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Studies on a Cholesterol-Lowering Microcrystalline Phytosterol Suspension in Oil

by

Anna von Bonsdorff-Nikander

Academic dissertation

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To my mother, Irja

ABSTRACT

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Some cholesterol-lowering drugs have lately caused severe side-effects in humans and the potential use of phytosterols as an optional method of lowering serum cholesterol has been given considerable attention. Although the positive effect of phytosterols has been known for decades, their unpleasant gritty texture and the poor solubility has prevented their widespread use.

In the present study, an oral phytosterol suspension was prepared by adding water to a supersaturated sterol in oil solution. The addition of water decreased the solubility in oil and microsized sterol crystals were formed. By changing the amount of sterol and/or water it was possible to control the crystal form, habit and size distribution. In the presence of water phytosterol recrystallised as needle-shaped hemihydrate or monohydrate crystals. Without added water, anhydrous plate-like crystals were achieved. Higher sterol concentrations resulted, due to supersaturation, in the formation of small crystals. By optimised process parameters, i.e. cooling temperature and stirring, it was also possible to achieve the desired crystal size for a so-called creamy suspension. Hardly any changes in crystal habit, size distribution or form were observed during storage of these suspensions for four months.

Incorporation of the suspension into cholesterol-lowering products includes heating and thus the knowledge of structural and mechanical changes of the suspension during heating is of importance. Dehydration of phytosterol crystals in an oil suspension was, similarly to plain crystals, a two-phased process. The suspension became less elastic and the crystals started to dissolve at relatively low temperatures.

A clinical study performed earlier using a similar microcrystalline suspension revealed a significant reduction of serum cholesterol levels. A dynamic *in vitro* study was performed to understand the mechanism by which phytosterols can inhibit cholesterol absorption in the small intestine. In addition to phytosterol, the choice of lipid in the suspensions was observed to have a significant effect on the solubilisation of sterols into the mixed micelles. The *in vitro* studies, in which medium chain length (MCT) and long chain length (LCT) lipids were compared, showed that phytosterols formulated in MCT efficiently displaced cholesterol from mixed micelles. Solubilisation into intestinal mixed micelles is a prerequisite for sterols to reach the site of absorption and thus cholesterol absorption is decreased.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which will be referred to by the Roman numerals I-V:

I. Christiansen, L.I., Rantanen, J.T., von Bonsdorff, A.K., Karjalainen, M.A. and Yliruusi, J.K., 2002. A novel method of producing a microcrystalline β-sitosterol suspension in oil. *Eur. J. Pharm. Sci*. 15 (3): 261-269.

II. von Bonsdorff-Nikander, A., Karjalainen, M., Rantanen, J., Christiansen, L. and Yliruusi, J., 2003. Physical stability of a microcrystalline β-sitosterol suspension in oil. *Eur. J. Pharm. Sci.* 19: 173-179.

III. von Bonsdorff-Nikander, A., Rantanen, J., Christiansen, L., Yliruusi, J., 2003. Optimizing the crystal size and habit of β-sitosterol in suspension. *AAPS PharmSciTech*., 4 (3): 349-356.

IV. von Bonsdorff-Nikander, A., Lievonen, S., Christiansen, L, Karjalainen, M., Rantanen, J. and Yliruusi, J., 2004. Physical changes of β-sitosterol crystals in oily suspensions during heating. *AAPS PharmSciTech* (submitted).

V. von Bonsdorff-Nikander, A., Christiansen, L., Huikko, L., Lampi, A-M., Piironen, V., Yliruusi, J. and Kaukonen, A.M., 2004. A comparison of the effect on medium versus long chain triglycerides on the *in vitro* solubilization of cholesterol and/or phytosterol into mixed micelles. *Lipids* (submitted).

1 INTRODUCTION

The presence of cholesterol in human and other mammals is vitally important for the cell membrane function. However, an excessively high serum cholesterol concentration is a risk factor for cardiovascular diseases (CVD). In today's world CVD is the leading cause of death in developed countries and is becoming one of the leading causes of death in developing countries as well (www.who.int, 2003). This means that despite the successful prevention of atherosclerosis, cardiovascular diseases are still responsible for one of every three cases of death. The combination of changed eating habits, the use of tobacco, and less physical activity are the main causes of the wide spread distribution of CVD. Genetic factors may also be a reason for enhanced serum cholesterol levels (Fuentes et al., 2000; Lind et al., 2002; Zuliani and Fellin, 2003). It has been demonstrated that a 10% decline in total cholesterol is associated with a 20% risk reduction of coronary heart disease at the age of 70 and even lowers the risk by 50% at the age of 40 (Law et al., 1994). Traditionally, high serum cholesterol levels have been normalised using cholesterol-lowering drugs. At the same time, the importance of dietary intake has been emphasised by the nutritionists.

Selective inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA or statins), the rate-controlling enzyme of cholesterol synthetic pathway, are effective drugs but a very expensive method to treat hypercholesterolemia. Statins (e.g. mevastatin, lovastatin, cerivastatin and simvastatin) are able to lower serum total cholesterol by decreasing low density lipoprotein (LDL) cholesterol by 20%, even up to 55% (Chong et al., 2001; Blasetto et al., 2003; Endo, 2004). Some statins have, however, lately caused severe side effects which have resulted in major estimated revenue decreases for the pharmaceutical industry (Clark, 2003; Jamal et al., 2004). In August 2001, Bayer AG had to withdraw their cholesterol-lowering drug Lipobay®/Baycol® (cerivastatin) worldwide due to reports of side effects involving muscular weakness (rhabdomyolysis) (Maggini et al., 2004; www.investor.bayer.com, 2004). In less than a month, Bayer's share had lost over 42% of its value (Bloomberg Terminal, 2004). The FDA (Food and Drug Administration) has received reports of serious muscle toxicity of another statin drug, Crestor® (rosuvastatin) by AstraZeneca (www.fda.com, 2004). At the moment the FDA is evaluating these reports and comparing the frequency of reports to the reports of other

statins. The importance of looking for optional methods of lowering cholesterol is therefore imminent.

Studies of phytosterols, which are structurally related to cholesterol, date back to the 1950s, when large amounts of phytosterols (10-15 g/day) were administrated in the form of a powder (Pollack, 1953; Farquhar et al., 1956). The unpleasant texture of phytosterols and the poor solubility in oil or water has caused several problems in preparation and administration, and thus prevented their widespread use (Miettinen, 2001).

High-fat foods such as margarines and butters appear to be ideal vehicles for phytosterols and its saturated form phytostanol because of their strong hydrophobic nature (Mattson et al., 1982). Finnish science is at the forefront of development in sterols/stanols dietary products. The Raisio corporation launched the first commercialised phytostanol ester-containing cholesterol-lowering margarine, Benecol®, in 1995 (Miettinen et al., 1995). High-fat foods are contradictory to the current approaches of maintaining healthy diets and a healthy lifestyle. Therefore, the attempt has been to incorporate phytosterols into lower-fat foods (St-Onge and Jones, 2003). Because of the hydrophobic nature of phytosterols, the cholesterol-lowering efficacy in low-fat foods was thought to be minor. Studies, however, show that the effect of low-fat foods have a significant cholesterol-lowering effect (Volpe et al., 2001; Nestel et al., 2001).

In the middle of the 1990s a new method was developed, to make the use of phytosterols more accessible. A microcrystalline suspension in oil allows incorporation of up to 30% of phytosterols into a food product without any chemical reactions or additives. The extent of cholesterol-lowering *in vivo* is similar to those examined using phytosterol or phytostanol esters dispersed in high fat spreads. In 2003, the European Union's Novel Foods Regulator gave its approval to begin marketing this suspension (Diminicol®) throughout the EU. Diminicol® received GRAS status (Generally Recognised As Safe) earlier the same year in the US by the FDA. The FDA also granted products containing Diminicol® the right to use the approved sterol heart health claim.

Functional foods have no precise, universally accepted definition in general, but they can be considered food components (being nutrient or not), which affects one or a limited number of function of the body in a positive way, providing a health benefit beyond traditional nutritional value (Roberfroid, 2000; Palou et al., 2003).

The combination of statins and sterols/stanols has only been tested on a small scale so far. It appears that for patients who are taking statins and are in need of additional cholesterol lowering, the addition of sterols/stanols into the diet is more effective than the increase of statin doses (Katan et al., 2003). A wide study carried out in Finland (FINRISK 2002) revealed that of all patients who were aware of their high cholesterol levels, 19% used cholesterol-lowering medicines, 11% used cholesterol-lowering bread spreads and 5% combined both therapies (de Jong et al., 2004).

2 REVIEW OF THE LITERATURE

2.1 Phytosterols as cholesterol-lowering agents

2.1.1 Composition, sources and consumption of phytosterols

Phytosterols are a natural mixture of sterols containing a minimum 70% β-sitosterol (Ph.Eur. suppl. 4.1, 2001). They differ from cholesterol by the presence of an extra methyl or ethyl group on the cholesterol side chain at the C-24 position. The planar sterol structure with a hydroxyl group and a hydrophobic tail is characteristic for the molecular structure. Over 200 different types of phytosterols have been identified, of which β-sitosterol, campesterol and stigmasterol are the major dietary sterols (Fig. 1) (Moreau, 2002). Phytostanols are saturated phytosterols, that is, they have no double bonds in the sterol ring.

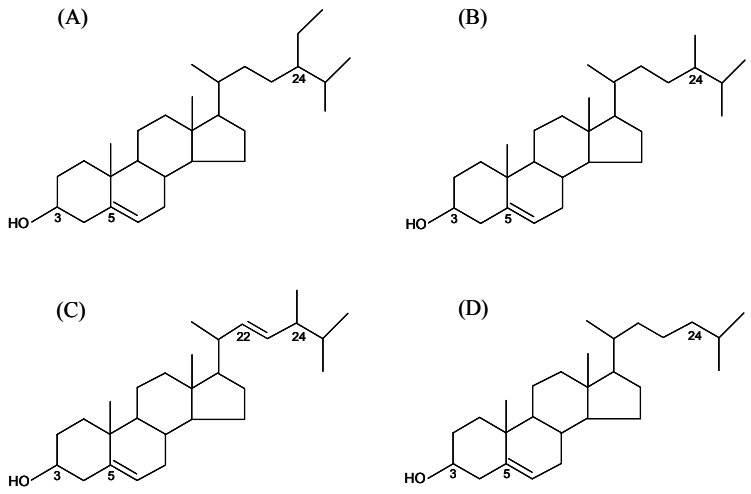

Figure 1. Structure of β-sitosterol (A), campesterol (B), stigmasterol (C) and cholesterol (D)

Phytosterols are important structural components of plant membranes, and they play a key role in plant cell membrane function just as cholesterol does in animal cell membranes (Quílez et al., 2003). Phytosterols are found in significant amounts in seeds, nuts, fruits and vegetables; however, the most concentrated source is vegetable oils (Ostlund, 2002). Since humans are not able to synthesize phytosterols, all phytosterols in the human body originate from dietary intake. As part of a normal healthy diet, most people eat 100-500 mg of phytosterol each day (Ostlund, 2002). Most of the phytosterols or phytostanols currently incorporated into foods are esterified to unsaturated sterol/stanol esters to increase lipid solubility, thus allowing maximal incorporation into a limited amount of lipid. Phytosterol or phytostanol intake from functional foods (e.g. bread spreads) is usually 1.5-3g/day. Phytosterol and phytostanol products reduce the serum concentration of total cholesterol by up to 15% and that of LDL cholesterol by up to 22% (Moghadasian and Frohlich, 1999; Ostlund, 2002; Christiansen et al., 2001a) (see also Table 1).

Cholesterol derives the intestinal tract from two major sources. A normal Western diet provides 300-600 mg cholesterol per day to the intestine and an additional 1000-1500 mg/day is derived from endogenous sources, mostly from the bile (Trautwein et al., 2003).

Table 1. Examples of different clinical studies with phytosterols incorporated in bread spread and the reduction in serum LDL cholesterol.

Phytosterol (carrier)	Dose (g/d)	Time (wk)	Changes in LDL cholesterol (%)	Reference
Sterol ester (margarine)	1.8	3	-6.5	Mussner et al., 2002
Sterol (butter)	1.8	3	-11.3	Vanstone et al., 2002
Sterol (margarine)	1.5 3.0	26 26	-11.6	Christiansen et al., 2001a
Sterol (butter)	1.0	4	-6.2	Volpe et al., 2001
Sterol ester (fat spread)	2.5	8	-10.0	Neil et al., 2001
Sterol ester (butter)	2.4	4	-12.2	Nestel et al., 2001
Sterol ester (margarine)	2.1	4	-10.4	Hallikainen et al., 2000

At first, animal and human studies showed that phytostanols inhibit cholesterol absorption and lower serum cholesterol more effectively than phytosterols (Sugano et al., 1977, Heinemann et al., 1986; Heinemann et al., 1991). However, more recent studies

reveal no difference between phytosterols and phytostanols in the lowering of the absorption or the serum concentration of cholesterol (Hallikainen et al., 2000; Law, 2000).

The daily dose of sterol/stanol food products is often divided into 2-3 portions, although it has been shown that a large single daily dose is equally effective (Plat et al., 2000). It is likely that part of the phytosterol is precipitated in the intestine and re-solubilised during lipolysis of lipids contained in the food ingested at a later time. Lately it has been revealed that phytosterols may also have an effect on cholesterol metabolism inside the enterocytes.

2.1.2 Safety of phytosterols and phytostanols

The current opinion is that phytosterols are safe when added to the diet because they are found in natural foods. Phytosterol and phytostanol-enriched foods, which decrease serum cholesterol levels, are among the first examples in Europe of functional foods whose safety has been evaluated following the legislation applied to Novel Foods (SCF 2000, 2003).

However, some observations of decreased levels of the absorption of other lipid soluble components such as vitamins and antioxidants are of concern (Plat et al., 2000; Mensink et al., 2002). Some randomised studies report that phytosterols and phytostanols lower serum concentration of β-carotene by up to 25%, concentrations of α-carotene by 10%, and concentration of vitamin E by 8% (Weststrate and Meijer, 1998; Hallikainen et al., 1999; Gylling and Miettinen, 1999). The serum concentration of vitamin D and retinol has been observed to be unaffected (Gylling and Miettinen, 1999). Contrary to the above, several studies report no significant effects on serum lipid soluble vitamins (Christiansen et al., 2001a; Davidson et al., 2001; Volpe et al., 2001 Raieni-Sarjaz et al., 2002). Thus the relevance of the observed changes is not yet adequately known and warrants further attention. A part of the reported reduction can be explained by a decreased amount of LDL particles in the circulation, which transports these lipid-soluble antioxidants (de Jong et al., 2003). Phytosterols and phytostanols may also interfere with the incorporation into the mixed micelles. Furthermore, phytosterol esters did not substantially affect vitamin K-dependent hemostasis on individuals that underwent warfarin therapy, suggesting that, at least in short term studies, vitamin K status was not affected by the consumption of phytostanols (Nguyen, 1999). Phytosterols have shown no evidence of *in*

vitro mutagenic activity (Wolfreys and Hepburn, 2002). Safety data of phytosterols and phytostanols collected for a period of more than five years in Finland and two years in the US show no evidence of hazard adverse effects (Katan et al., 2003).

2.1.3 Phytosterol absorption

The amounts of absorption vary among the individual phytosterols due to both the sterol nucleus and the length of the side chain (Ostlund, 2002). In humans, less than 2% of the ingested phytosterols is absorbed. β-sitosterol, which is the most extensive phytosterol, is the least well absorbed (0.5%) due to its high hydrophobicity. The absorption of phytosterols, therefore, is considerably smaller than that of cholesterol, which can be up to 60% (Ostlund et al., 2002). Total serum phytosterol concentrations in healthy adults range from 7 μmol/L to 24 μmol/L (Moghadasian, 2000).

Due to the hydrophobicity of the sterols, solubilisation into intestinal mixed micelles is a prerequisite for reaching the site of absorption. Phytosterols are solubilised under the same conditions that exist for cholesterol and other lipids (Ros, 2000; Moghadasian, 2000). Solubilisation into mixed micelles is greatly enhanced in the presence of products of triglyceride digestion (monoglycerides (MG) and fatty acids (FA)) (Fig. 2) (Carey and Small, 1970; Ros, 2000).

Mechanism of dietary mixed micelle formation

The mechanical digestion of lipids starts as early as in the mouth and continues in the stomach with emulsification. Chewing breaks down large pieces of fat into smaller sizes whereafter the peristaltic movements further grind the smaller pieces into a creamy emulsion (chyme). The purpose of emulsification is to increase the surface area of the lipid droplets, thereby increasing the area on which the digestive enzymes can act effectively. Lingual lipase probably has a minor effect on lipolysis, unlike gastric lipase, which hydrolyses approximately one out of four triglycerides molecules during digestion of a meal (Embleton and Pouton, 1997; Ros, 2000). The gastric (and lingual) lipase is responsible for the hydrolysis of triglyceride (TG) to the corresponding diglyceride (DG) and fatty acid (FA). The presence of lipolysis products (mainly FA) in the duodenum stimulates the release of the hormone cholecystokinine, which then induces the secretion of bile acids from the gallbladder and the release of pancreatic juice containing lipases. The sudden increase in pH in the duodenum causes an abrupt change in the physical

behaviour of the fatty acids contained in the emulsion droplets. These become partly charged and migrate to the interface of the emulsion droplet, inhibiting the binding of the principal lipolytic enzyme, pancreatic lipase. The effect of co-lipase is to anchor pancreatic lipase to the surface of the emulsified lipid droplet. By the enzymatic action of pancreatic lipase on TG droplets, the corresponding 2-monoglyceride and two fatty acids are produced.

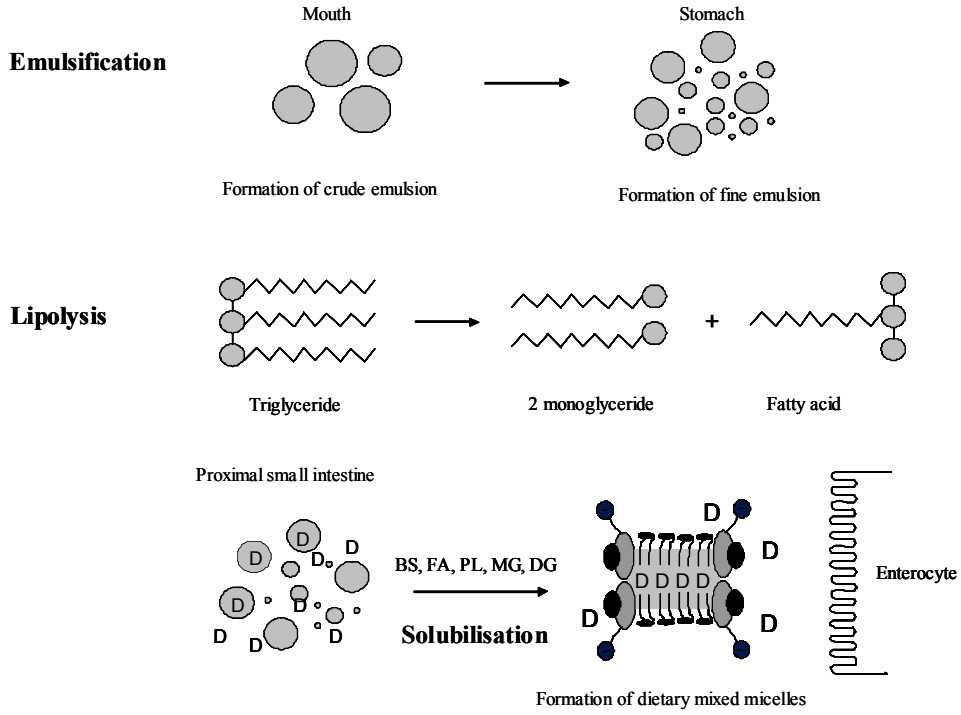

Figure 2. Schematic model of intestinal lipid digestion and trafficking of lipolysis products. D=drug or other compound, BS= Bile salts, FA= Fatty acid, PL= Phospholipid, MG= Monoglycerides, DG= Diglyceride (partly adapted from Porter et al., 2004).

Bile, containing a mixture of bile salts and phospholipids, plays a fundamental role in the solubilisation of lipid digestion products and other poorly water-soluble compounds. A typical concentration of bile salts in the fasted intestine is 4-6 mM, compared to post-prandial concentrations of 10-20 mM (Ladas et al., 1984; Armand et al., 1996; Humberstone and Charman, 1997). When the concentration of bile salts in the intestine exceeds critical micellar concentration (cmc), lipids and bile salts interact spontaneously to form polymolecular aggregates, micelles. Lipids (digestion products) promote micellar swelling and therefore increase the capacity of the micelles to solubilise lipophilic/hydrophobic compounds (Porter and Charman, 2001). When the amount of lipid digestion products in the aqueous phase increases, while the amount of bile does

not, the lipolytic products accumulate on the surface of the lipid droplet. This results in the shedding of lipolytic products in the form of multilamellar liquid crystalline structures. These structures are normally dissolved quickly into mixed micelles at the appropriate bile salt concentration (Ros, 2000). Thus, higher bile salt concentrations improve lipid digestion through effective solubilisation of lipolytic products, leading to an abundance of swollen micellar structures. This promotes the solubilisation of sterols or other lipophilic compounds.

Influx/efflux of sterols in the enterocytes

The transfer of sterols/stanols from the mixed micelles into the enterocytes has long been considered a passive process. Recently it has been suggested that a specific protein in jejunal enterocytes called Niemann-Pick C1 Like 1 (NPC1L1) shuttle system facilitates sterol absorption from the intestinal lumen through the brush border membrane of the enterocyte (Davis et al., 2004; Altmann et al., 2004). The ATP-binding cassette (ABC) transporters, ABCG5 and ABCG8, are two quite recently discovered half-transporters, strongly present in the liver and the intestines. They are involved in the excretion of sterols back into the intestinal tract (Berge et al. 2000; Chen, 2001; Albrecht et al., 2002; Trautwein et al., 2003, Ostlund, 2004). ABC transporters are proteins that use ATP as a source of energy to transport substrates between different cellular compartments and out from the cell (Oram and Lawn, 2001). In a similar way another ATP-binding cassette A1 (ABCA1) is involved in the transport of excess tissue sterol from the enterocytes into the lumen. Mutations in any of the ABCG5 and ABCG8 genes are associated with sitosterolemia. Sitosterolemia (also known as phytosterolemia) is a rare genetic disorder, (one in 6 million people), where patients who fail to excrete sterols into bile have extremely high concentrations of serum phytosterols, particularly sitosterol, in serum and tissues (Moghadasian, 2000; Lu et al., 2001). Patients with sitosterolemia also hyperabsorb cholesterol and are usually hypercholesterolemic (Berge et al., 2001; Lee et al., 2001).

Esterification and incorporation into chylomicrons

Absorbed free cholesterol is esterified by acyl-coenzyme A cholesterol acyltransferase (ACAT) inside the enterocyte. Only the esterified form is incorporated into chylomicrons and excreted into the circulation. There are several potential explanations for the low

phytosterol (phytostanol) absorption compared to that of cholesterol. Phytosterols have a low affinity for ACAT and are therefore poorly esterified (Field and Marthur, 1983). Since only the esterified form is incorporated into chylomicrons, and further excreted into the circulation, the concentration in circulation remains low.

2.1.4 Phytosterol mechanisms of action on intestinal cholesterol absorption

The mechanism of the cholesterol-lowering activity of phytosterols is not completely understood but several theories have been proposed (Fig. 3). Phytosterols are claimed to inhibit the absorption of dietary and reabsorption of endogenous cholesterol from the gastrointestinal tract. Additionally, phytosterols seem to enhance the efflux of excess absorbed cholesterol. Consequently, the excretion of cholesterol in the faeces leads to decreased serum levels of this sterol.

Competition between cholesterol and phytosterols for solubilisation in mixed micelles

Cholesterol has to be solubilised within mixed micelles, containing bile-salts and phospholipids, in order to reach the site of absorption and further be absorbed into circulation. Mixed micelles have a limited capacity to solubilise hydrophobic molecules. *In vitro* and *in vivo* studies suggest that phytosterols have a greater affinity for micelles and can therefore displace cholesterol from the mixed micelles (Ikeda and Sugano, 1983; Mel´nikov et al., 2003b; Trautwein et al., 2003). Armstrong and Carey reported that phytosterols, due to their increased hydrophobicity compared to cholesterol, have a lower solubility in, but a higher affinity for bile acid micelles than does cholesterol (1987).

Co-crystallisation of phytosterols and cholesterol

One potential mechanism of lowering the intestinal cholesterol absorption is co-crystallisation of cholesterol and phytosterols in the gastrointestinal tract, forming poorly absorbable mixed crystals (Christiansen et al., 2001b; Christiansen et al., 2003). Both phytosterol and cholesterol are, in a free form, sparingly soluble in oil (3 g/100 ml at 37 °C in presence of water) and practically insoluble in water (~0.2 mg/100 ml) (Jandacek et al., 1977). As early as in the 1950s Davis noticed that cholesterol and β-sitosterol formed a new crystal form when the sterols were precipitated in methanol (1955). Co-crystallisation of phytosterols and cholesterol in the gastrointestinal tract should lead to the reduction of intestinal cholesterol uptake since the solubility of the new crystal is

considerably lower than that of cholesterol. Recently, however, Mel´nikov et al. (2003a; 2003b) reported that it is unlikely that the formation of mixed crystals largely affects the intestinal absorption of cholesterol *in vivo*. This is due to the relatively high solubility of cholesterol, phytosterol and phytostanol in products of fat lipolysis.

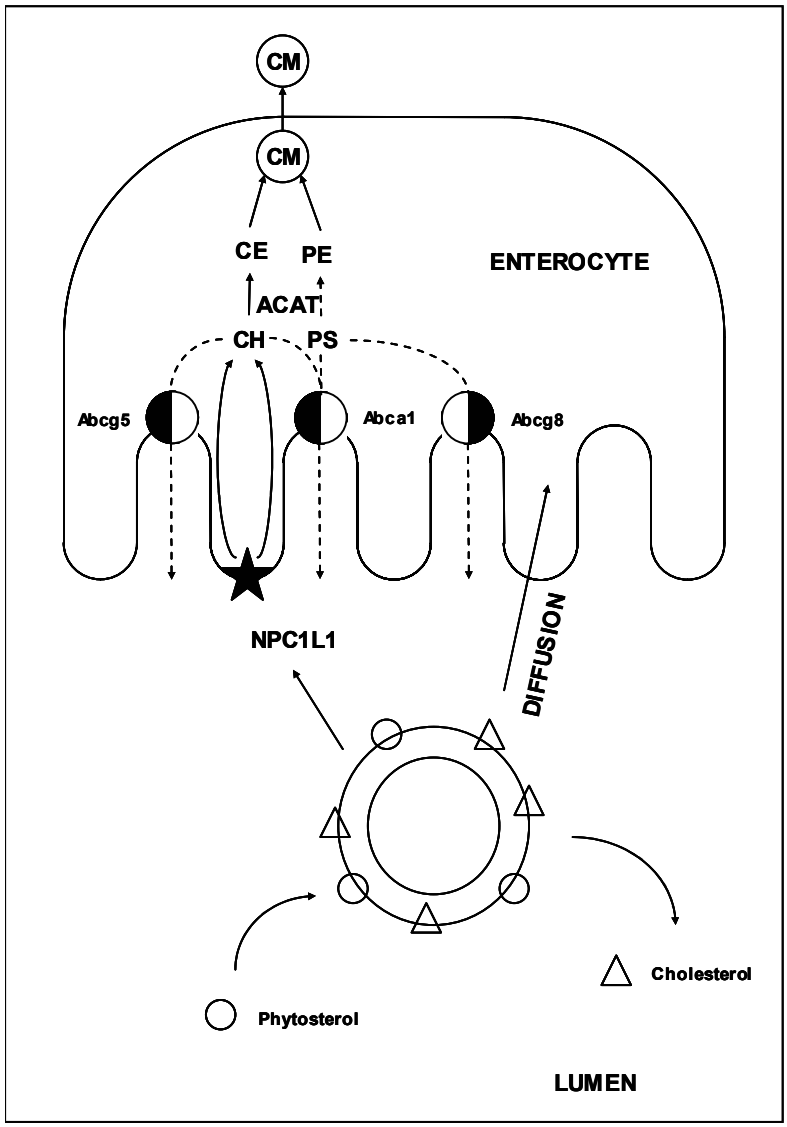

Figure 3. Phytosterols displace cholesterol from intraluminal intestinal micelles, reducing cholesterol absorption by the brush border membrane. The exact mechanism by which sterols are absorbed to enterocytes is not fully known. The transfer of sterols/stanols has long been considered a passive process. Recently the Niemann-Pick C1 Like 1 (NPC1L1) shuttle system was found to be the intestinal phytosterol and cholesterol transporter. The ATP-binding cassette transporters (ABCG5, ABCG8 and ABCA1) are similarly involved in the efflux of excess sterol from the enterocytes back into the intestinal lumen. Phytosterols may also reduce the esterification rate of cholesterol inside the enterocytes through inhibition of acyl-coenzyme A cholesterol acyltransferase (ACAT). Only the esterified form (cholesterol ester CE, phytosterol ester, PE) is incorporated into chylomicrons and secreted into circulation.

Decreased cholesterol influx from and increased cholesterol efflux back into the intestinal lumen

Recently it has been stated that the Nieman Pick C1 L1 (NPC1L1) shuttle system plays a fundamental part in the regulation of cholesterol influx into the enterocytes. However, it appears that these transport systems are unable to differentiate between cholesterol and phytosterols. Thus an increased amount of phytosterol in the intestine results in reduced

cholesterol in the enterocyte and further reduced cholesterol in circulation. In the same way the cellular ATP-binding cassette transporters (ABCG5, ABCG8, ABCA1) are involved in the efflux of excess sterol from the enterocytes back into the intestinal lumen (Plat and Mensink, 2002; de Jong et al., 2003).

Inhibition of ACAT activity

Another proposed cholesterol-lowering mechanism of phytosterols is the possible reduction of the esterification rate of cholesterol inside the enterocytes through inhibition of acyl-coenzyme A cholesterol acyltransferase, ACAT (Chen, 2001; de Jong et al., 2003; Trautwein, 2003). ACAT reduces the intracellular free cholesterol concentration by transforming it into cholesteryl ester. Phytosterols might challenge ACAT activity and reduce the absorption of cholesterol because of the high intracellular free sterol concentration. Cholesterol has to be incorporated into chylomicrons before it is secreted into the lymph. Up to 80% of the cholesterol incorporated in the chylomicrons is in an esterified form (Ikeda et al., 1988; Dawson and Rudel, 1999).

2.2 Theory of suspensions and crystal properties

A suspension can be defined as a heterogeneous system in which one phase, a solid, is finely divided and dispersed in the other, generally a liquid (the dispersion medium). Drugs and other compounds are dispensed as suspensions for different reasons, the most common being poor solubility in water, oil or an organic liquid (Falkiewicz, 1988). The large surface area of the suspended compound ensures a high degree of bioavailability for absorption. Suspensions may also be used to mask the taste of a bitter or unpleasant compound. Pharmaceutical suspensions are prepared mainly for oral use, topical administration, parenteral use or inhalation therapy (Nash, 1996). Other applications for suspensions are e.g. cosmetics, foods and household products.

2.2.1 Properties of solid particles

The number of particles and different crystalline modifications of the solid material, such as *crystalline, amorphous* or *solvated* forms, can affect certain properties of a suspension, for example solubility, dissolution rate and chemical stability (Haleblian, 1975). Crystalline solid is a state where structural units, formed by molecules, atoms or ions, are

arranged in a regular, repeating array (Vippagunta et al., 2001). Within a specific crystal, each *unit cell* is the same size and contains the same number of molecules. The unit cell can be thought of as a box, which when stacked together three-dimensionally, produces the *crystal lattice*. The simplest and most symmetric of these is the cubic system. The other six systems are the hexagonal, tetragonal, trigonal, orthorhombic, monoclinic and the triclinic systems.

When a material is in solid state but consists of disordered arrangements of molecules, it is said to be amorphous (glass state). Amorphous solids have properties very different from the crystal form, no melting point and no definitive x-ray pattern. Consequently, they have zero crystallinity. The crystallinity can be described as the *degree of crystallinity* which is the ratio of the crystalline to amorphous parts in a substance.

Crystals in a suspension are larger than those in colloids or solutions; they are visible under a microscope, and some can be seen with the naked eye. The *particle size distribution* of the suspended phase is one of the most important parts in the formulation and stability of a suspension (Nash, 1996). A fine particle size is important to ensure a slow rate of sedimentation of the suspended particles and better resuspendability. While most of the existing suspensions have particle sizes in the micron range, technology development in recent years has extended the range down to the submicron region. One of the advantages of nanosized crystals is the possibility of intravenous administration (Merisko-Liversidge et al., 1996). When particle size grows beyond ~1 μm, the system is called coarse suspension (Nash, 1988). The upper limit for a suspendable solid particle in coarse pharmaceutical suspensions is from 50 to 75 μm. However, particles larger than 5 μm will cause a gritty texture when administered in the eye and particles over 25 μm might block the needle in parenteral use. The optimum particle size for a so-called creamy suspension administered orally lies, according to the food industry, between 10 μm and 50 μm (Viaene and Januszewska, 1999; Kilgast and Clegg, 2002).

Research has focused more on crystal size distribution than on *crystal habit*, which is another critical property of a crystalline solid in suspensions. The crystal habit particularly affects the stability of suspensions and their usage. Regarding the physical stability, needle-shaped crystals are preferred, since they stay suspended better than prismatic crystals (La Manna, 1985). The crystal habit, or overall shape of the crystal, depends on internal factors (e.g. structure and bonds) and external factors (e.g.

supersaturation and solution composition) (Boistelle and Astier, 1988). The shapes of particles are classified into ten classes according to British Standards (BS 2955). Crystals are, however, usually classified according to the seven general systems as acicular (needle), prismatic, pyramidal, tabular, equant, columnar and lamellar types (Fig. 4). Acicular, prismatic and plate-like crystals are common habits for recrystallised compounds (Anwar et al., 1989; Agafonov et al., 1991).

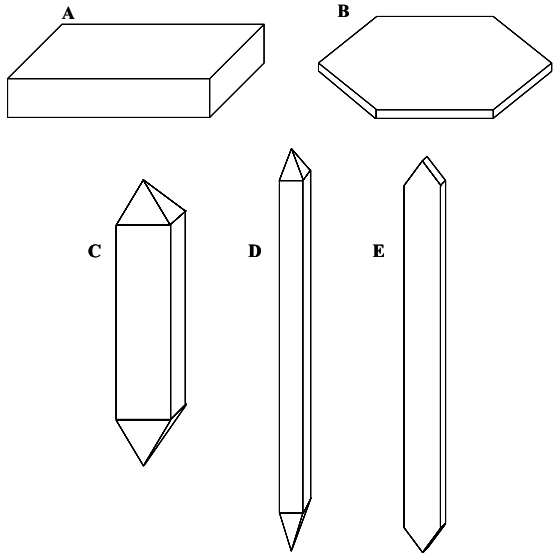

Figure 4. Some crystal habits A. tabular, B. platy, C. prismatic, D. acicular and E. bladed.

Different crystal structures of the same compound (polymorphism) can also be a factor in suspension technology. *Polymorphism* is defined as the ability of a compound to crystallise in at least two different crystal forms (Haleblian and McCrone, 1969). Polymorphs of a certain substance usually have different physicochemical properties, such as melting point, solubility and density. Polymorphism may thus have an effect on the bioavailability, manufacturability, and stability of the product. *Pseudopolymorphs*, also known as solvates, are crystalline solids that include solvent molecules within the crystal structure (Vippagunta, 2001). The differences between polymorphs and pseudopolymorphs are significant. Whereas polymorphs are different crystal structures of the same molecules, pseudopolymorphs, on the other hand, are crystals of the same molecule with different numbers of solvent molecules. If the solvent molecule is water, these crystals are termed hydrate. The liberation of water molecules or organic solvents from crystals is dependent on environmental conditions such as temperature, humidity and pressure. Crystalline hydrates can be classified into three categories (Morris and

Rodrigues-Hornedo, 1992). The first category (class 1) is the isolated lattice site hydrates, where the water molecules are not in direct contact with each other. The second category (class 2) is channel hydrates, where the water molecules lie next to other water molecules forming channels through the crystals. The third category (class 3) is the ion-associated hydrates, in which the metal ions coordinate with the water molecules, and are included in the growing lattice structure. Today more than ever before, the ability to develop a suitable salt form of a new compound during development and preformulation has to be considered. Evaluating the physical properties of potential salt forms is of major importance as they may cause changes in dissolution rates and solubility. Changes in the hygroscopicity and stability of different salt forms have to be taken into consideration during formulation.

2.2.2 Properties of the dispersion medium

The dispersion medium (i.e. external or continuous phase) is generally a liquid or a semisolid. The medium is selected on the basis of safety, density, viscosity, taste and stability (Nairn, 1990). Water is the most commonly used medium in pharmaceutical suspensions. One factor to consider in the preparation of suspensions is the degree of interaction between the internal phase and the dispersion medium. If there is little interaction between the internal phase and the dispersion medium, the dispersion is lyophobic (or hydrophobic if water is used). The internal phase consists of either organic or inorganic compounds and thus has little interaction with the aqueous phase. As a result, the suspensions are physically unstable and should be prepared by the methods discussed below (section 2.2.3). If there is considerable interaction between the internal and external phases, the dispersion is lyophilic (or hydrophilic). In this type of dispersion (e.g. gels), the internal phase has polar groups with a hydrophilic character.

By increasing the viscosity of the dispersion medium, a slower particle sedimentation rate can be achieved and thus an increase in stability. The most common method of increasing the viscosity is by adding a viscosity enhancer (e.g. natural, semisynthetic, or synthetic hydrocolloids, and clays) (Ofner et al., 1989). Ideally, the system should be pseudoplastic, i.e., it should have high viscosity during storage and low viscosity during shaking.

2.2.3 Formulation of a suspension

Since the specific properties of various suspended compounds differ, there is no single procedure that will always lead to a successful suspension product; however, certain principles are fundamental in all formulations. Suspensions can be prepared either by dispersing finely divided powders in an appropriate vehicle or by precipitation.

Dispersion method

The preparation of suspensions by dispersion method consists of three main steps: first it is necessary to ensure that the internal phase is of fine particle size, then the internal phase is dispersed in the dispersion medium, and finally, the product is stabilised (Nairn, 1990). Common methods of particle size reduction include e.g. dry milling, spray drying and recrystallisation from supercritical fluids (SCF) (Nash, 1988; Mullin, 2001). When the dispersion method is utilised for suspension preparation, the vehicle must be formulated so that the solid phase is easily wetted and dispersed. Certain solids are readily wet by the dispersion medium whereas other are not. The use of surfactants (e.g. sodium lauryl sulfate, polysorbates or sorbitan esters) is then desirable to ensure uniform wetting of hydrophobic solids as they decrease the solid-liquid interfacial tension, because of their dual affinity for both oil and water. Once the particles have been wetted, they must be separated and distributed uniformly throughout the liquid vehicle. Sufficient agitation of the mixture of solid and liquid must be provided initially to obtain a high degree of dispersion (the extent to which particles are separated and distributed throughout the vehicle). Sometimes shearing forces from mixers are used to break up particle aggregates for better wetting and distribution of the compound. The final step is to maintain the stability of the dispersed state. This is mainly done by increasing the viscosity of the dispersion medium or by reducing the particle size (see sections 2.2.2 and 2.2.8).

Precipitation method

The more seldom used method to suspend an insoluble compound is by precipitation from a solution. There are several common methods for the production of solids in the pharmaceutical industry. Water-insoluble compounds can be precipitated by first dissolving them in water-miscible organic solvents and subsequently adding water under

standard conditions. The technique where changing the pH causes the precipitation is only applicable to those compounds in which solubility is dependent on the pH value. When a solution contains a solute (solid e.g. drug) at the limit of its solubility at any given temperature and pressure, it is said to be *saturated*. If the saturation limit is exceeded, the solution is *supersaturated*. Supersaturation is the thermodynamic driving force for both crystal nucleation and growth. A supersaturated solution can also be achieved by evaporating, cooling or heating the solution or by adding a precipitant (Boistelle and Astier, 1988).

In Fig. 5, point A shows the zone where the solution is undersaturated and any crystals present in the system would dissolve. When the concentration increases at a constant temperature, the solution reaches the saturation point B. At concentrations greater than B, the solution is supersaturated.

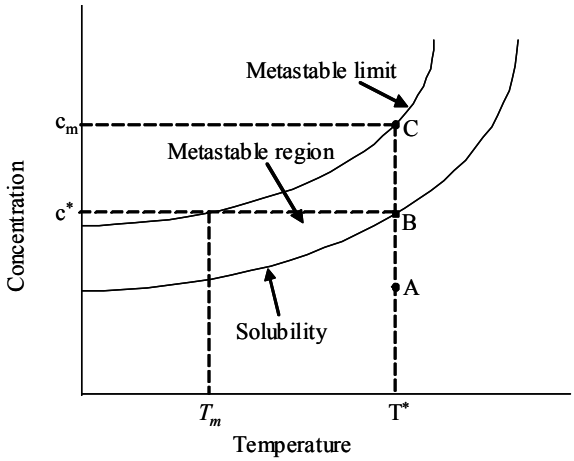

Figure 5. The solubility/supersolubility diagram.

Nucleation (see section 2.2.4) will, however, not occur until the concentration reaches point C, which defines what is called the metastable limit. The metastable region (c_m-c^* or T_m-T^*) varies from one substance to another and also within one substance because of impurities.

2.2.4 Crystal nucleation

Primary nucleation

Nucleation is the process that precedes crystallisation. Within this process, a nucleus, onto which a crystal can grow, develops. If a solution does not contain any solid foreign

particle or any roughness of the walls of its container, nuclei can be formed by homogeneous type (Myerson and Ginde, 1993). In primary homogeneous nucleation, the molecules randomly collide with each other forming small aggregates (Boistelle and Astier, 1988). The formation of the aggregates continues up to a critical size above which the nuclei transform into crystals.

Most primary nucleation is of the heterogeneous type as it is almost impossible to remove foreign bodies completely from crystallising systems. Still homogeneous nucleation does form the basis of several nucleation theories (Myerson and Ginde, 1993; Mullin, 2001). Heterogeneous nucleation takes place on foreign surfaces, e.g. on the walls of the crystallisation vessel, on the surface of the stirrer or any other added seed crystal, or on dust present in the solution (Boistelle and Astier, 1988). The presence of foreign substrates catalyses the nucleation and can induce nucleation at a lower supersaturation.

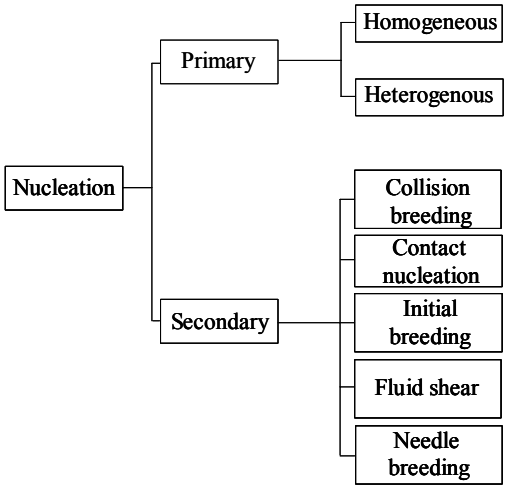

Figure 6. Classification of types of nucleation

Secondary nucleation

All nucleation that occurs after primary nucleation is called secondary nucleation. When crystals of the solute are present or deliberately added, nucleation occurs at a supersaturation lower than the one needed for primary nucleation (Mullin, 2001). Secondary nucleation can occur through several different mechanisms (Davey and Garside, 2000; Myerson and Ginde, 1993). The most common mechanism of secondary nucleation, *contact nucleation,* can result from a disturbance occurring on the surface of a growing crystal. Possible contact sources are the crystalliser, impeller or another crystal. *Collision* or *attrition breeding* results from crystal fragments that serve as nucleation sites. The collision breeding is dependent on e.g. crystal hardness (Myerson and Ginde, 1993). In *initial* or *dust breeding,* the secondary nuclei originate from the seed crystals. Small crystallites are formed on the seed crystal surface and when placed into solution the crystallites become new centres for growth (Myerson and Ginde, 1993; Davey and

Garside, 2000). At a high level of supersaturation crystals are needle-like. They are often fragile and may break into small parts. These crystal fragments serve as nucleation sites and the process is called *needle breeding*. *Shear nucleation* has also been presented as one mechanism of secondary nucleation, when shear forces due to solution flowing past crystals may cause crystal breakage and produce secondary nuclei on the surface (Davey and Garside, 2000).

2.2.5 Crystal growth

When stable nuclei, i.e. particles larger than the critical size, have been formed, they begin to grow into crystals of visible size (Mullin, 2001). When a growth unit reaches the crystal surface, it either integrates into the lattice or returns to the fluid phase. The ability of a surface to capture arriving growth units is dependent upon e.g. the structure, bonds and defects (Garside, 1984; Boistelle and Astier, 1998; Davey and Garside, 2000).

There are several possible pathways by which a molecule passes from the solution to become integrated into lattice position on a growing-phase crystal. Crystals are thought to grow in a *layer-by-layer* fashion. Fig. 7 shows the three possible sites for the molecule to incorporate into the crystal surface. At site A, the molecule is only attached to the surface of a growing layer (*flat*), at site B, the molecule is attached to both the surface and the growing layer (*stepped*), while at site C, the molecule is attached at three surfaces (*kinked*). Molecules tend to bond at locations where they have the maximum number of nearest neighbours as these are the most energetically favourable sites (Myerson and Ginde, 1993). The growth units are believed to adsorb onto the crystal surface (A) followed by diffusion along the surface to a step site (B), and then further diffused to a kink (C), where the incorporation takes place (Mullin, 2001; Myerson and Ginde, 1993).

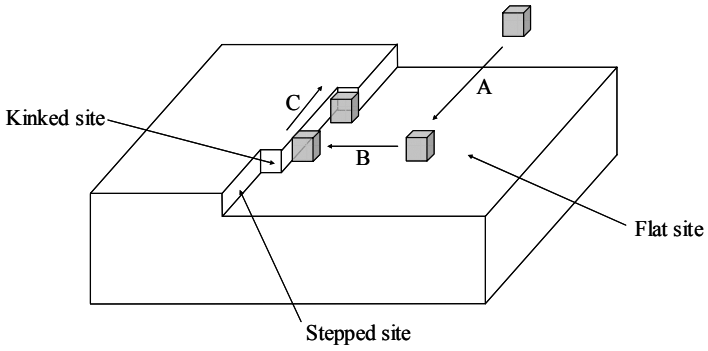

Figure 7. Kossel´s model of a growing crystal surface

When the surface of a crystal is rough there are many potential kink sites, and new growth units arriving at the surface will find a growth site (Fig. 8) (Garside, 1984). The crystal growth is therefore *continuous*. On the other hand, when the interface is smooth, growth is more difficult. If the growth units do not find a growth site, they either return to the liquid phase or form two-dimensional circular clusters on a flat surface (Fig. 8) (Davey and Garside, 2000). These islands act as new kink sites where additional growth units can join the surface. When a complete layer has been formed, the crystal has grown by one monolayer (Boistelle and Astier, 1988).

Crystal growth like this is referred to as *two-dimensional growth* or *surface nucleation*. Two-dimensional growth can be mononuclear when there is only one cluster which spreads across the surface at the same time. In the polynuclear model several clusters simultaneously spread on the crystal surface. In contrast to the mononuclear growth where the whole crystal surface is covered before the next cluster is formed, this model allows several layers to grow at the same time.

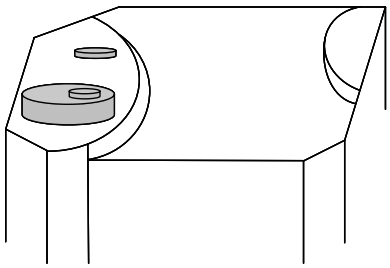

Figure 8. Two-dimensional crystal growth on a flat site.

Most crystals contain imperfections (see section 2.2.6); most commonly screw dislocations, providing one or more steps which can spread over the surface (Boistelle and Astier, 1988; Myerson and Ginde, 1993). Molecules absorb on the crystal surface and diffuse to the top step of the dislocation and the surface becomes a spiral staircase. When one layer is complete, the dislocation still exists and the *spiral growth* can continue. Growth can continue to a certain maximum determined by the supersaturation in the medium in which the crystal is growing (Mullin, 2001).

2.2.6 Imperfections in crystals

In a crystal, each atom or molecule has a precise location, forming a continuous structure (Mullin, 2001). If the structure is disrupted in any way, the crystal is said to have imperfections. Crystals are always imperfect in some sense, and although the defects usually are small, they might have an impact on some important chemical and physical factors, e.g. dissolution, solubility, and melting point (Burt and Mitchell, 1981). The defects in organic crystals can be divided into point, line and surface defects.

Point defects

A point defect is a defect that occurs at a specific lattice point. The most common point defects are presented in Fig. 9. *Vacancies* are lattice sites from which units are missing (Myerson and Ginde, 1993; Mullin, 2001). The missing units may be atoms, molecules or ions. A foreign unit occupying positions between the regular lattice sites of the crystals is called *interstitial impurity*. When a foreign unit occupies a regular lattice site, taking the place of the host atom, it is a so-called *substitutional impurity*. These defects are called point units, as they involve a single unit of the crystal structure.

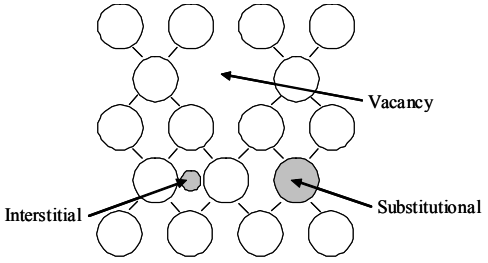

Figure 9. A schematic model of vacancy, interstitial and substitutional point defects

Line defects (dislocations)

Line defects are defects that extend through the crystal along a one-dimensional boundary, such as a line or a curve. These defects are formed when planes of atoms are out of place. The two main types of line defects are the *edge* and *screw dislocations* (Mullin, 2001). An *edge* dislocation is illustrated in Fig. 10. The figure shows that half of the vertical row of atoms is missing. The position of the dislocation is marked by an arrow. The lattice points are displaced in the region of the dislocation but get smaller and finally return to normal. The other type of line defect is the screw dislocation. In screw dislocations, atoms have moved one lattice unit higher at the edge forming a cotinuous spiral-formed step. Without dislocation, the movement of atoms relative to each other would be very difficult, and no plastic deformation would be possible. The enormous number of dislocations is also the reason why metallic parts bend during manufacturing. Ceramic materials, for exmple, are brittle as they do not contain as many dislocations.

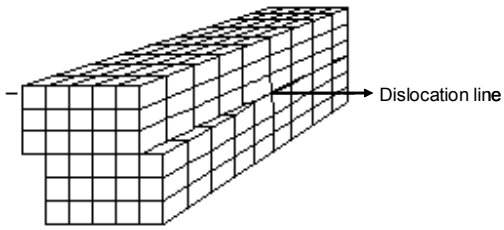

Figure 10. Line dislocation

Surface defects

Surface defects occur wherever the crystalline structure of the material is not continuous across a plane. Surface, planar, or interfacial defects are divided into *crystallite, tilt* and *twist boundaries*. These boundaries are formed in crystalline materials as the result of mechanical or thermal stresses or irregular growth (Mullin, 2001). Crystallite boundaries, for example, can be created when the crystalline structure does not match perfectly with the crystalline structure in the neighboring area (Fig.11). The area between these crystalline areas (crystallites) is called a crystallite boundary.

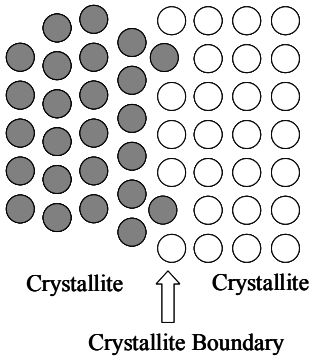

Figure 11. Crystallite boundary between two crystalline areas.

2.2.7 Factors affecting crystal properties

The *degree of saturation* is the critical parameter controlling the rate of nucleation (Myerson and Ginde, 1993). At a high supersaturation, the presence of fragments that serve as a source of secondary nuclei, is greater and results in a larger number of nuclei. The number of crystals is dependent on the nucleation and thus "one nucleus, one large crystal; a billion nuclei, a billion tiny crystals" (Byrn et al, 1999). As the degree of saturation increases, the crystal size distribution tends to reduce and additionally the habit tends to change towards acicular-shaped (Haleblian, 1975).

When crystal growth takes place in the presence of *impurities*, the growth rates of the crystal can be strongly affected. Impurities, such as ions and surface-active agents, may decrease the amount of molecules incorporating onto the crystal surface by selective adsorption (Dirksen and Ring, 1991). Impurities may affect the crystal morphology even at a very low concentration (ppm), but usually they become more efficient with increasing concentration (Davey and Garside, 2000; Mullin 2001). Sometimes impurities are deliberately added to achieve the desired crystal morphology.

The cooling of a solution is one of the most widely used methods for achieving the supersaturation essential for crystallisation (Jones and Mullin, 1974). At low cooling temperatures the crystal size decreases owing to a higher level of supersaturation and an

increase in nucleation rate (Myerson and Ginde, 1993). The cooling rate also affects the crystal habit through an effect on the degree of supersaturation. Rapid cooling results in needle-shaped or thin plate-shaped crystals.

The crystal size can be altered by varying the *stirring* rate owing to its influence upon nucleation rate (Mackellar et al. 1994). Generally, rapid stirring is said to enhance the nucleation rate resulting in smaller crystal size, although this is not always the case (Mullin, 2001). In some cases, stirring might also lead to a thinning of the absorbed layer (in secondary nucleation), causing fewer fragments and thereby leading to lower nucleation rates (Myerson and Ginde, 1993). The rate of stirring must be strong enough to provide a complete contact between the crystals and the solvent. When a particular stirring rate is reached, it should not be exceeded as this could cause a mechanical breakage of the crystals.

2.2.8 Physical stability of suspensions

Since a suspension exists in more than one state (liquid and solid), it possesses some disadvantages relative to other dosage forms. Suspensions are thermodynamically unstable systems, i.e. they always tend toward ultimate loss of stability. The primary disadvantage is their physical instability, such as the difficulty of redispersability of the sediment and crystal growth (Akers et al., 1987). A suspension must remain sufficiently homogeneous for at least the period of time necessary to remove and administer the required amount after shaking.

The rate of sedimentation of a suspended phase depends on several factors which may be controlled by pharmaceutical manipulation. For example, by reducing the particle size or by increasing the viscosity and density of the dispersion medium (see sections 2.2.2 and 2.2.8), the rate of sedimentation can be retarded. Even though the particle size of a compound is small when the suspension is first prepared, a certain degree of crystal growth always occurs during storage. Over a period of time the small crystals will diminish further, whereas the larger particles will increase in size, owing to a difference in solubility rates of particles of different sizes (Ostwalt ripening). A number of additives such as polymers and surfactants have been suggested to prevent crystal growth (Motawi et al., 1982). A change from one pseudopolymorphic form to a thermodynamically more stable crystal form, or a change in the crystal habit due to the degree of hydration of the

compound may lead to crystal growth in the suspensions. This may cause caking and deflocculation of the suspension (Khankari and Grant, 1995). The effect of temperature changes is important when measuring physical stability as it might affect both the solubility and the recrystallisation of the suspended compound. Chemical and microbial stability factors are also relevant when studying the stability of suspensions.

2.2.9 Methods for analysing suspensions

Microscopy

Optical microscopy is the only technique by which it is possible to achieve information about both the morphology and size distribution of the crystals under study (Brittain, 1999). This widely used method is an important tool for characterising different polymorphs and pseudopolymorphs as the crystal habit usually changes with the structure. The magnification of an optical microscope is usually not beyond 600x, which might limit its use when observing microcrystalline materials. When a higher magnification level is needed, the electron microscope can be used as a complementary method on account of its very high level of magnification (90 000x).

Structure of the solid material

X-ray powder diffraction (XRPD) has been used in two main areas, for the fingerprint characterisation of crystalline materials and the determination of polymorphs (Cullity, 1978).

Fig. 12 illustrates the principle of x-ray diffraction technique which is based on Bragg's law. This describes the diffraction of monochromatic x-ray radiation impinging on a plane of atoms. The two paths, A and B for the incident and diffracted beams, differ in length by $n\lambda = 2d \sin\theta$ where d is the distance between atomic layers in a crystal and θ the angle of beam diffraction. The variable lambda (λ) is the wavelength of the incident x-ray beam, and n is the order of the diffraction pattern. Quantitative determination of the

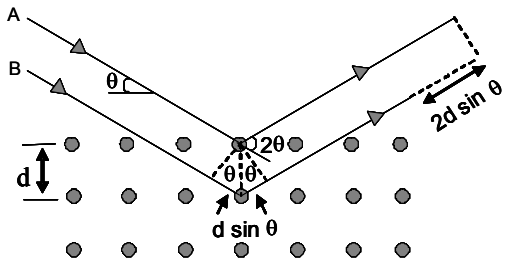

Figure 12. Schematic diagram for determining Bragg's law

estimated amount of different polymorphs can be difficult due to preferred orientation. Preferred orientation can be defined as a condition where the distribution of crystal orientation is non-random (Cullity, 1978). The changes in the intensity of the diffraction maximum can be explained by this. Needle-shaped and plate-like particles are prone to preferred orientation. Yet these effects can be minimised by reducing the particle size (Byrn et al., 1999). The presence of overlapping reflections can also make the determination of intensity more difficult (Agatonovic-Kustrin et al., 1999). XRPD has also been used to quantify sample crystallinity, even though no clearly defined diffraction peaks can be recorded with amorphous materials.

Near-infrared (NIR) was discovered in 1800 as the first non-visible region in the absorption spectra (Blanco et al., 1998). Near-infrared spectroscopy covers the electromagnetic spectrum from 760 to 2600 nm. Like the other vibrational spectroscopy methods, NIR spectroscopy measures stretchings and bending of bonds between atoms. All organic bonds have absorption bands in the NIR region but the method is particularly used for quantitative measurements of O-H, N-H, and C=O bonds. NIR spectroscopy is widely used for quantification of water and it can also be used to examine the state of water. The most intense absorption bands of pure water in the NIR region are found around 1450 and 1940 nm (Osborne et al., 1993). NIR spectroscopy offers a number of important advantages, being a fast, non-destructive method of high precision that requires minimal sample preparation. Raw data often needs to be mathematically processed before removed in the spectra. The first and the second derivatives are most commonly used.

Thermal methods

By using a hot stage on the microscope, any changes in a solid can be related to temperature by direct observation (Byrn et al., 1999). Hot stage microscopy with imaging facilities is an important supportive tool for the characterisation of melting, desolvation, crystallisation and solubility as a function of temperature (Brittain, 1999). Changes in crystal morphology during heating may indicate a change from one crystal form to another, while a change in the transparency may be caused by dehydration prior to melting (Byrn et al., 1999).

Differential scanning calorimetry (DSC) is a routinely used thermal analytical technique for measuring phase transitions (Giron, 1998; Clas et al., 1999; Bond et al., 2002). DSC is used to measure the heat flow into and out of a sample cell with respect to a reference cell in a controlled atmosphere and over a wide temperature range (Byrn et al., 1999; Giron, 1998). The result of a DSC analysis is a thermogram, where the endotherms represent processes in which heat is absorbed (e.g. desolvation, melting, and phase transitions) (Brittain, 1999). The exotherms represent processes where energy is released, such as crystallisation. This method together with thermogravimetric analysis (TGA) is particularly useful in the study of hydrates with dehydration steps at low temperatures (Giron, 1995).

In TGA, the measured parameter is the weight loss of the material as a function of the applied temperature (Brittain 1998). This may involve controlled heating or cooling or a maintained constant temperature. The TGA is most commonly used to study the desolvation processes of hydrates and other solvates and can be used as an adjunct to Karl-Fisher titrations for the determination of moisture.

Rheology and mechanical properties

Rheology is used to define the consistency and can be described by viscosity (thickness) and elasticity (stickiness) of a product (Blomstedt, 2000). Rheology is a viscosity measurement by which it is possible to characterise the flow behaviour and determine the structure of a material. Gases and liquids are usually described as viscous fluids and solids as elastic materials. Several materials (e.g. food products) show both viscous and elastic properties (i.e. viscoelastic), as they are able to store some of the deformation energy while some of it is lost. The main types of viscometers are rotational and capillary. The cone-plate viscometer, an example of a rotational viscometer, consists of a flat circular plate with a wide-angle cone placed centrally above it. The cone just touches the plate and the sample is loaded into the gap.

Dynamic mechanical analysis (DMA) supplies information about the viscoelastic properties of pharmaceutical and biomedical systems (Menard, 1999; Jones, 1999). It enables the measurement of the viscosity of the material during applied force and stiffness (modulus) from the sample recovery (Menard, 1999). These properties are often described as the ability to lose energy as heat and the ability to recover from deformation.

DMA is a versatile technique that may be used to simultaneously characterise both rheological and thermal properties of a wide range of sample types (Jones, 1999). Dynamic mechanical testing methods have been widely used in the characterization of viscoelastic materials, particularly in the polymer sciences (Craig and Johnson, 1995; Giron, 1998).

3 AIMS OF THE STUDY

The unpleasant gritty texture of phytosterols and the poor solubility in oil or water has caused several problems in their preparation and administration. In the present study, a non-esterified phytosterol suspension in oil was prepared for oral administration. To avoid the gritty sensation in the mouth, the preparation of this suspension was optimised in order to achieve a microcrystalline particle size. As this phytosterol suspension is intended for addition into cholesterol-lowering food and pharmaceutical products, the knowledge of its thermal changes during process is of importance as well as the physical stability during storage. A clinical test performed earlier using a similar microcrystalline suspension revealed a significant reduction of cholesterol levels. A dynamic *in vitro* study has been performed to understand the mechanism by which phytosterols interfere with cholesterol absorption.

The specific aims were

- to prepare a microcrystalline phytosterol suspension in oil by precipitation method and to describe how different processing parameters affect crystal properties of phytosterol,
- to investigate the different pseudopolymorphic forms of phytosterol in the presence and absence of water,
- to evaluate physical properties of the microcrystalline suspension during storage and to characterise the changes that occurred during heating, and
- to study the effects of the microcrystalline phytosterol suspension on cholesterol solubilisation *in vitro*, and to compare the effect of medium and long chain length lipid on the solubilisation.

4 EXPERIMENTAL

A more detailed description of the materials and methods is given in the respective original publications (I-V).

4.1 Materials

β-sitosterol (β-sitosterol for biochemistry) was purchased from Merck, Germany (I,V), DRT (Les Dérivés Résiniques et Terpéniques), France (II,III) and from Calbiochem (Biosciences Inc., La Jolla, USA) (IV). According to our GC-MS analyses, the phytosterol from Merck contained 79% pure β-sitosterol (14% β-sitostanol, 6% campesterol and 2% campestanol). The wood-based phytosterol from DRT contained ≥78.5% β-sitosterol (10% β-sitostanol, 8.7% campesterol and campestanol 1%) and the Calbiochem phytosterol with 75.5% pure β-sitosterol (13.0% β-sitostanol). The cholesterol was a Sigma Chemical Co. (St. Louis, USA) product (V). According to the GC-MS analyses the cholesterol was 99% pure.

The MCT oil (medium chain triglyceride) was from SHS International Ltd., UK (I-IV). The purified MCT oil that was used contained mainly caprylic and capric acid-based triglycerides. The other oils used were soybean oil (LCT; ≥ 85% linoleic, oleic and linolenic acid), which was a Sigma product and Captex 355 (MCT; ≥ 95% caprylic and capric acid) a product from Abitech Corporation (Janesville, USA) (V). Polyoxyethylene 20 sorbitan mono-oleate (polysorbate 80), (Tween 80® for parenteral use, ICI Surfactant, Germany) was added when studying the effect of surfactant on the crystal size distribution (III).

4.2 Preparation of phytosterol crystals (I)

Anhydrous phytosterol was prepared by crystallising the sterol from supersaturated acetone and hydrated from crystals from acetone-water 95:5 (v/v) mixture. Anhydrous crystals were dried over night at 80 °C and stored over silica gel at 20±2 °C. Hydrated phytosterol was stored over a saturated solution of K_2SO_4 at 20±2 °C, which corresponded to 98% relative humidity.

4.3 Preparation of the suspensions (I-V)

The suspensions were prepared by heating phytosterol or cholesterol and oil (MCT, Captex 355 or Soybean oil) in a vessel while stirring. Phytosterol/cholesterol was dissolved at about 100 °C and a clear solution was formed.

Suspensions were prepared by heating the phytosterol (I-IV) or cholesterol (V) in medium chain triglyceride (I-IV) oil or long chain triglyceride (V) up to 100-110 °C until a clear solution was formed. During cooling, at about 90 °C, purified water of the same temperature was added. The suspension was stirred until it reached room temperature (I-III). Additionally, the suspensions were prepared by rapid cooling (II-V) in order to achieve a smaller crystal size. The first part of the process was the same as explained earlier. However, following the addition of water (90°C) to the clear solution, in this case, the vessel was then immediately immersed in ice. The suspension was subsequently stirred until it reached room temperature (+25 °C). After preparation the samples were kept at room temperature for half an hour. The samples were then stored in airtight plastic containers at +4 °C (I-V) and -19 °C (II). Compositions of the sterol suspensions are presented in Table 2.

Table 2. Compositions of the studied suspensions in publications I-V.
The proportions of phytosterol: oil: water (w/w).

I	II	III	IV	V
20:80:00	05:90:05	17:70:13	05:82:13	17:70:13
20:80:01	05:75:20		17:70:13	
20:80:05	17:78:05		17:83:00	17:83*
20:80:10	17:63:20		30:57:13	
20:80:15	30:65:05			
10:80:05	30:50:20			
05:80:05				

* Cholesterol: MCT/LCT

When studying the effect of surface-active agent on the crystal size, polyoxyethylene 20 sorbitan mono-oleate (polysorbate 80), was added to the water before combining the water and phytosterol-oil mixture (III). Batches with surface-active agent contained 1% (w/w) polysorbate 80. The effect of cooling temperature was studied by immersing the vessel in which the suspensions were made in an ice/water bath at six different levels of

temperatures: 0°C, 10°C, 20°C, 30°C, 40 °C and 50 °C (±2 °C). The cooling temperature was measured every 30 seconds for four minutes. By this time the suspensions immersed in either 0 °C or 10 °C had reached at least room temperature. To investigate the effects of stirring time and stirring rate, the batches were prepared with a mixer (Kenwood Chef Classic KM 400, Great Britain). The metallic bowl was covered with a frozen shell to cool down the suspension in the same way as was done when immersing the beaker in ice. The rotation speed of the mixer was 0, 60, 120, 200 and 250 rpm. Stirring time was, in this case, 3 minutes. At this point the mass had reached room temperature and visually the system was equally well agitated. Every 30 seconds the bowl was cleaned on the sides with a spatula. The effect of stirring time was studied at 250 rpm at the following times: 30, 60, 120, 240 and 360 s. In these cases also, the bowl was cleaned on its sides every 30 seconds.

4.4 Analysis of phytosterol crystals and suspensions

Optical Microscopy (I-IV)

The size distribution and the habit of the crystals were evaluated by optical microscopy (Leica DMLB, Leica Mikroskopie und Systeme GmbH, Germany). The samples were prepared by taking a small amount of the suspension and diluting it with a small amount of MCT oil, because of the high viscosity. The crystal size of 300 particles per sample was measured manually using a measuring rod. Observations regarding crystal habit during storage were made on a visual basis (II). Crystal size and habit determinations were carried out on the samples stored at +4°C, initially, and after 1, 2, 4, 8, 12 and 16 weeks of storage. Samples stored at -19 °C were determined initially and after 12 weeks of storage.

X-Ray Powder Diffraction (XRPD) (I-IV)

The crystal structure of the phytosterol suspensions was measured using an x-ray powder diffractometry (XRPD) with a theta-theta diffractometer (Bruker AXS, D8 Advance, Germany) in a symmetrical reflection mode with Cu K_α radiation (1.54 Å) using Göbel mirror bent gradient multilayer optics. The scattered intensities were measured with a scintillation counter. The angular range was from 3° to 30° using a step size of 0.05° and the measuring time was 1s/increment. The x-ray diffraction measurements for the thermal

analyses were made as described although each sample was measured at several temperatures (VT-XRPD) between 25 and 60 °C (IV).

Differential scanning calorimetry (DSC)(IV)

A differential scanning calorimeter (Mettler DSC30 with TC15 TA processor, Mettler-Toledo AG, Switzerland) was used in the study. The analyses were made by STARe Thermal Analysis System version 3.1 (Mettler-Toledo-AG). The DSC was calibrated using the melting temperature of n-pentane, n-hexane, mercury, ion changed water, gallium and indium. In addition, the melting point of indium was checked once during the determinations. Each sample was first heated from 25 to 60 °C/min. This was followed by cooling down to 25 °C and heating back to 60 °C, 5 °C/min. The samples (10-30 mg) were hermetically sealed in 40 µl aluminium pans ($n \geq 2$; analysed the day after preparation). An empty aluminium pan was used as reference sample.

Dynamic mechanical analysis (DMA) (IV)

A dynamic-mechanical analyzer (DMA 242, Netzsch-Gerätebau GmbH, Selb, Germany) was used to observe the storage modulus, loss modulus and tan δ as a function of temperature. The DMA was equipped with a DMA 242 measuring unit, a cooling gas controller, a DMA 242 and TASC 414/3 controller, and Netzsch DMA 242 software version 1.5. The instrument was used with a disk-bending sample holder. The sample holder consisted of three stainless steel layers with circular holes of 30 and 26 mm in diameter. The layers were separated by two 0.05 mm thin PET plastic films and the sample was located between these two films. During the analysis, sinusoidally varying stress was applied to the material observed as a function of temperature. The samples were analysed on the day after preparation, at frequencies of 0.1, 0.5, 1, 2.5 and 5 Hz and the amplitude was 7.5 µm. The samples were heated at a heating rate of 2 °C/min from 30 °C to 60 °C. These analyses were used to obtain three major parameters: (1) the storage modulus, G′, which measured the amount of energy stored in the material during deformation; (2) the loss modulus, G″, which is proportional to the amount of energy dissipated per cycle; and (3) the loss tan δ, which corresponds to the ratio of energy stored per cycle (Menard, 1999).

Thermogravimetric (TG) analysis (I)

The thermogravimetric (TG) analyses of the crystalline phytosterol samples were performed with a Mettler TGA/SDTA analyzer (model 851^e, Mettler Toledo, Switzerland). Samples (5 mg) were analysed in open aluminium pans under nitrogen flow (50 ml/ min) at 25-150 °C with a heating rate of 10 °C/min. The temperature scale of the equipment was calibrated with zinc and indium, while the microbalance was calibrated with calcium carbonate.

Near-infrared spectroscopy (NIR) (I)

Near-infrared (NIR) spectra were measured with a Fourier transform (FT-NIR) spectrometer (Bomem MD-160 DX, Hartman&Braun, Quebec, Canada) using Bomem-GRAMS software (version 4.04, Galactic Industries, Salem, NH, USA). The spectra were measured through the bottom of a glass vial containing the sample. FT-NIR spectra of the crystals and suspensions were recorded over a range of 4000 and 10 000 cm^{-1} with a resolution of 16 cm^{-1}. Standard reflection was measured using a Teflon background (Labsphere, SRS-99-070, North Sutton, NH, USA). The samples were scanned 40 times, and each spectrum was reported as the average of these scans.

Cone and plate rheometer (I, II)

The viscosity of the suspensions was measured, as a function of shear rate, with a cone and plate rheometer (CP 5/30, Bohlin VOR Rheometer, Bohlin Reologi, Sweden). The gap between the lower and the upper plate was 0.5 mm. The measuring temperature was 20°C.

Karl Fisher Analysis (I, III)

The water content of the phytosterol starting material was determined by Karl Fisher titrimetry (Mettler DL35, Mettler-Toledo, Switzerland). Hydranal®- Titrant 2 (Sigma, USA) was used as the titer and Hydranal® Solvent CM (Sigma, USA) as the solvent.

4.5 Dynamic *in vitro* lipolysis method (V)

In vitro lipolysis

In *in vitro* lipolysis, a pH-stat continuously titrates the fatty acids that are liberated via lipolysis. The lipolysis is carried out in a reaction medium with a low buffering capacity, thereby ensuring that fatty acids liberation causes the pH to drop. The number of moles of neutralising hydroxyl ions present in this volume of titrant is parallel to the fatty acid liberation caused by lipolysis (MacGregor et al., 1997).

The *in vitro* lipolysis experiments were performed in accordance to the previous studies described earlier by Professor Charman and his team (Sek et al., 2001; Sek et al., 2002; Kaukonen et al., 2004a; Kaukonen et al., 2004b). Cholesterol and/or phytosterol suspensions were dispersed in 9 mL of bile salts (BS)/phospholipids (PL) and mixed micelles prepared in a pH 7.5 buffer. All digests contained a total of 50 mg of LCT or MCT, i.e. 25 mg of pure triglyceride was added when only cholesterol or phytosterol suspensions were studied. Two levels of BS/PL concentrations were used to represent fed or fasted state small intestinal conditions. The amount of phytosterol contained in the dose of suspension was either 14 μmol or 28 μmol while the amount of cholesterol was 15 μmol. Cholesterol-loaded micelles with 1.5 mM cholesterol were also used to simulate the presence of endogenous or pre-solubilised cholesterol under fed state conditions. Lipolysis experiments were performed at 37°C in a stirred and thermostatted glass vessel and initiated by the addition of 1 mL of 20% pancreatin suspension. The pancreatin extract contained 10 000 TBU/ml of pancreatic lipase conferring physiologically relevant lipase activity. The lipolysis was performed over 60 min using a pH-stat titration unit (Radiometer, Copenhagen, Denmark) which maintained the pH at 7.5. The fatty acids produced by triglyceride lipolysis were titrated with 0.2 M NaOH. At the end of each experiment a lipolysis inhibitor was added to the digestion mixture to stop further digestion. Two 4 mL aliquots of the post-digestion mixtures were then ultracentrifugated in order to separate the digests into an oil phase, an aqueous phase, and a precipitated pellet. The aqueous phase was aspirated into a syringe by penetrating the side of the tube and transferred into glass vials. Finally, the samples were dried overnight in a lyophilisator (HETO LyoPro 3000, Freeze dryer) prior to analysis by gas chromatography (GC, Agilent technologies 6890N Network GC with a RTX®-5w/INTEGRA fused silica column).

Solubility determination

The equilibrium solubility of phytosterol was determined by adding anhydrous phytosterol crystals (dried over night at 80 °C, 0% RH) into the aqueous phase obtained after *in vitro* lipolysis of 50 mg of LCT or MCT in fed state micelles pre-loaded with 1.5 mM cholesterol. A total of four lipolyses were performed (both for MCT and LCT) whereafter the aqueous phase was separated by ultracentrifugation. Solid phytosterol was added, after which the samples were incubated at +37 °C for the entire period of the solubility studies and vortexed periodically. Sterol concentrations were analyzed from samples taken at the start of the experiment and after 6, 24, 48, and 120 hours of incubation by GC. The samples were filtrated prior to determination and the filtrate was collected for analysis. In order to assess the potential loss of solubilised sterol by filtration, aqueous phase samples from digests containing both cholesterol and phytosterols were analysed prior to and after filtration. The loss of sterols was ≤ 1.7%. No apparent visual changes were observed during the solubility study.

5 RESULTS AND DISCUSSION

5.1 Crystal forms of phytosterol (I-IV)

Phytosterol was observed to exist in three different crystal forms; anhydrous, hemihydrate and monohydrate crystals (I). When phytosterol was crystallised in the absence of water from acetone, anhydrous flaky-like sterol crystals were formed (I, Fig. 6a). In the presence of water phytosterol precipitated from acetone-water as needle-shaped monohydrate crystals (I, Fig. 6c-d). This crystal form is unstable and a more stable hemihydrated phytosterol was formed when approximately half of the water left the monohydrated structure. The main x-ray reflections of the three pseudopolymorphic forms are presented in Table 3.

Table 3. The main x-ray reflections of the three different pseudopolymorphic forms of phytosterol (I)

Crystal form	(Å)										
Anhydrous	17.6	11.7	8.8	7.07	5.87	5.23	3.92				
Hemihydrated	18.8	12.4	7.45	6.22	5.90	5.70	5.32	5.05	4.81	4.74	4.61
Monohydrated	17.6	11.7	7.05	5.86	5.03	4.81	4.56	3.92			

Phytosterol crystal forms in oil suspensions

The formation of phytosterol crystal structure in oil suspension is dependent on the water content (I, Fig. 8). When anhydrous phytosterol was used, oil suspensions without added water produced x-ray reflections that corresponded well to those of anhydrous phytosterol. However, if phytosterol was used as received and no water was added, the crystal form was mostly anhydrous but reflections corresponding to a hydrated phytosterol crystal form were also apparent (IV, Fig.2). When the added amount of water was 1%, both the hemihydrated and monohydrated crystal forms were observed (I, Fig. 8). As the amount of water was beyond 5%, the x-ray diffraction patterns showed that the monohydrated form was in majority (I,IV).

Further analysis indicated that the amount of phytosterol, in addition to the water content, can affect the crystal structure (II). When the suspension contained less sterol (5%), the monohydrated phytosterol was in majority, but as the sterol concentration increased (30%), so did the hemihydrated crystal form. This implies the possibility of an increased penetration of water into the crystals when sterol content was low. The suspension that

contained 5% sterol was mostly amorphous, from the large amount of oil, and thus the estimation was difficult.

Different preparation methods (i.e. changes in temperature, stirring, addition of a surface-active agent) were observed not to affect the crystal structure of phytosterol in oil-water-suspensions (III). Despite the preparation, the diffraction patterns of the suspensions included reflections of both hemihydrated and monohydrated forms indicating that the suspensions contained a mixture of both crystal forms.

Stability of phytosterol crystal forms

The sterol crystal forms, containing different amounts of phytosterol, were stable in suspensions during a storage period of 16 weeks in +4 °C and in -19 °C (II). Since the suspensions contained water (5%), the initial crystal form was a mixture of hemihydrate and monohydrate forms. A minor increase of the monohydrate form was observed, although an accurate quantitative determination of different phytosterol crystal forms in suspensions is difficult because of preferred orientation and due to the presence of some overlapping reflections.

However, the degree of crystallinity changed during the storage period and caused a typical sigmoid-shaped curve (II, Fig. 7) (Avrami, 1939). The amorphous form increased during the first four weeks whereafter it began to decrease and the degree of crystallinity returned to the initial level in twelve weeks. During the last four weeks, some decrease in the degree of crystallinity was observed again. An increase in oil content reduced the amount of crystalline form while water content had no effect. A clear increase in the degree of crystallinity was observed with growing phytosterol concentrations.

5.2 Dehydration from phytosterol

5.2.1 Dehydration from hydrated phytosterol crystals (I)

Thermal analysis (DSC, TG) of phytosterol crystals showed that the dehydration of monohydrated phytosterol was a two-step process. The monohydrated form contained approximately 1 mol water/mol phytosterol, while the dehydration to the hemihydrated form reduced the amount to 0.5 mol water/mol phytosterol. The evaluation of the phytosterol crystals using near-infrared (NIR) reflectance spectroscopy revealed the presence of two different energy states of water in the spectra of the different

pseudopolymorphs (I, Fig. 4). The less tightly bound water (absorbance maxima at 1901 nm) of the monohydrate form dehydrated at temperatures below 60 °C and a hemihydrated structure was formed. The more tightly bound water (absorbance maxima at 1949 nm), which appeared both in monohydrate and the more stable hemihydrate structure, dehydrated after further being heated up to 90 °C. The early onset of dehydration during heating is characteristic of channel hydrate dehydration (Morris and Rodrigues-Hornedo, 1992). The explanation for the ease with which phytosterol dehydrates is the migration of the water molecules along tunnels where they lie (Vippagunta et al., 2001).

5.2.2 Dehydration from phytosterol crystals in oil suspensions (IV)

The dehydration of phytosterol crystals in oil suspensions was studied at temperatures from 25 to 60 °C. The main changes in the suspensions were detected around 40 °C. In accordance to the previous section (4.2.1) the water molecules were weakly bonded to phytosterol, as the dehydration from monohydrate to hemihydrate crystal form occurred at such low temperatures. The somewhat earlier onset of dehydration compared to plain crystals is explained by the surrounding oily vehicle and the crystal size distribution, which in this study was considerable smaller. The broad endotherms, resulting from the DSC measurements (IV, Fig. 3), are typical for crystals with weakly bonded water molecules and strengthen the assumption of channel hydrates (Morris and Rodrigues-Hornedo, 1992). At the end of the study, at 60 °C, the dehydration process was not completed, since there were still both mono- and hemihydrated crystal forms left. Further heating was, however, impossible due to rapid dissolution of the crystals. With the DMA it was possible to follow the dehydration from a rheological point of view. At increasing temperatures the dehydrated water molecules were immiscible in the surrounding oil phase and acted as a lubricant between the solid phase and the oil phase (IV, Fig. 4). In addition to dehydration, an increase in temperature caused the dissolution of the smallest phytosterol crystals and larger crystals were formed at the expense of the smaller ones (Ostwald ripening). This was recognised as increased elasticity in terms of DMA measurements and as a growth in the intensities of some reflections during x-ray measurements (IV, Fig. 2). At temperatures beyond 50 °C, even the larger crystals started to dissolve, and the suspension became less elastic again. Due to the dissolution of the crystals, the x-ray reflections also started to diminish. As the water was immiscible in the

surrounding oil phase, the water molecules were available for phytosterol crystals during cooling. The DSC results suggested a reversible dehydration process.

5.3 Crystal habit and size distribution of phytosterol

For poorly soluble compounds, particle size distribution is one of the most important factors which affects bioavailability in relation to the physicochemical properties of a compound. Thus the large differences in the phytosterol crystal length and the habit modification produced by various process parameters are likely to have an effect on bioavailability of the suspension. The use of phytosterols in functional foods has faced several problems since phytosterols have an unpleasant texture. To avoid the gritty sensation in the mouth, a microcrystalline particle size is desirable. The optimum particle size for a so-called creamy product lies between 10 μm and 50 μm (Tyle, 1993; Viaene and Januszewska, 1999; Kilgast and Clegg, 2002). The crystal size is of greater significance if the crystal habit is hard and sharp than if it is flat and rounded. Crystal can be even up to 80 μm, if the habit is soft, and still have a creamy oral texture. Additionally, small crystals have a high free surface area, which facilitates the saturation of the sterol solubility in the intestinal lumen and interference with cholesterol absorption.

5.3.1 *The effect of the composition on crystal modifications (I,II)*

Phytosterols are practically insoluble in water and the solubility in MCT-oil was between 3.5-4.0% (w/w) and 1.5-2.0% (w/w) when water was present (I). Due to the low solubility in oil or a mixture of oil and water, β-sitosterol was saturated in all the studied compositions. The phytosterol crystal appearance in oil suspensions was affected by the presence of water in the suspension (I). The absence of water resulted in large platy-like particles (I, Fig. 6a). When the water content was between 5 and 20%, the crystals were acicular (I, Fig 6c-e and II). Crystal length varied according to the concentration of the sterol. The length of the acicular-shaped crystals in the water-containing oil suspensions decreased with an increasing phytosterol concentration (I, Fig. 7 and II Fig. 2). A high sterol concentration resulted in high supersaturation and thus the formation of small crystals. At a high level of supersaturation the needle-shaped crystals can be fragile and break into small parts (Myerson and Ginde, 1993). These parts act as secondary nuclei onto which molecules can attach and new crystals can be formed.

It is thought that adsorption of an impurity onto the crystal surface most likely has an inhibitory effect on crystal growth. The addition of a surface-active agent (Polysorbate 80) in the suspension did, however, only have a minor effect on the phytosterol crystal growth (III). The crystal length was reduced only if the crystals were larger, due to the preparation method. By visual examination it was recognised that these phytosterol crystals grew in width rather than in length. It seems that the surface-active agent (1%, w/w) adsorbs selectively onto a specific crystal face and only retards, into some degree, the growth in length. Similar observations of specific adsorption have been made when crystallising e.g. carbamazepine (Luhtala, 1992). Usually only a small amount of a surface-active agent is required to bring noticeable changes to crystallisation phenomena, but, for some purposes, more than 1% is needed to achieve the desired effect (Canselier, 1993).

The differences in the compositions in phytosterol suspensions in oil not only affected the crystal size distribution and the habit but also the viscosity of the suspensions (I, Fig. 5 and II). Phytosterol suspensions in oil in the absence of water caused nearly Newtonian flow behaviour since the anhydrous platy-like crystals have a tendency to align in the direction of the applied shear (Pena et al., 1995). Addition of 1% water did not affect the rheology of the suspension, even though the crystals were partially hydrated. The viscosity of the suspensions containing 5-13% water was considerably higher compared to the suspensions with plate-like crystals whereafter the viscosity systematically increased with higher phytosterol concentrations. Needle-shaped crystals have a tendency to aggregate. Yet a drastic decrease in viscosity was discovered with increasing shear rate due to rearrangement of the particles. This resulted in lower flow resistance and consequently lower viscosity (Pena et al., 1995; Zitoun et al., 2001). The viscosity of the suspension was also dependent on the phytosterol concentration. An increase in solid concentration limited the movement of the particles and increased the interactions between the particles, resulting in higher viscosity (Zitoun et al., 2001). The diminished viscosity with higher shear rates showed that the network formed by the particles was rather weak.

5.3.2 The effects of process parameters on crystal modifications (I,II, III)

Effect of cooling temperature

Cooling temperature turned out to have a remarkable effect on phytosterol particle size distribution in oil suspension due to the effect on supersaturation. Rapid crystallisation of phytosterol, produced by sudden cooling, resulted in the formation of needle crystals with a median crystal length of 23 μm. However, the suspensions still contained a distinct number of larger crystals (Fig. 12 and III, Fig. 2). With an increase in cooling temperature the crystal habit also changed to some degree. At low temperatures (≤10 °C), when the crystals were small, they were needle-like and even in shape and length, while they grew in width to a certain extent in increased temperatures (≥20 °C). Additionally the crystals were uneven in shape and length (III, Fig. 1).

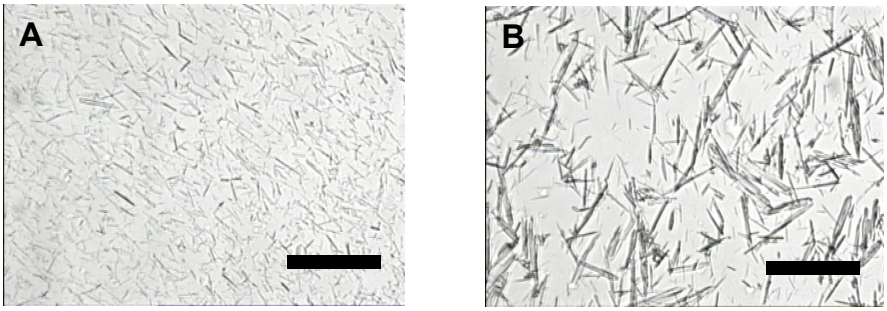

Figure 12. Microscope images of phytosterol suspensions prepared by cooling at (A) 0 °C (immersed in ice) and (B) 50 °C (bar =100 μm).

A similar decrease in crystal length was observed when crystallising porcine insulin at different temperatures (Feldmeier et al., 1991). By increasing the cooling temperature from 0 °C to 30 °C, the average crystal length increased from 15 μm to 24 μm. Similarly, Motawi et al. showed that raising the temperature from 20 to 30 °C increased the rate of sulfathiazole crystal growth about 4.5 times (1982). At low cooling temperatures, the supersaturation level is high, resulting in an increased nucleation level and a large number of small crystals (Myerson and Ginde, 1993). The increase in the viscosity of the oil as the temperature decreased was a minor factor in our study. The diminished molecular movement slowed down both nuclei formation and the growth rate of the nuclei formed (Ebian et al., 1973; Mullin, 2001).

Effect of stirring rate and time

Crystallisation at a low stirring rate (60 rpm) produced a few needle-shaped but mostly large platy-like crystals (median size 40 μm) (III, Fig. 3). By increased stirring (up to 250 rpm), it was possible to achieve a nearly two-fold decrease in the median crystal size. The reduced phytosterol crystal size observed with stronger stirring was the result of increased secondary nucleation since crystal fragments acted as nucleation sites (Myerson and Ginde, 1993). Stirring is known to have a strong influence upon the nucleation rate until a particular stirring rate is reached. Similar results were obtained by Feldmeier et al. as increased stirring (from 0 rpm to 200 rpm) reduced the crystal size of porcine insulin roughly three-fold (1991).

In addition, the stirring time has an influence on the crystal habit and size distribution. With increased stirring time, the length of the phytosterol crystals decreased (III, Fig. 4). The main recrystallisation in the oil suspension appeared at around 60 °C. If the agitation was stopped at higher a temperature, large crystals with a median crystal length of 38 μm were obtained. When stirring continued until the phytosterol suspension had reached room temperature (25 °C), the median crystal size was less than 20 μm.

5.3.3 Changes in crystal size distribution and crystal habit during storage (II)

Suspensions containing different amounts of phytosterol were stored at two different temperatures (+4 °C and -19 °C) for 16 weeks (II). The stability study showed that the viscosity of the suspensions seemed to have an influence on crystal length during storage although the crystal habit remained the same. Suspensions containing 5% (w/w) phytosterol had a much lower viscosity than the two other suspensions made with higher concentrations (II, Fig. 4). In 16 weeks the median phytosterol crystal length in this suspension increased from ~40 μm to ~60 μm when stored at +4 °C. With increasing sterol concentration and consequently at higher viscosity (see also section 4.3.1), the diffusion-controlled process involved in the crystal growth was retarded. Thus, there was no change in crystal size distribution in suspensions containing 17% and 30% phytosterol.

On the other hand, phytosterol crystals in suspensions stored at -19 °C did not change in size during the period of storage at any concentrations. Equally, lower temperatures

decreased the molecular diffusion onto the surface of the particle, thereby affecting the crystal growth (Motawi et al., 1982).

5.4 Effects on cholesterol solubilisation *in vitro (V)*

Previously an *in vivo* study with 155 hypercholesterolemic volunteers has been performed to evaluate the cholesterol-lowering effect of the microcrystalline phytosterol suspension (Christiansen et al., 2001a). The *in vitro* study was performed to understand the mechanism by which phytosterols interfere with cholesterol absorption. The study was performed using a similar suspension as in the *in vivo* study, containing either a medium chain or long chain triglyceride.

In vitro digestion of cholesterol and/ phytosterol during lipolysis

The prediction of the solubilisation profile for a poorly water-soluble compound in a lipid formulation is complex and cannot be accurately achieved using simple dispersion methods. Recently various *in vitro* lipolysis methods have been used as models of solubilisation of water-insoluble substances (Trautwein et al., 2003; Zangenberg et al., 2001a; Zangenberg et al., 2001b). Digestion experiments with LCT or MCT lipids containing either cholesterol or phytosterol were made to establish their individual solubilisation behavior.

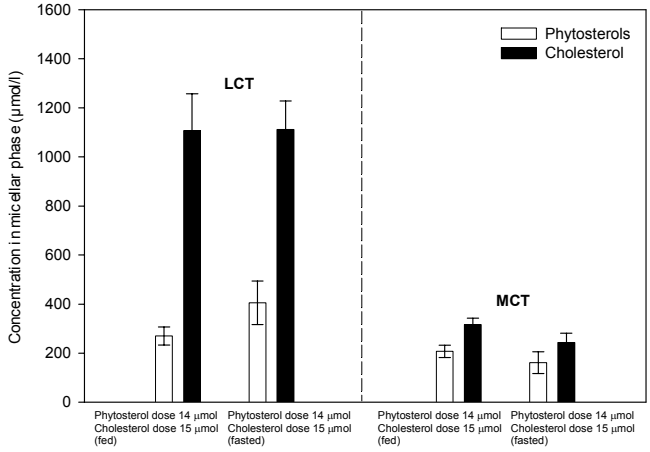

Figure 13. Solubilisation of co-administered phytosterol and cholesterol into mixed micelles. The sterols were suspended in either LCT or MCT (n=3) (V, Figure 2).

The mixed micellar phase containing digestion products of LCT was able to incorporate significantly higher amounts of cholesterol than those obtained after digestion of MCT (IV, Fig. 1a). Replacing the long chain length triglyceride with one containing medium chain length fatty acids decreased the amount of solubilised cholesterol by 50% in the aqueous phase. Similar serum reduction has been observed earlier *in vivo* with both cholesterol and fat-soluble vitamins following ingestion of MCT compared to LCT (Kritchevsky and Tepper, 1965; Borel et al., 1998).

Co-administration of phytosterol and cholesterol, particularly when suspended in MCT, showed a significant reduction of solubilised cholesterol in digests using cholesterol-free micelles (Fig. 13 and IV, Fig. 2). The amount of cholesterol solubilised into the micellar phase was further reduced to ~24% of the administered dose. Digestion of co-administered phytosterol and cholesterol suspensions in either LCT or MCT was performed to simulate dietary intake of both sterols and to study the capacity of phytosterol to reduce cholesterol solubilisation. Despite the promising results with co-administered sterols, it was not possible to displace pre-solubilised cholesterol during digestion in either MCT or LCT systems (IV, Fig. 3). These results were supported by the solubility studies (IV, Fig 4). Mel'nikov et al. showed in their study that phytosterols and phytostanols were able to compete with and displace pre-solubilised cholesterol from mixed micelles (2003b). This is explained by the saturated micelles ascribable to higher concentrations of lipolysis products. The pre-solubilised micelles were used to simulate the presence of endogenous cholesterol.

When cholesterol and phytosterols are simultaneously present in the intestinal lumen during lipid digestion, it seems that both will be solubilised by the mixed micelles. However, it is obvious that phytosterols, together with MCT, act as a more efficient limiting factor of the micelle's capacity to solubilise cholesterol. Thus, the results suggest that micelles, containing MCT digest products, would provide a more effective cholesterol-lowering agent compared to LCT. In addition to the transporter-associated mechanisms, the dynamic competition between these two sterols is one of the mechanisms contributing to the reduction of cholesterol absorption.

6 CONCLUSIONS

In the present study, preparation of a microcrystalline phytosterol suspension in oil and water is described. By changing the amount of sterol and water, it was possible to influence the crystal habit and size distribution. Without added water, anhydrous plate-like crystals were achieved. Addition of water to the composition produced mostly needle-shaped monohydrated crystals. Monohydrated crystal form is unstable and dehydrates to a hemihydrated form at a low temperature. Dehydration was similar both from plain crystals and crystals suspended in oil. The early onset of dehydration is characteristic of channel hydrates and it appears that water molecules are weakly bonded. Due to supersaturation, higher sterol concentrations resulted in the formation of small crystals. No changes in crystal habit, size distribution or crystal form were observed during storage of these small crystal sized suspensions, at either +4 °C or -19 °C for four months. A microcrystalline particle size was desirable to avoid a gritty feeling in the mouth. In addition to the saturation level, optimisation of the preparation process had a great impact on the crystal size distribution and crystal habit as well. By changing the process parameters, i.e. cooling temperature and stirring, it was possible to achieve the optimum crystal size for a so-called creamy suspension. In addition to phytosterol, the choice of lipid in the suspensions was observed to have a significant effect on solubilisation of sterols into the mixed micelles. The dynamic *in vitro* studies, in which medium and long chain length lipids were compared, showed that phytosterols formulated in MCT efficiently displaced cholesterol from mixed micelles, thereby contributing to the reduction of intestinal cholesterol absorption. Solubilisation into intestinal mixed micelles is a prerequisite for sterols to reach the site of absorption. As a result, the cholesterol absorption is decreased, which agrees with previous *in vivo* studies with a similar suspension.

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