appeared to be more liable to break down than others; e.g. although the content of brassicasterol is low, the content of oxybrassicasterols is higher compared to other sterols and oxysterols. The structural arrangement of the brassicasterol molecule may provide a greater possibility for oxidation than the other sterols. The formation of SOPs is affected not only by the chemical nature of the sterols, but also by their quantity. The amounts of sitosterol and oxysitosterols are higher in these samples (Figures 5.6 and 5.7). There is a positive correlation between the total sterols and the total POPs ($0.01 < p \leq 0.05$). The variation in the content and composition of sterols and SOPs may be due to the collection of the samples from different locations and facilities, various sources and diverse refining conditions.

5.4 Paper IV-V

The tissues of chickens and rabbits from three levels of both TFA (*trans* fatty acid) and OXL (oxidized lipid) feeding trials were analyzed for their content of total cholesterol and oxycholesterols (COPs), as presented in Papers IV-V.

In general, the effect of TFA- and OXL-enriched feeds was a consistent increase in the levels of cholesterol in the meat, liver and plasma of chickens and rabbits. The inclusion of TFA and OXL showed a strong positive correlation and a significant effect at $0.01< p \leq 0.05$ on the content of cholesterol and COPs in tissues (meat, liver and plasma) of both animals (Figures 5.8A-D and 5.9 A-D).

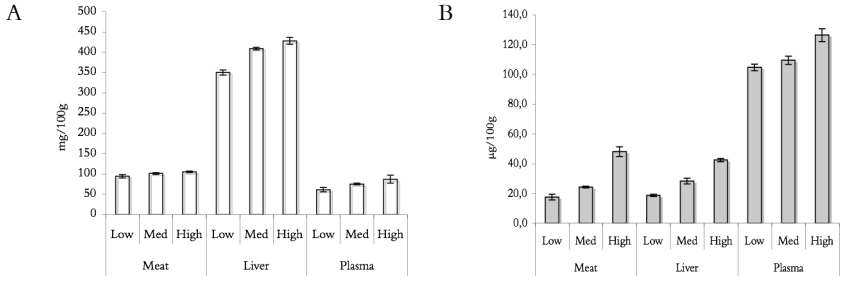


Figure 5.8. A, Content of cholesterol; B, Content of COPs; in meat, liver, and plasma (dL) of chickens fed with low, medium, and high levels of TFA (means of 4 replicates per treatment).

The most common COPs were identified in both studies: β-ring oxidation products such as 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), 7-ketocholesterol (7-KC) and cholesteroltriol (CT), epoxy isomers such as α-epoxycholesterol (α-CE) and β-epoxycholesterol (β-CE), and common side-chain COPs such as 20α-hydroxycholesterol (20α-HC) and 25-hydroxycholesterol (25-HC). However, none of the samples contained quantifiable amounts of 20α-HC (<0.1 μg/100 g sample).

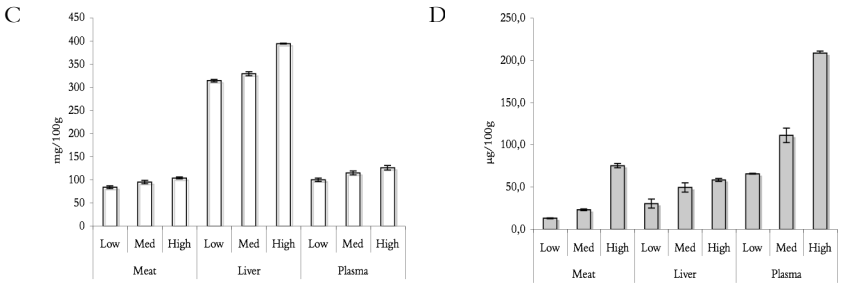


Figure 5.8. C, Content of cholesterol; D, Content of COPs; in meat, liver, and plasma (dL) of chickens fed with low, medium, and high levels of OXL (means of 4 replicates per treatment).

In all the meat samples from chickens and rabbits, 7α- and 7β-hydroxycholesterol, β-epoxycholesterol and 7-ketocholesterol were

quantified. However, both the chicken and the rabbit meat samples contained considerably higher amounts of total and individual COPs after the high TFA and OXL feeding trials (Figures 5.8 B & D and 5.9 B & D).

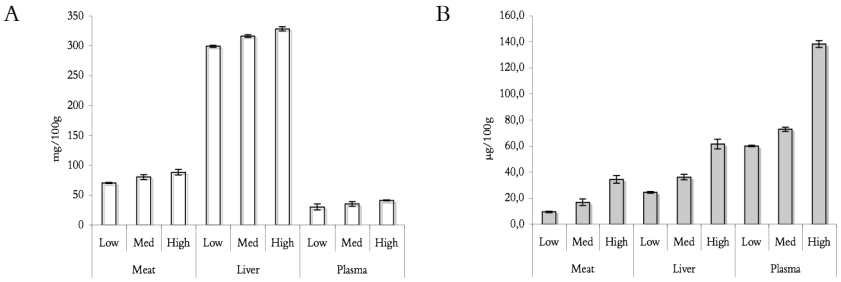


Figure 5.9. A, Content of cholesterol; B, Content of COPs; in meat, liver, and plasma (dL) of rabbits fed with low, medium, and high levels of TFA (means of 4 replicates per treatment).

This is the first study on the effects of dietary TFA and OXL on the cholesterol level in chicken and rabbit tissues to my knowledge. However, it has been reported that dietary beef tallow, fresh and oxidized sunflower oil and linseed oil do not generate a significant difference in the cholesterol level in raw chicken meat (Grau *et al.*, 2001). The present study showed that the content of COPs found in chicken meat increased consistently with TFA and OXL feeds.

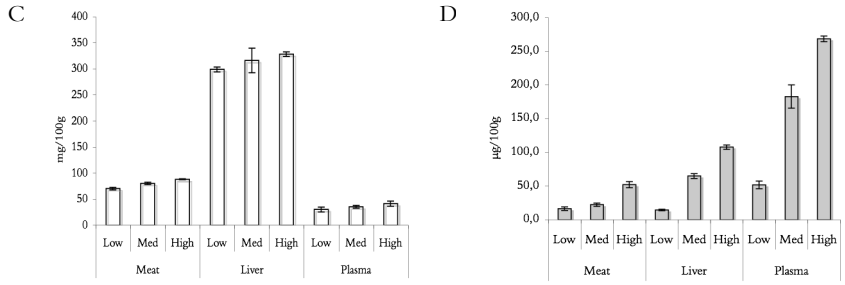


Figure 5.9. C, Content of cholesterol; D, Content of COPs; in meat, liver, and plasma (dL) of rabbits fed with low, medium, and high levels of OXL (means of 4 replicates per treatment).

This was possibly due to the higher levels of cholesterol in these meats, which resulted from the increased amount of TFA and OXL in the chicken and rabbit diets. There are other factors which may affect cholesterol oxidation in meat from animals fed with high TFA or OXL feeds; e.g. the content of tocopherols in feeds decreases from high to low in TFA- and OXL-enriched diets possibly because of hydrogenation.

Tissue components in animals, such as fat (mainly triacylglycerols), phospholipids, cholesterol and other polar compounds, are susceptible to oxidation. The larger amounts of peroxy and alkoxyl radicals in a high OXL diet may facilitate COPs formation *in vivo*. The COPs in liver can be formed both from enzymatic and non-enzymatic degradation. The non-enzymatic production of COPs might be facilitated by high-energy free radicals from the high OXL diets in both animals. In both animals, the content of cholesterol in the liver is higher than that in the other tissues (Figure 5.8 and 5.9). The liver is a main organ that regulates cholesterol anabolically and catabolically, which causes its higher content in the liver. However, high levels of COPs were found in plasma of chickens and rabbits compared to the other tissues. Dietary TFA is known to increase the total plasma cholesterol in animals and humans (Combe *et al.*, 2007; Gatto *et al.*, 2001). It has been reported that TFA activates cholesteryl ester transfer protein (CEPT) in rabbit, thereby increasing the plasma cholesterol level. Our results from both animal studies agree with the above conclusion, which has also been shown in rabbits after feeding them with TFA (Gatto *et al.*, 2001).

The amount of dietary antioxidants is low in the oxidized lipid feed, resulting in higher *in vivo* lipid autoxidation and subsequently in the increased generation of COPs. Additionally, α-tocopherol can be destroyed in the gastrointestinal tract by the free radicals present in OXL feeds or those present in the tissues, which probably have been absorbed from the feeds. The effect of a lower intake and lower deposition of tocopherol in tissues can be clearly seen when comparing the amounts of COPs in TFA and OXL trials in both animals. The levels of tocopherol were lower in TFA feeds than in OXL feeds, but the levels of COPs were higher in chicken from the TFA and OXL trials, except for the high OXL level. In the case of the higher OXL level the high pro-oxidative effect of the lipid oxidation products may have lowered the COPs level. Epoxycholesterols were prominent compared to C7-oxycholesterols. A considerably higher amount of 25-HC was present in rabbit plasma and a comparatively high amount of β-CE was present in chicken plasma at all levels of the TFA feeding trials. To my knowledge, there are no earlier reports, which can be compared with these findings. Feeds containing fats with moderate amounts of TFA and OXL enhance the levels of cholesterol and COPs in the chicken and rabbit meat, liver and plasma. However, more studies are crucial to confirm these observations.

6 Conclusions

- The cold saponification method with 95% ethanol had higher recovery percentages compared to transesterification and this method may be more suitable for analyzing oxysterols at low levels.
- A two-dimensional capillary GC column with varying selectivity is more suitable than a single GC capillary column for improving separation efficiency in the analysis of complex mixtures of oxysterols.
- The contents and composition of sterols and oxysterols vary to a greater extent in the by-product feed fat samples collected from different locations in Europe from chemical and physical refining processes.
- There is a positive correlation between the amounts of total sterols ($0.01 < p \leq 0.05$) and the amounts of total oxysterols in the analyzed feed fat by-products collected from chemical and physical refining processes.
- In the present study, the ranges of the oxycholesterols were quite high in chickens and rabbits fed with a high level of *trans* fatty acids and oxidized lipids.
- The results of the present study which show that high levels of *trans* fatty acids and oxidized lipids in animal feeds can facilitate cholesterol biosynthesis and cholesterol oxidation in animals.
- Achieving high quality in meat and other animal food products for human consumption requires high-quality feed fats, which need to be continually controlled and regulated. This is particularly important when using fat by- and co-products, recycled fats and other fatty sources from the food chain in feed formulation in order to protect animal welfare and to confirm safe food for consumers.

7 Future Prospects

- Further improvement of the work-procedure needs to be carried out to decrease the analytical variations of oxysterols at low levels in different matrices, particularly for transesterification. The importance of carefully validating the in-house method is also emphasised.

- Further development of the separation of complex oxysterols by capillary GC columns is required, to reduce the analysis time by using different column selectivity and dimensions.

- It is necessary to perform more studies using wider ranges of feeds enriched with *trans* fatty acids and oxidized lipids, to examine their long-term effects in chickens and rabbits on the oxidative stability of their edible tissues.

- It is necessary to perform more studies using wider ranges of *trans* fatty acids and oxidized lipids, to examine their long-term implications in various animal species. Further studies are also required to determine the oxidative stability of meat during processing, cooking and storage from the animals fed with such feeding fats.

- Future studies are of interest on the effect of high levels of phytosterols and POPs in animals for human consumption.

- The present research emphasizes the importance of conducting further studies to achieve better consumer confidence in environmentally friendly and sustainable high-quality meat production using by-products and recycled feed fats from the food chain.

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