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EFECTE DE LA INGESTA DE FITOSTEROLS I PECTINES SOBRE EL PERFIL LIPÍDIC EN CONILLS D'ÍNDIES

Gemma Brufau Donés

Barcelona, 28 de Setembre del 2006

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EFECTE DE LA INGESTA DE FITOSTEROLS I PECTINES SOBRE EL PERFIL LIPÍDIC EN CONILLS D'ÍNDIES

Memòria presentada per Gemma Brufau Donés, per optar al títol de Doctor en Farmàcia

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Barcelona, 28 de Setembre del 2006

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CERTIFIQUEN

Que la memòria titulada "EFECTE DE LA INGESTA DE FITOSTEROLS I PECTINES SOBRE EL PERFIL LIPÍDIC EN CONILLS D'ÍNDIES" presentada per GEMMA BRUFAU DONÉS per optar al grau de Doctor en Farmàcia, ha estat realitzada sota la seva direcció. Així mateix, consideren finalitzat aquest treball i autoritzen la seva presentació, per a que sigui jutjat per la Comissió corresponent.

I per a que així consti, firmen aquest certificat a Barcelona amb data 28 de Setembre del 2006.

Dra. Magda Rafecas Dr. Miguel Ángel Canela Dr. Rafael Codony

For you, Pascal

Als meus pares i a l’Anna

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ABBREVIATIONS

ABC	ATP-Binding Cassette
ACAT	Acyl-coenzime A: cholesterol acyl transferase
AHA	American Heart Association
Apo	Apolipoprotein
CE	Cholesterol esters
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
Chol	Cholesterol
CM	Chylomicron
CVA	Cerebrovascular accident
DM	Degree of methylation
FA	Fatty acids
FFA	Free fatty acids
GalA	Galacturonic acid
GC	Gas-chromatography
HDL	High density lipoprotein
HG	Homogalacturonan
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzime A
IDL	Intermediate density lipoprotein
INC	Instituto Nacional de Consumo
LCAT	Lecitin: cholesterol acyl transferase
LDL	Low density lipoprotein
Lp	Lipoprotein
LPL	Lipoprotein lipase
MUFA	Monounsaturated fatty acids
NCEP	National Cholesterol Education Program
NPC1L1	Niemann-Pick C1 Like 1 protein
PL	Phospholipids
PUFA	Polyunsaturated fatty acids
RG	Rhamnogalacturonan
SCFA	Short chain fatty acids
SFA	Saturated fatty acids

SR-BI	Scavenger receptor class B type I
tFA	Trans fatty acids
TG	Triacylglycerol
UFA	Unsaturated fatty acids
VLDL	Very-low density lipoprotein

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INTRODUCTION

1 CARDIOVASCULAR DISEASE

Cardiovascular disease is currently the leading cause of death and illness in developed countries (Murray & Lopez, 1997). For instance, in 2001, 31.4% of total Spanish mortality was due to cardiovascular diseases (Instituto de Salud Carlos III, 2006). The term cardiovascular disease covers any disease of the heart and blood vessels, including heart diseases, stroke and peripheral vascular disease. Atherosclerosis is the single most important contributor of cardiovascular disease.

Atherosclerosis is characterized by a deposition of cholesterol-rich plaques in the endothelium that provokes a decrease in the diameter and elasticity of the vessel wall. The chronic accumulation of lipids and the inflammation in the arterial wall due to the cellular proliferation are the main reasons for the changes in vessel wall properties (Ross, 1999). This process starts with the damage of endothelium, due to mainly three causes: elevated levels of cholesterol and triacylglycerols in blood, high blood pressure and cigarette smoke. Because of the damage a lipid accumulation on the arterial walls occurs, and this accumulation is called atherosclerotic plaque. These plaques can be found in arteries throughout the body and reduce the blood flow in the peripheral extremities. The initial lesions, called fatty streaks, are characterized by the accumulation, on the arterial wall, of macrophages with large contents of fat and cholesterol (foam cells). The result of this process depends on the nature of the affection and the role of the vessels affected. Thus, coronary artery lesions provoke the appearance of coronary cardiopathy, which may take place in acute form, like ischemic thoracic pain (such as angina pectoris) or myocardial infarction. Lesions in cerebral arteries may end with cerebrovascular affections [e.g. cerebrovascular accident (CVA)], whereas those in femoral and iliac artery or aorta may cause peripheral arterial disease, such as ischemic pain in the extremities, gangrene or aneurysm.

The most important modifiable risk factors of cardiovascular diseases are smoking, hypercholesterolemia, hypertension, obesity and diabetes (Grundy *et al.*, 2000). Some other risk factors are age, male sex, familiar stories, low HDL-cholesterol concentrations and hypertriglyceridemia. These risk factors have an important role in all cardiovascular diseases, although some of them have stronger associations with particular diseases. For instance, high total and LDL-cholesterol concentrations are associated with coronary cardiopathy and hypertension to CVA, whereas smoking,

diabetes mellitus and hypertriglyceridemia are important risk factors for the development of coronary cardiopathy and peripheral arterial disease. Since hypercholesterolemia is known to be one of the major risk factors for cardiovascular disease, we focus this section on the cholesterol metabolism. First, we discuss some basic principles of cholesterol metabolism and the relation between cholesterol concentration and cardiovascular risk, to further briefly show how cholesterol concentration may be modulated through dietary approaches.

1.1 CHOLESTEROL METABOLISM

Cholesterol is an essential component of animal bodies, since it is one of the building blocks of cell membranes and the precursor of a number of compounds, such as steroid hormones and bile acids. A normal western diet provides 300-600 mg of cholesterol a day to the intestine, with a small proportion in esterified form (like cholesterol fatty acid esters) (Grundy, 1983). In addition, 1000-1500 mg are provided to the intestine through endogenous sources, predominantly from the bile (Homan & Krause, 1997; Ellsworth & Starr, 1998). Approximately 30-60% of total cholesterol is absorbed (Bosner *et al.*, 1999). The remnants of cholesterol are excreted as bacterial degradation products, mainly coprostanol and coprostanone (Li *et al.*, 1996; 1998).

Due to its low solubility in the hydrophilic circulation environment, cholesterol (that from diet and that from synthesis *de novo*) is transported through the blood stream by means of lipoproteins. Lecithin cholesterol acyl transferase (LCAT) is an enzyme that esterifies free-cholesterol to cholesterol esters, accounting for 2/3 of total blood cholesterol (Dobiasova & Frohlich, 1999).

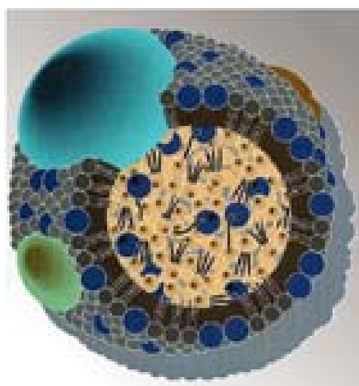

Fig. 1. Lipoprotein particle (Instituto Químico Biológico, 2006)

A lipoprotein (Fig. 1) consists of a hydrophilic coat, containing free cholesterol, phospholipids and apolipoprotein, and a hydrophobic core consisting of cholesterol esters and triacylglycerols. Each particle is associated with one or more protein molecules, called apolipoproteins, which have hydrophobic parts located in the lipoprotein core and hydrophilic parts located in the surface.

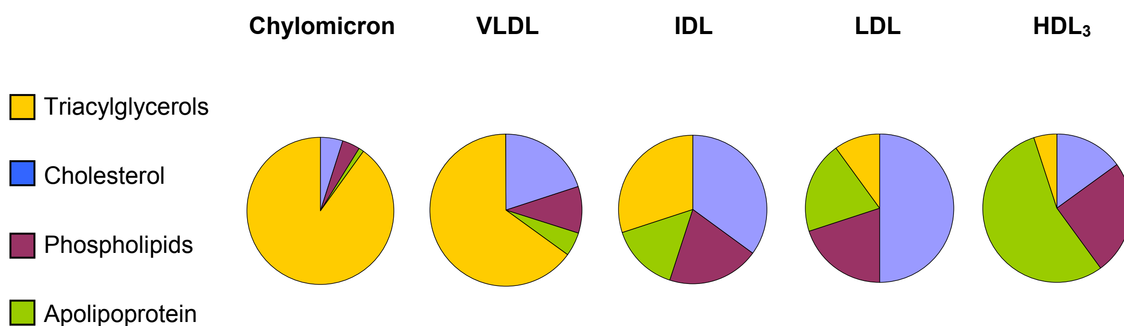

Fig. 2. Lipoprotein composition; the proportions are approximated (based on Frayn, 1998)

Lipoproteins are a heterogenic group of particles with different size and chemical composition. The most common classification is based on the flotation index in ultracentrifuge (Fig. 2). The composition of the main lipoproteins is shown in Table 1. Chylomicrons and very-low-density lipoproteins (VLDL), rich in triacylglycerols, are involved in triacylglycerol transport. The smaller lipoproteins, low-density lipoproteins (LDL) and high-density lipoproteins (HDL), are more related to cholesterol transport. Cholesterol in lipoproteins is derived from diet (exogenous pathway) and from endogenous synthesis (endogenous pathway).

Exogenous pathway

Dietary cholesterol esters and triacylglycerols are first hydrolysed in the small intestine. This results in the formation of free cholesterol, free fatty acids (FFA), monoglycerides, diglycerides and glycerol. These components, along with phospholipids and bile acids, are incorporated into mixed micelles. The individual components of the micelles can be taken up by the enterocytes. The main sites of

cholesterol absorption are the duodenum and the proximal jejunum, the absorption occurring through the intestinal mucosal cells (enterocytes) (Wilson & Rudel, 1994).

Table 1. Chemical and physical properties of lipoprotein (based on Frayn, 1998)

Lipoprotein	Density range	Diameter (nm)	Main lipids	Main apolipoproteins	Composition Weight percentage			
					Proteins	TG	Chol	PL
CM	<0.950	500	Dietary TG	B48, A1, A2, C, E	1	90	5	4
VLDL	0.950-1.006	43	Endogenous TG (from the liver)	B100, C, E	10	65	20	10
IDL	1.007-1.019	27	CE, TG	B100, E	15	30	35	20
LDL	1.019-1.063	22	Chol, CE	B100	20	10	50	20
HDL	1.063-1.090	8	CE, PL	A1, A2, C, E	55	5	15	25

Abbreviations used: CM, chylomicrons; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; PL, phospholipids; TG, triacylglycerol; Chol, cholesterol; CE, cholesterol ester.

Notes: (I) Concentrations are on average and depend on each lipoprotein type; (II) C means presence of C1, C2 y C3 apolipoproteins. Most of the time, these apolipoproteins are found together.

Initially, cholesterol absorption was thought to occur by passive diffusion, aided by the concentration gradient of free cholesterol created between the extra- and intra-cellular space (Wilson & Rudel, 1994) and by the cholesterol in bile (Sehayek *et al.*, 1998). However, recent data suggest that several receptors and transporters are involved in cholesterol uptake by facilitating cholesterol influx into enterocytes or cholesterol efflux back to the intestinal lumen. The Niemann-Pick C1-like 1 (NPC1L1) protein, located in the brush border membrane, induces an active influx of cholesterol from the intestinal lumen into the enterocyte (Altmann *et al.*, 2004; Davis *et al.*, 2004). Furthermore, recent studies show that ATP binding cassette (ABC) transporters, ABCG5 and ABCG8, may work together as an apical export pump promoting active efflux of cholesterol, back from the enterocyte into the lumen for excretion (Yu *et al.*, 2002a,b; 2003).

Once cholesterol enters the mucosal cells, most of it is re-esterified by an enzyme called ACAT (acyl-coenzyme A: cholesterol acyl transferase) (Sugiyama *et al.*,

1995; Chang *et al.*, 1997). However, monoglycerides and diglycerides are first hydrolysed into free fatty acids (FFA) and glycerol and then used for the formation of new triacylglycerols (Levy *et al.*, 1995; Hegele, 1998). These new triacylglycerols and cholesterol esters are, together with apolipoproteins and phospholipids, used for the synthesis of chylomicrons.

New chylomicrons are composed by a core of cholesterol esters and triacylglycerols (with about 90% of lipids) and a surface of non-esterified cholesterol, phospholipids and apolipoproteins B48 and A1 (synthesized in the intestine). Apolipoproteins C, E and A are also found in the surface of chylomicrons (Kane *et al.*, 1980). The lipoproteins are secreted into the lymph, reaching the circulation by the vena subclavia. Triacylglycerols from the chylomicrons are taken up by various peripheral tissues (e.g. adipose tissue and muscle cells) after hydrolysis into FFA and glycerol through the action of the enzyme lipoprotein lipase (LPL).

The particles formed by the gradual delipidation, now called chylomicron remnants, are relatively rich in cholesterol. They are taken up by the liver, which can store cholesterol as cholesterol esters through the action of ACAT, or resecrete it together with endogenously synthesised cholesterol, into circulation, in very-low-density lipoprotein (VLDL) particles (Frayn, 1998).

Endogenous pathway

Of the total daily cholesterol synthesis, 10% takes place in the liver and 15% in the intestine. Cholesterol synthesis occurs in the cytoplasm and microsomes from the acetil-CoA group, which comes from the oxidative decarboxilation of piruvate or from the FA oxidation. Delivery of cholesterol from chylomicrons to the liver can modulate hepatic cholesterol biosynthesis via feedback suppression of 3-hydroxy-3-methyl-glutaril Coenzime A (HMG-CoA) reductase (a rate-limiting enzyme in cholesterol synthesis).

The liver produces and secretes VLDL particles, containing mainly triacylglycerols, cholesterol, apolipoprotein B100 and also small amounts of apolipoproteins E and C (Table 1). Once VLDL are in the blood stream, initial metabolic

steps are similar to those described for chylomicrons. Lipoprotein lipase hydrolyses triacylglycerols to obtain energy, or to further synthesize structural (like phospholipids) or bioactive (like leucotrienes and tromboxanes) compounds. They can also be stored like triacylglycerols. The remaining particle, now called intermediate density lipoprotein (IDL), can be taken up through hepatic low-density lipoprotein (LDL) receptor in liver. IDL particles not taken up are converted into cholesterol-rich LDL particles that are also cleared from the circulation by LDL receptors.

LDL uptake in the liver is a key event in cholesterol homeostasis since it has three important functions: (i) inhibiting the activity of HMG-CoA reductase, an important enzyme in cholesterol synthesis, (ii) reducing apoB-100 receptors synthesis and therefore, also LDL uptake in cells and (iii) increasing ACAT activity, decreasing thus the concentration of free cholesterol in the cytosol.

In addition, some cells, especially macrophages, express some receptors different from those described previously, which can also take up LDL. However, these receptors, called scavenger receptors, are not under the regulation of intracellular cholesterol levels. This process takes place with normal LDL concentrations, but it is enhanced by: (i) increase in LDL concentrations or (ii) modification of these particles, like oxidation (Heinecke, 1997; Dhaliwal & Steinbrecher, 1999; Yla-Herttuala, 1999). The presence in the vessel walls of macrophages rich in these lipoproteins is one of the first steps in the development of atherosclerosis.

In contrast to LDL particles, high- density lipoproteins (HDL) are involved in the reverse transport of cholesterol from peripheral cells to the liver (Kwiterovich, 1998). HDL is a heterogenic group, varying in apolipoprotein composition as well as in particle size (Table 1). However, all of them contain apoA-I (Bonnefont-Rousselot *et al.*, 1999), as well as apoA-II, A-IV and C. The newly HDL particles acquire some surface lipids (cholesterol and phospholipids) from VLDL and chylomicrons. Subsequently, HDL acquires free cholesterol from the cell membranes of various peripheral tissues. The catabolism and conversion of HDL is dependent on plasma lecitin: cholesterol acyl transferase (LCAT). LCAT esterifies free cholesterol at the surface of HDL particles to form cholesteryl esters, which will be transferred into the core of HDL particles. Cholesteryl esters in HDL can be transferred to apoB containing particles by cholesterol ester transfer protein (CETP) (Lusa *et al.*, 1996). Furthermore, scavenger receptor class B type I (SR-BI) is thought to be involved in direct uptake of cholesteryl

esters from HDL particles to the liver (Acton *et al.*, 1996; Zannis *et al.*, 2006). Intracellular cholesterol in the liver can be used for bile acid synthesis, or secreted as free biliary cholesterol.

1.2 CHOLESTEROL CONCENTRATIONS AS A RISK FACTOR FOR CARDIOVASCULAR DISEASE

High blood lipid concentrations, especially those from total- and LDL-cholesterol, are an important risk factor of cardiovascular mortality (Pekkanen *et al.*, 1990). First evidences of the association between blood lipid concentrations and cardiovascular risk arose in statin studies (Slater & MacDonald, 1988). Statins inhibit HMG-CoA, the rate-limiting enzyme for endogenous cholesterol synthesis. Primary and secondary intervention trials have shown that statin therapy reduce serum total- and LDL-cholesterol by approximately 20 and 30% respectively. This hypocholesterolemic activity is associated with a 30% reduction in risk of major coronary events, with a greater benefit for patients with higher baseline risk (Maron *et al.*, 2000). Moreover, it has been proved that these drugs may be used with a wide range of patients: (i) individuals with or without prior myocardial infarction, (ii) female or male, (iii) with or without diabetes, (iv) patients with peripheral vascular or cerebrovascular disease, as well as those with coronary artery disease, (v) adults or elderly people, and (vi) patients with unstable or stable cardiovascular disease (Larosa *et al.*, 1999; Maron *et al.*, 2000; Serruys *et al.*, 2002).

The presence of several LDL components, like unsaturated fatty acids (UFA) from phospholipids, which are easily oxidized, may be the reason why LDL-cholesterol is atherogenic. The formation of oxidized LDL is an important step in the development of atherosclerosis. Oxidized LDL is not recognized by the LDL receptor, but it is efficiently cleared out by scavenger receptors from macrophages found in the arterial walls. This causes that macrophages develop into foam cells. Many of these foam cells form a fatty streak that can ultimately turn into an atherosclerotic plaque.

Nowadays, there is still no agreement about the adequate LDL-cholesterol concentration to reduce the risk of cardiovascular diseases. The European Society of Cardiology (De Backer *et al.*, 2003) recommends lowering LDL-cholesterol concentration down to 115 mg/dL; however, the United States (Grundy *et al.*, 2004)

have more strict recommendations, with a limit below 100 mg/dL. Spanish recommendations are in agreement with those of United States, the upper limit being 100 mg/dL (Villar-Alvarez *et al.*, 2000).

In contrast to high serum LDL-cholesterol concentrations, high HDL-cholesterol levels are negatively associated with the risk for cardiovascular disease (Pekkanen *et al.*, 1990; Despres *et al.*; 2000). For instance, a 10% reduction in HDL-cholesterol is associated to a 13% increase in ischemic heart disease (Despres *et al.*, 2000). The protective effect of HDL is related to its role in reverse cholesterol transport. Cholesterol from extrahepatic tissues, including the vessel wall, is taken up by HDL particles that transfer cholesterol directly or via LDL and VLDL particles to the liver for the final secretion.

1.3 MANAGEMENT OF CHOLESTEROL CONCENTRATIONS THROUGH NUTRITIONAL APPROACHES

It is known that dietary factors may modulate some traditional cardiovascular risk factors, such as plasma lipids (Hu *et al.*, 2001), as well as blood pressure and relative body weight (De Backer *et al.*, 2003). For instance, a high fat intake, saturated fatty acids (SFA), *trans* fatty acids (tFA) and high amounts of dietary cholesterol are associated with increased risk of cardiovascular disease (Heinonen & Piironen, 1991; Johansson *et al*., 1996; Dietschy, 1998; Elmstahl *et al*., 1999, De Roos *et al*., 2001; Dixon & Ernst, 2001), whereas unsaturated fatty acids (UFA), especially n-3 polyunsaturated fatty acids (PUFA), dietary fiber and moderate intakes of alcohol may decrease the risk (Liu *et al*. 2002). Selenium and antioxidant vitamins A, C and E are also negatively correlated with the risk of cardiovascular disease (Gerber *et al*., 2000; Osler *et al*., 2001).

The Spanish nutritional objectives (Serra-Majem *et al.*, 2001) (Table 2) claim for a reduction in total lipid intake, but especially the consumption of saturated fat and cholesterol should be decreased (Mann, 1987; Grundy & Denke, 1990; Grundy *et al.*, 2002). In the following section, we discuss how some nutritional compounds may modulate the blood lipoprotein profile.

Table 2. Daily nutrient intake in Spain

	Nutrient objectives (Serra-Majem *et al.*, 2001)	Real intake (INC*, 2006)
Energy (Kcal)	---	2634
Caloric content. Carbohyd. (% energy)	50-55	41.9
Caloric content Protein (% energy)	---	14.2
Caloric content Lipids (% energy)	30-35	41.5
- Caloric content SFA (% energy)	7-8	11.9
- Caloric content MUFA (% energy)	15-20	18.9
- Caloric content PUFA (% energy)	5	6.8
Cholesterol (mg/day)	<300	440
Total fiber (g)	>25	21
- Insoluble fiber (g)	---	12.4
- Soluble fiber (g)	---	8.0

* Instituto Nacional de Consumo

Cholesterol

Dietary cholesterol has little effect on blood cholesterol concentrations, though it may increase LDL- and total-cholesterol concentrations (Mattson *et al.*, 1972; Beynen & Katan, 1985; Howell *et al.*, 1997). This effect is due to the fact that an increase in liver cholesterol content depresses the expression of LDL receptors.

The magnitude of this effect depends on each subject, since there is a high variation on cholesterol absorption and hepatic cholesterol homeostasis between subjects (Beynen & Katan, 1985; Katan *et al.*, 1986; Katan & Beynen, 1987). Nutritional recommendations of American organizations, the *American Heart Association* (AHA) and the *National Cholesterol Education Program* (NCEP) claim to reduce cholesterol intake to less than 300 mg/d for healthy subjects and less than 200 mg/d in hypercholesterolemic subjects with pre-existent coronary heart disease (Expert Panel on Detection, 1993; Krauss *et al.*, 2000).

Saturated Fatty Acids

SFA raise, more than dietary cholesterol, blood cholesterol concentration (Elmstahl *et al.*, 1999). However, this effect depends on FA length (Aro *et al.*, 1997; Ginsberg *et al.*, 1998). For instance, short-chain fatty acids (SCFA) such as butanoic (4:0), hexanoic (6:0), octanoic (8:0) and medium-chain FA decanoic (10:0) are rapidly oxidized to acetyl CoA in the liver. These FA do not alter the composition of the lipid pool in the liver, or the concentration of free or esterifed cholesterol in the hepatocyte or hepatic LDL receptor activity. Thus, they are biologically neutral with respect to the regulation of the LDL-cholesterol concentration. Interestingly, the long-chain SFA, stearic acid (18:0), also appears to belong to this biologically neutral group (Kris-Etherton & Yu, 1997). However, lauric (12:0), myristic (14:0) and palmitic (16:0) acids inhibit LDL receptor activity (Woollett *et al.*, 1992; Dietschy, 1998), enhance LDL-cholesterol production and increase the concentration of LDL-cholesterol in the serum (Temme *et al.*, 1996; Kris-Etheron & Yu, 1997), with the result that blood LDL-cholesterol concentrations increase.

The explanation for the effects of SFA on cholesterol metabolism is that SFA in liver (especially medium chain SFA) reduce the transformation of non-esterified cholesterol to esterified cholesterol. Taking into account that the expression of LDL receptors is regulated for hepatic non-esterified cholesterol concentration, high hepatic SFA concentration decreases the expression of hepatic LDL receptors, increasing thus blood LDL concentration (Woollett *et al.*, 1992; Dietschy, 1998).

AHA guidelines recommend limiting saturated fat to less than 10% of total energetic intake in healthy subjects (Expert Panel on Detection, 1993; Krauss *et al.*, 2000). However, the limit should be below 7% for those subjects with high LDL-cholesterol concentrations or other coronary heart disease risk factors (Expert Panel on Detection, 1993). Although several campaigns have been carried out to make the population aware about the health risk of high SFA consumption, the average intake is still about 12% (INC, 2006). Therefore, it seems obvious that changing dietary behaviour is a difficult task.

Unsaturated fatty acids

There are three main homologue series of unsaturated fatty acids (UFA) – monounsaturated (MUFA), n-6 and n-3 polyunsaturated (PUFA). When replacing UFA by SFA, total and LDL-cholesterol concentrations are reduced (Hegsted *et al.*, 1965; Mensink & Katan, 1992; Howard *et al.*, 1995). These effects may be explained by the simple fact of the substitution of saturated by unsaturated fat. However, some studies point to some direct hypocholesterolemic effects of UFA (Howard *et al.*, 1995).

It has been suggested that oleic acid (the most important MUFA) reduces blood cholesterol concentrations by means of two mechanisms. First, it increases hepatic uptake of LDL particles, blocking the depression of hepatic LDL receptors provoked by high cholesterol concentrations. Second, it enhances the activity of CETP, which increases intracellular esterified cholesterol concentration, enhancing thus the expression of LDL receptors in cellular membranes (Kurushima *et al.*, 1995).

There are mainly two mechanisms underlying the hypocholesterolemic effects of n-6 PUFA. They increase the expression of hepatic LDL receptors, as well as the activity of 7α-hydrolase (the enzyme responsible for the conversion of cholesterol to bile acids) (Fernandez & West, 2005).

Finally, oils rich in n-3 PUFA, especially fish oils, can also reduce blood cholesterol and triacylglycerol concentrations, due to their action in several pathways. They reduce lipogenesis and secretion of VLDL. They also increase LPL activity and enhance cholesterol reverse transport through HDL lipoproteins (Nestel, 2000; Fernandez & West, 2005). Nevertheless, intakes too high of n-3 PUFA might increase the oxidability of plasma lipids, though such effect has not been found when these oils are part of an equilibrated diet (Kratz *et al.*, 2002).

Trans *fatty acids*

Epidemiological studies have unveiled the association between tFA and coronary heart disease mortality in USA and Europe (Willett *et al.*, 1993; MacDonald *et al.*, 1995; Ascherio *et al.*, 1996; Hu *et al.*, 1997; Pietinen *et al.*, 1997; Oomen *et al.*, 2001). These FA may increase LDL-cholesterol concentration (Lichtenstein *et al.*, 1993; Judd *et al.*, 1994) and decrease HDL-cholesterol (Mensink & Katan, 1990; Judd

et al., 1994), compared with an isocaloric intake of *cis* UFA. Further, tFA increase lipoprotein(a) concentration (Lp(a)) (Nestel *et al.*, 1992), an important cardiovascular risk factor (Utermann, 1989). tFA also increase plasma triacylglycerol concentrations (Stampfer *et al.*, 1996). In addition, they may disturb essential FA metabolism (linoleic and linolenic) and prostaglandin balance. Finally, some data suggest that a high tFA intake is associated to an increase in the diabetes type 1 (Lovejoy, 1999).

Total fat and carbohydrates

Epidemiological studies over the past 25 years have shown that the level of dietary fat intake is positively correlated with the average serum cholesterol value and mortality from cardiovascular disease (Dietschy, 1998). By this reason, one of the main dietary recommendations is to reduce total fat, especially saturated fat. Moreover, it is also recommended to decrease the consumption of simple carbohydrates (such as sugars) by increasing the monounsaturated fat intake (De Lorgeril *et al.*, 1999).

Low-fat diets with a high content in carbohydrates may provoke potentially adverse metabolic responses, especially, in diabetic type II subjects. For instance, they may increase plasma triacylglycerols, as well as reduce HDL-cholesterol concentration. Moreover, high carbohydrate diets can also modify the size and density of LDL particles, becoming smaller and heavier and, thus, more atherogenic (Krauss & Dreon, 1995; Krauss, 1998; Grundy, 1999). However, these side effects may be counteracted by partial substitution of carbohydrates for monounsaturated fats (Mensink & Katan, 1987).

2 FUNCTIONAL FOODS

As mentioned in the previous section, a large part of the population from developed countries (Grundy *et al.*, 2002), including Spain (Capita & Alonso-Calleja, 2003), should decrease the intake of total fat, and especially the consumption of SFA, tFA and cholesterol, to reduce the risk for cardiovascular disease. Although changes in dietary habits should be encouraged, functional foods can be a very useful tool to reduce the incidence of cardiovascular diseases. However, functional foods must be consumed as part of a recommended diet, and should not be used to counterbalance the effects of an unhealthy diet.

One of the definitions of functional food is: “foods can be regarded as functional if they can be satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way relevant to an improved state of health and well-being and/or reduction of risk of disease” (Contor, 2001). It must be taken into account that functional foods, in the amounts they are usually included in our diet, must keep their beneficial effects on determined organic functions (Roberfroid, 2000).

In the next sections we summarize the actual knowledge about plant sterols and pectins (soluble fiber), two compounds used as functional ingredients to improve blood lipoprotein profile.

2.1 PLANT STEROLS AND STANOLS

2.1.1 INTRODUCTION

Plant sterols and stanols are non-nutritive compounds with the same basic functions in plants as cholesterol in animals; that is, they regulate membrane fluidity of plant cells. Their chemical structure differs from that of cholesterol because of the presence of a modified side chains at carbon C-24.

More than 250 different plant sterols (or phytosterols) and related compounds in various plant and marine materials have been identified. Depending on their structure and biosynthesis, plant sterols can be divided into 4-desmethyl sterols, 4α-monomethyl sterols and 4,4-dimethylsterols. However, the 4,4-dimethylsterols and 4α-methylsterols are present in at lower concentrations than 4-desmethyl sterols. Therefore, the most abundant plant sterols belong to the group of 4-desmethyl sterols, and they are β-sitosterol, campesterol and stigmasterol. On average they account for 65, 30 and 3%, respectively, of dietary phytosterol intake. β-sitosterol and campesterol have an ethyl and methyl group at C-24, respectively, whereas stigmasterol is identical to β-sitosterol except for a double bound at C-22 (Fig. 3). Stanols are saturated sterols, with no double bonds in the sterol ring, are less abundant in nature than sterols.

Plant materials, especially vegetable oils, contain free and esterified steryls and steryl glycosides, which can be esterified to acylated steryl glycosides (Piironen *et al.*, 2000).

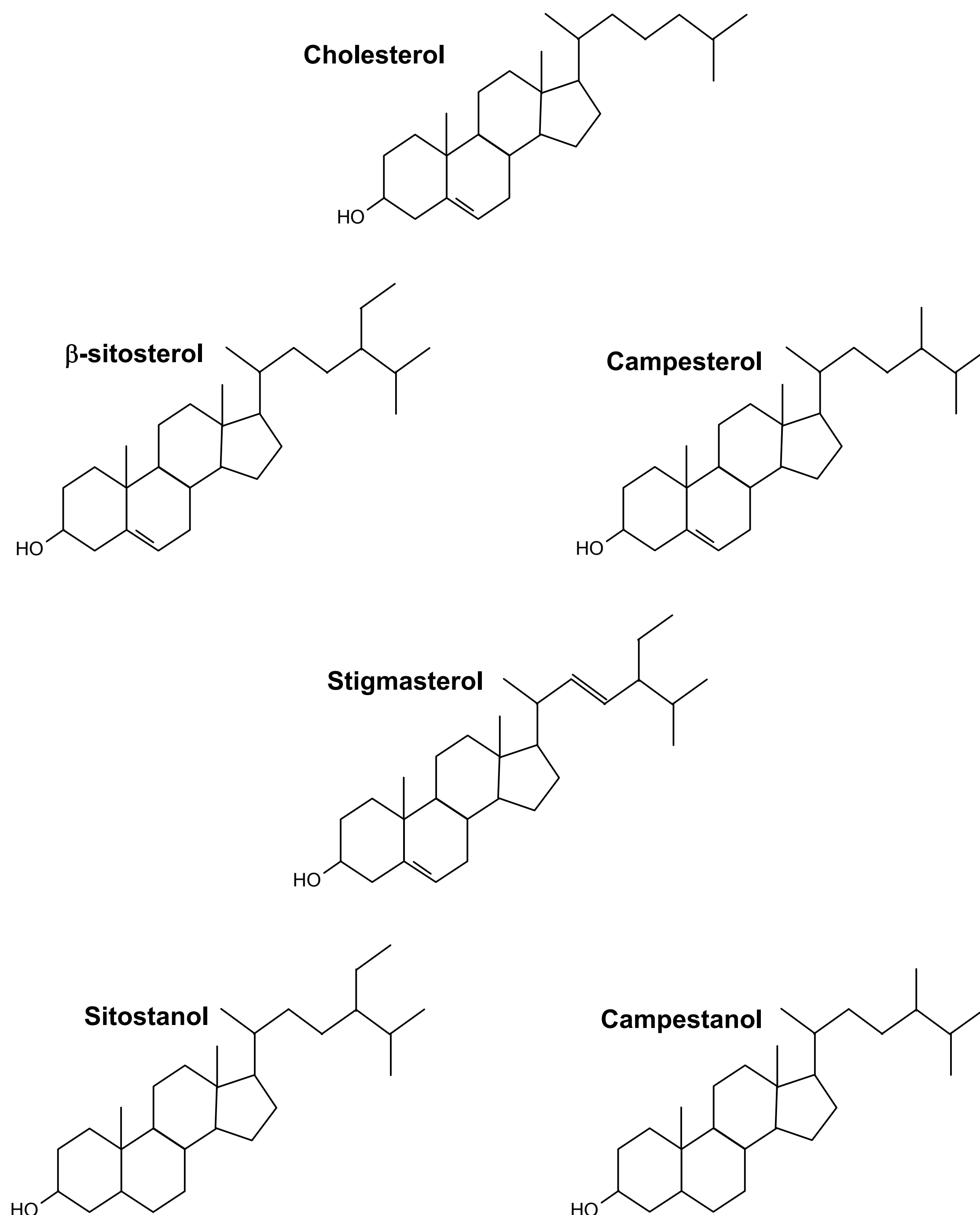

Fig. 3. Chemical structures of cholesterol, 4-desmethyl sterols and hydrogenated plant sterols

4-desmethyl sterols have demonstrated their hypocholesterolemic effects in a great number of studies (Katan *et al.*, 2003; Quílez *et al.*, 2003a). In contrast, 4,4-dimethylsterols, like lupeol, α-amyrin and cycloartenol, hardly lower serum cholesterol (Sierksma *et al.*, 1999). The structures of 4-desmethyl sterols resemble the structures

of cholesterol more than those of 4,4-dimethylsterols. This might be the reason of the difference in cholesterol-lowering effect. Usually the term phytosterols is restricted to 4-desmethyl sterols.

Plant sterols occur naturally in vegetable products (such as fruits and nuts) and oils (Piironen *et al.*, 2000). There is a rich variety in plant sterol content, which fluctuates from 8-9 g/kg in corn oil to 0.5 g/kg in palm oil, although most consumed oils have intermediate contents (Phillips *et al.*, 2002). Table 3 gives values of the total phytosterol content of some representative foods (Weihrauch & Gardner, 1978). Besides interspecies differences, genetic factors, growing and storage conditions, may affect the sterol content of oil seeds, and further variations may be caused by different processing conditions (Piironen *et al.*, 2000). A Western diet provides between 170 and 360 mg/d of phytosterols, depending on dietary habits and geographical location (Ling & Jones, 1995; de Vries *et al.*, 1997).

Table 3. Phytosterol contents (Weihrauch & Gardner, 1978)

Product	Edible portion (g/kg)
Corn oil	9.52
Sunflower oil	7.25
Soybean oil	2.21
Olive oil	1.76
Almonds	1.43
Beans	0.76
Corn	0.70
Wheat	0.69
Palm oil	0.49
Lettuce	0.38
Banana	0.17
Apple	0.12
Tomato	0.07

Plant sterols and stanols are nowadays added to a wide range of products, such as margarines, yoghurts, salad dressings, milk and snack bars. Phytosterols are generally extracted from by-products of either pulp and paper industry (wood-derived: “tall oil soap”) or from vegetable oil industry (vegetable derived), using organic solvents (hexane and acetone). The final product is a mixture of various plant sterols, whose

composition may vary, depending on the plant sterol source. Plant stanols are mainly produced by hydrogenation of plant sterols. Chemical saturation of the double bond of β-sitosterol and campesterol results in the formation of sitostanol and campestanol respectively (Fig. 3) (Moghadasian, 2000).

2.1.2 HYPOCHOLESTEROLEMIC EFFECT

It was found in the 1950s that plant sterols from soybeans lowered serum cholesterol concentration (Peterson, 1958). Plant sterols were used as capsules, syrups or suspensions, so that large doses were needed to achieve substantial hypocholesterolemic effects (Lees *et al.*, 1977). Because of their poor water solubility and bioavailability, it was not possible to use them as pharmaceutical agents, and they were consequently abandoned. However, a new interest arose when the esterification of these compounds facilitated the inclusion of these compounds into some food products (Weststrate & Meijer, 1998; Hendriks *et al.*, 1999).

Since then, many studies have shown that both plant sterol and stanol esters lower serum LDL-cholesterol concentrations, with no effect, either on serum HDL-cholesterol concentrations or in triacylglycerol levels (de Graaf *et al.*, 2002; Gylling & Miettinen, 2002, Temme *et al.*, 2002, Vanstone *et al.*, 2002; Quílez *et al.*, 2003b), although some studies did not find such effect (Denke, 1995; Jones *et al.*, 2003; AbuMweis *et al.*, 2006). In recent years, also free plant sterols and stanols have been incorporated into food products (Vanstone *et al.*, 2002). Their effects on serum lipoprotein concentrations are the same as when esterified plant sterols and stanols are used.

In a meta-analysis of 41 trials with various enriched food products, Katan and co-workers (2003) found that the optimal daily dosage of sterols or stanols is 2g/d, resulting in a 10% reduction in LDL-cholesterol. Higher doses may not improve efficacy and may produce adverse effects, except in the case of stanols, due to their negligible absorption. In general, it can be said that cholesterol absorption, both from diet (between 200 and 500 mg/d) and from enterohepatic circulation (around 1 g/d) (Grundy, 1983), is reduced by phytosterols (Normen *et al.*, 2000), and non-absorbed cholesterol is removed by faeces. The main mechanism of action is shown in Fig. 4.

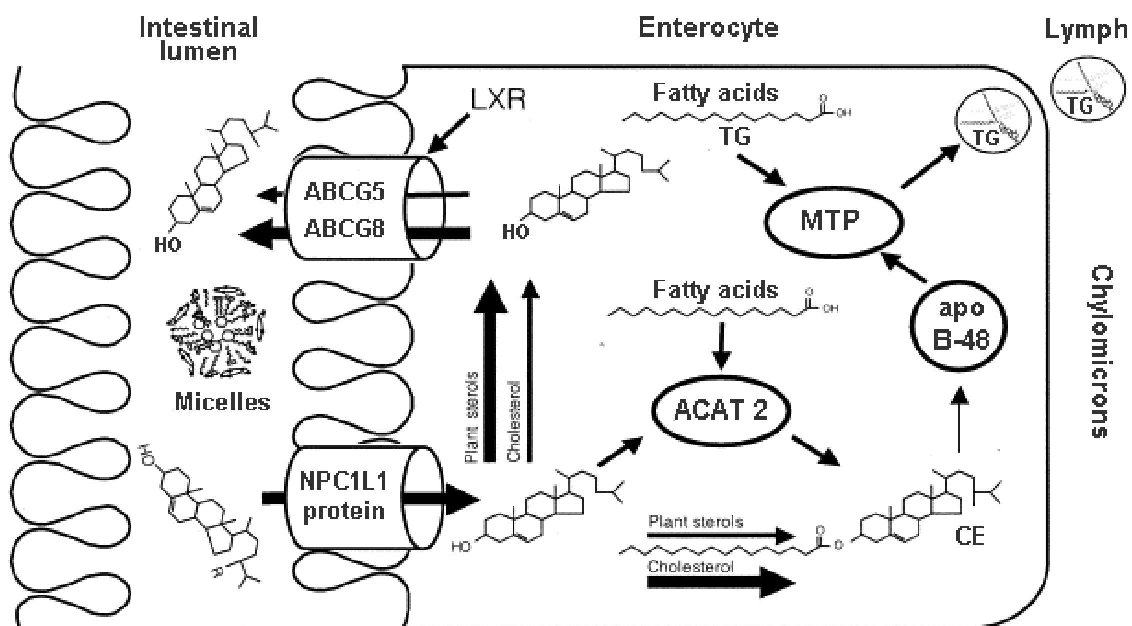

Fig. 4. Simplified model of mechanisms of action of phytosterols (based on von Bergmann *et al.*, 2005)

In the first studies, it was assumed that plant sterols should be taken together with foods containing cholesterol to achieve the maximum effect (Mattson *et al.*, 1982). However, in 2000, a study showed that a daily intake of 2.5 g of plant stanols is as effective as the same daily amount of plant stanols divided over three meals (Plat *et al.*, 2000). Moreover, it has been later suggested that it is not necessary for the plant sterol intake to come with the main meals (Quílez *et al.*, 2003a). The fact that plant sterol/stanol esters may reduce cholesterol-LDL concentration effectively when consumed only once a day, suggests that there are several mechanisms of action underlying these effects.

The most common and known mechanism of action of plant sterols and stanols is based on their high hidrophobicity. They are better incorporated into the mixed micelle than cholesterol. Then, cholesterol is displaced from the mixed micelles and precipitates together with non-solubilized phytosterols, leading to an increase in cholesterol excretion. However, it has also been suggested that cholesterol absorption may be regulated inside the enterocyte by regulating the expression on transfer proteins. To date, the data available are conflicting. Plat *et al.* (2002b, 2005b) reported that in *in vitro* studies, plant sterols may activate LXR (nuclear receptor which controls

the expression of ABCG5/8), thereby increasing the expression of ABC transporters and the transport of cholesterol from the enterocytes back into the intestinal lumen. In contrast, other authors (Calpe-Berdiel *et al.*, 2005; Plosch *et al.*, 2006) concluded that the effects of plant sterols on cholesterol absorption are not caused by LXR-mediated induction of intestinal gene expression in mice. If finally this secondary mechanism of action is confirmed, it might explain why a single daily intake of phytosterols is enough to reveal their cholesterol-lowering properties.

The inhibition of cholesterol absorption produces a state of relative cholesterol deficiency, followed by an upregulation of cholesterol biosynthesis and LDL receptors activity (Ling & Jones, 1995) (Fig. 5). After chronic phytosterol feeding, whole body cholesterol biosynthesis rises to 46% (Gylling *et al.*, 1999) and LDL receptor expression increases 25-42%. Also, changes in LDL levels are correlated with LDL receptor expression in peripheral cells (Plat & Mensink, 2002a). However, the net result is a decrease in cholesterol concentrations after phytosterol feeding.

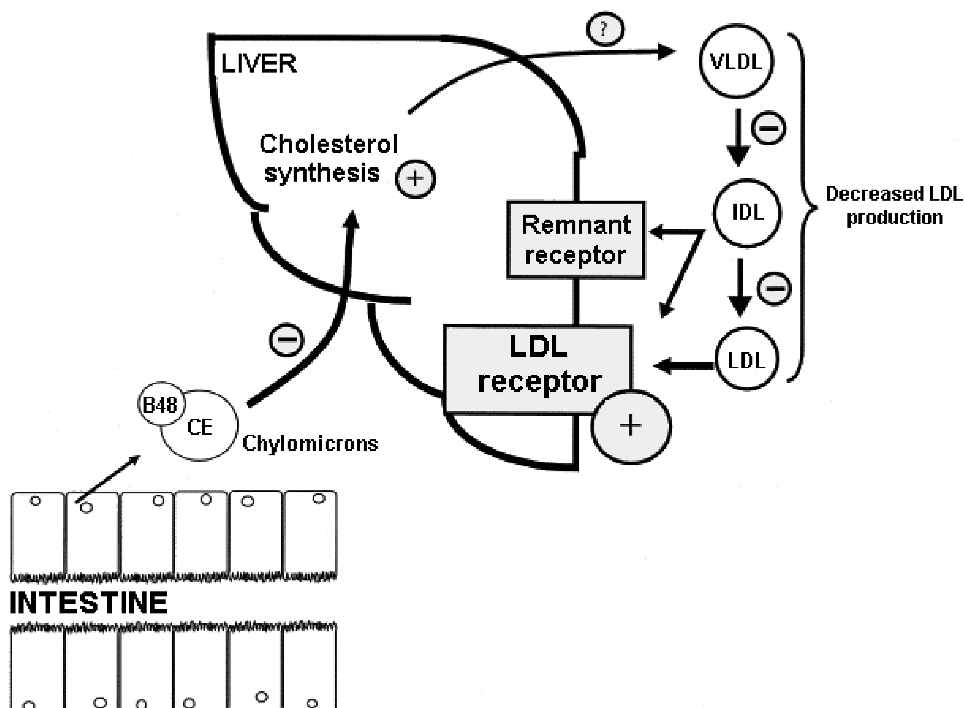

Fig. 5. Cholesterol hepatic metabolism (based on Plat & Mensink, 2005a)

Plant sterols and stanols have shown their efficacy like hypocholesterolemic agents in hypercholesterolemic subjects (De Graaf *et al.*, 2002; Gylling & Miettinen, 2002; Temme *et al.*, 2002; Vanstone *et al.*, 2002), normocholesterolemics subjects (Mensink *et al.*, 2002; Quílez *et al.*, 2003b) and diabetic subjects (Gylling & Miettinen, 1994; Lau *et al.*, 2005), as well as in children (Amundsen *et al.*, 2002; 2004). Moreover, it has been shown that they are effective as part of an equilibrated diet (Hallikainen *et al.*, 1999). Phytosterol-induced reduction in blood LDL-cholesterol varies among patients (Jones *et al.*, 1999b). This variation appears to be unrelated to variation in dietary cholesterol intake, probably because the biliary excretion of cholesterol via the gut (approximately 2g daily) is considerably greater than the average dietary cholesterol intake (approximately 500 mg/d) (Jones *et al.*, 1999b). LDL-cholesterol reduction by phytosterols is greater in patients with enhanced cholesterol absorption, especially if they have simultaneously reduced hepatic cholesterol production (Patel & Thompson, 2006).

Plant sterols used together with hypocholesterolemic drugs, such as statins (Blair *et al.*, 2000) and fibrates (Nigon *et al.*, 2001), have an additional effect. For instance, three servings a day of 5.1 g phytosterols can reduce LDL-cholesterol 10% more than placebo and statin therapy alone (Blair *et al.*, 2000). Moreover, in a recent study, the hypocholesterolemic effect of a triple therapy (statins, phytosterols and cholesteramine) was evaluated, finding a reduction in LDL-cholesterol of about 67% (Gylling & Miettinen, 2002). Thus, phytosterols may be useful in secondary prevention of heart disease when greater targeted reductions in LDL-cholesterol are needed.

2.1.3 PLANT STEROLS AND STANOLS METABOLISM

Although cholesterol and phytosterols are structurally similar, their metabolism differs in some aspects. For instance, mammals do not synthesise phytosterols (Salen *et al.*, 1970), in contrast to what happens for cholesterol. The absorption of these compounds is also different from that of cholesterol. Humans absorb and retain 55-60% of dietary cholesterol, but the net absorption of non-cholesterol compounds is below 5% (Gould *et al.*, 1969; Salen *et al.*, 1970; Kudchodkar *et al.*, 1973). Estimated uptake of campesterol (1.89%) is higher than that of β-sitosterol (0.51%) and campestanol absorption (0.16%) is higher than that of sitostanol (0.04%) (Ostlund *et al.*, 2002). The different absorption rate found among plant sterols and stanols may be

explained by the structure of side chain in C24. Increasing the complexity of the side chain increases hydrophobicity of the molecule, thereby reducing their absorption (Heinemann *et al.*, 1993). Saturation of the plant sterols also lowers absorption. Therefore, absorption of β-sitosterol, with an ethyl group at position 24, is lower than that of campesterol (with a methyl group). Similarly, absorption of sitostanol is lower than that of campestanol. Cholesterol only contains a hydrogen atom at position 24 and has the highest absorption (Fig. 3).

Phytosterols must be solved in micellar forms to be absorbed. The sterol-laden micelle interacts with the intestinal brush border membrane thereby facilitating the uptake of sterols by enterocytes. The precise molecular mechanisms are not well defined yet, but both cholesterol and phytosterol require the Niemann-Pick Like 1 Protein (NPC1L1) for their absorption (Altmann *et al.*, 2004). NPC1L1 is expressed in the small intestine, mostly likely in the brush border membrane of enterocytes. In addition, ABCG5 and ABCG8 belong to the family ATP Binding Cassette (ABC) transporters and they play a role in the absorption of phytosterols and cholesterol into mucosal cells. ABCG5/8 form a full active transporter in the enterocytes and they are responsible for the efflux of absorbed phytosterols and cholesterol back into the intestinal lumen (Berge *et al.*, 2000).

Plant sterols and stanols are poor substrates for ACAT and after absorption, only a very small part is esterified. The esterification rates are 89, 79 and 34% for campesterol, β-sitosterol and stigmasterol, respectively, compared to that of cholesterol (Dobiasova, 1983). Unesterified phytosterols and cholesterol are transported back into the intestinal lumen by ABCG5 and ABCG8 (Yu *et al.*, 2002a,b). Unabsorbed phytosterols may undergo bacterial transformation by intestinal flora, to produce metabolites such as coprosterol and coprostanone (McNamara *et al.*, 1981; Lütjohann *et al.*, 1996).

Once taken up by the liver through lipoproteins, plant sterols and stanols are incorporated into VLDL or secreted via bile. ABCG5 and ABCG8 are also responsible for biliary excretion of phytosterols (Salen *et al.*, 1970; Miettinen *et al.*, 2000). Due to the low affinity of ACAT for the β-sitosterol in liver, biliary excretion is faster for β-sitosterol than for campesterol (Tavani *et al.*, 1982). This, together with the higher absorption of campesterol from the gut, explains why serum concentration of campesterol is higher than that of β-sitosterol. Serum concentrations of plant stanols

are lower than those of their respective unsaturated forms, which can be explained, at least in part, because the intestinal absorption of plant stanols is lower than that of plant sterol.

In humans, β-sitosterol and campesterol levels range from 0.10 to 0.14% of cholesterol concentration (Miettinen *et al.*, 1990). When dietary phytosterols are supplemented at 2-3 g/d, serum β-sitosterol and campesterol increases 34-73%, but mostly within the normal range (Weststrate & Meijer, 1998; Hallikainen *et al.*, 2000). Stanols fed in the same doses, reduce serum plant sterols concentration by 17-36%, presumably by inhibiting their absorption (Weststrate & Meijer, 1998; Hallikainen *et al.*, 2000). Baseline serum stanols levels are about 0.003-0.004% of cholesterol (Hallikainen *et al.*, 2000). Serum campestanol and sitostanol are unchanged after phytosterol feeding but increase 200-275% after supplementation with 2 g/d of mixed stanols. However, the absolute levels of total stanols remain below 0.02% of cholesterol concentrations.

2.1.4 SAFETY AND SIDE EFFECTS

Many studies have found no important side effects of plant sterols and stanols (Baker *et al.*, 1999; Slesinski *et al.*, 1999; Turnbull *et al.*, 1999a,b; Waalkens-Berendsen *et al.*, 1999; Katan *et al.*, 2003; Plat & Mensink, 2005a). Hepburn and co-workers (1999) conducted an ongoing evaluation of the safety of phytosterol sterols and their oxides. No evidence of toxicity or genotoxicity was found. Rats were fed mixed phytosterol esters [β-sitosterol (48.7%), campesterol (25.8%) and stigmasterol (26.7%)] at levels of 0.1, 1.0, 2.2 and 5.0 (wt/wt) for 90 days. Twenty-one blood clinical parameters were measured and the weights of a number of tissues compared. No changes related to the treatment were observed (Ayesh *et al.*, 1999; Baker *et al.*, 1999; Waalkens-Berendsen *et al.*, 1999; Weststrate *et al.*, 1999; Whittaker *et al.*, 1999; Sanders *et al.*, 2000; Wolfreys & Hepburn, 2002). A reproduction study found no adverse effects of orally administered phytosterols in two successive generations of Wistar rats (Sanders *et al.*, 2000). No indication of oestrogenic effects was observed nor effects on levels of reproductive hormones in females volunteers. Dietary phytosterol esters had no effect on faecal bacterial enzymes or faecal SCFA in human volunteers. The results of toxicological studies with phytostanol esters remind us those seen with phytosterol esters (Slesinski *et al.*, 1999; Turnbull *et al.*, 1999a,b).

Nevertheless, daily intake of plant sterols and stanols should be limited below 8.6 g/d, since detailed clinical information in humans about consumption levels exceeding this dose is lacking (de Jong *et al.*, 2004). However, as increasing consumer populations become exposed to a large variety of food products enriched with plant sterols and stanols the likelihood of rare adverse effects increases and surveillance is necessary (De Jong *et al.*, 2004; Tikkanen, 2005).

Thus, plant sterols and stanols have been considered safe and effective to be used as functional ingredients to reduce cholesterol concentrations. For instance, products enriched with these compounds can be found in the market in Finland since 1995. However, recently, some studies have found adverse reactions that should be taken into account (Plat *et al.*, 2000; Jordan *et al.*, 2004).

2.1.4.1 Liposoluble antioxidants

Since plant sterol and stanol esters reduce the solubility of cholesterol by displacing it from the mixed micelles, some other lipophilic compounds (like liposoluble antioxidants) may also be displaced.

Randomised trials have shown that plant sterols and stanols lower blood concentration of β-carotene by about 25%, concentration of α-carotene by 10%, and concentration of vitamin E by 8% (Weststrate & Meijer, 1998; Gylling & Miettinen, 1999; Hallikainen *et al.*, 1999; Hendriks *et al.*, 1999). Since these liposoluble antioxidants protect LDL-cholesterol from oxidation, and stanols and sterols reduce the amount of LDL-cholesterol, the changes in blood concentrations of these antioxidants should be adjusted for the concentration of LDL-cholesterol. With this adjustment, concentrations of vitamin E remained unchanged, but those of β-carotene were reduced by 8-19% (Law, 2000). Nevertheless, eating more food products rich in carotenes, such as fruits and vegetables, may counteract this side effect (Law, 2000; Noakes *et al.*, 2002). The blood concentrations of vitamin D, vitamin A and vitamin K were unaffected (Gylling & Miettinen, 1999; Hendriks *et al.*, 1999; Plat *et al.*, 2000; Judd *et al.*, 2002)

The mechanism underlying these effects is not fully understood, although phytosterols may reduce the incorporation of these compounds in the mixed micelles. β-carotene absorption is reduced after phytosterol intake, whereas that of oxygenated carotenoids (lutein/zeaxanthin, β-cryptoxanthin) is not modified. This may be due to the

distribution of these antioxidants in the mixed micelle (surface-core). It is thought that the apolar hydrocarbon carotenoids (α-carotene, β-carotene and lycopene) are solubilized in the core of the mixed micelle, whereas oxygenated carotenoids are mainly in the surface (Borel *et al.*, 1996). This suggests that plant sterols and stanols may replace not only cholesterol from the core of the mixed micelles, but also other compounds solved there.

2.1.4.2 Serum concentrations of plant sterols/stanols

One of the most known adverse effects of plant sterols and stanols occurs in sitosterolemic subjects. Sitosterolemia is a rare inherited disease characterized by tendon xanthoma and premature coronary disease (especially in young men), haemolytic episodes, arthritis and arthralgias (Salen *et al.*, 1985; 1992).

These subjects have elevated rates of plant sterol and stanol absorption and low biliary excretion, resulting in accumulation of these compounds in plasma and tissues. Studies with sitosterolemic patients demonstrated increased phytosterol absorption of between 16 and 63% (Lütjohann *et al.*, 1995, 1996). Patients with sitosterolemia have mutations in ABCG5 and ABCG8 (transporters expressed in intestine and liver), which may result in a reduced transport of plant sterols and stanols from enterocytes back into the intestinal lumen and in a reduced secretion of these compounds into bile (Berge *et al.*, 2000; Hubacek *et al.*, 2001). Plasma levels of phytosterols in these patients are 7 to 16% of the total cholesterol concentration in plasma. Typically, ranges for β-sitosterol are 0.3 to 1.6 mol/L, for campesterol, 0.2 to 0.5 mol/L, as compared to levels of 0.01 to 0.05 mol/L for both sterols in normal healthy subjects (Kritchevsky & Chen, 2005).

Therefore, it may be thought that high concentrations of phytosterols may be atherogenic; in fact, some studies have found an association between increased plasma phytosterol concentrations and increased risk of coronary heart disease (Glueck *et al.*, 1991; Sutherland *et al.*, 1998; Rajaratnam *et al.*, 2000; Sudhop *et al.*, 2002; Assmann *et al.*, 2006). Phytosterols have been found in human tissues including normal aortic wall and atheromatous tissues (Bhattacharyya & Connor, 1974; Mellies *et al.*, 1976). All of these studies support the possibility that elevated phytosterols could contribute to the development of premature coronary artery disease in certain families. In contrast, several studies have been carried out in apolipoprotein E-deficient mice -an

accepted model for atherosclerosis- and in hamsters and rabbits, showing them all reduction of either lesion formation or lesion size after phytosterol supplementation (Moghadasian *et al.*, 1997; Ntanios *et al.*, 1998; Moghadasian *et al.*, 1999b; Volger *et al.*, 2001; Ntanios *et al.*, 2003). Therefore, more data are needed to clarify this potential adverse effect of phytosterols.

Furthermore, oxidized LDL is recognized as a pro-inflammatory component of the atherosclerotic process (Salonen *et al.*, 1997; Zieden *et al.*, 1999). Interestingly, plant sterols are more readily oxidized than cholesterol, which could contribute to atherosclerotic risk (Plat *et al.*, 2001). Some studies have shown serum oxisterol concentrations in healthy subjects (Grandgirard *et al.*, 2004) and also in sitosterolemic patients (Plat *et al.*, 2001). The relevance of these results is not known, although there are some conflicting data concerning whether phytosterol oxides are as atherogenic as cholesterol oxides are (Brown & Jessup, 1999). *In vitro* studies, phytosterol oxides have shown similar citotoxic effects as oxisterols (Adcox *et al.*, 2001; Ryan *et al.*, 2005). However, transgenic animals fed with phytosterol oxides have shown a reduction in the formation of atherosclerotic plaques (Moghadasian *et al.*, 1999a). Therefore, since the oxides of phytosterols may be produced during food processing, more studies are warranted to find out the risk of these compounds for health.

Other important manifestations of sitosterolemic patients are the non-cardiac manifestations, like large abnormally shaped red blood cells, thrombocitopenia, haemolysis and chronic haemolytic anaemia. These effects may be presumably related to abnormal sterol content of the red cell membrane (Patel & Thompson, 2006), since it is known that plant sterols and stanols are easily incorporated into cell membranes (Child & Kuksis, 1982; Ratnayake *et al.*, 2000) and, therefore, they may interfere membrane functionality. In fact, Ratnayacke and co-workers (2000) found a decrease in the life span and deformity of red blood cells of the stroke-prone spontaneously hypertensive (SHRSP) rats, maybe due to phytosterols replaced cholesterol from the membrane. It has been speculated that this replacement may make erythrocytes less deformable and fragile, resulting in an impaired oxygen transport through the smaller vessels. However, it is not known whether these effects are due to plant sterol and stanol intake or to decrease in cholesterol concentration. It would be interesting to find out the effects of an intake of plant sterols in this animal model, but without modifications in blood phytosterol concentrations, since concentrations were much higher than in non-sitosterolemic subjects.

The importance of these studies in humans is not known, although recently in Canada, some episodes of haematological adverse reactions after sterolin and plant sterol consumption have been described in adults and children (Jordan *et al.*, 2004). However, de Jong and co-workers (2006) found that plant sterol and stanol ester consumption for 16 weeks did not modify osmotic fragility of erythrocytes in statin-treated patients. Thus, more studies are needed to clarify the role of high plasma phytosterol concentrations on erythrocyte properties.

2.1.5 OTHER POTENTIAL BENEFICIAL EFFECTS

Since the discovery of phytosterols, a number of scientific studies has been conducted, mainly focused on their cholesterol-lowering activities. It is only the last fifteen years that other biological properties have been associated with the consumption of these plant compounds. These include anti-inflammatory activity, antipyretic and anti-diabetic properties. Now, there is clear evidence that these compounds can be applied as supplements to combat life-threatening diseases where the immune system needs maturation. Their importance in cancer is still limited, and more research is needed, although some data from *in vitro* and *in vivo* studies suggest that they may be effective, not only preventing the condition, but also in its treatment (Awad *et al.*, 2000).

Some studies (Awad *et al.*, 1996; Awad & Fink, 2000) have used established human colon cancer cell lines, human breast cell lines or prostate cell lines to show that cell growth is inhibited at doses of phytosterols within physiological ranges. They have also showed some beneficial effects in benign prostate hyperplasia (Wilt *et al.*, 1999). Their effects as immunomodulators and their anti-inflamatories properties (Bouic, 2001) have been recently reported. However, most of these studies are preliminary.

2.1.6 CONCLUSIONS

Most of the studies available have demonstrated that foods enriched with plant sterols and stanols reduce intestinal cholesterol absorption (Hallikainen *et al.*, 2000; Davidson *et al.*, 2001). Daily intakes of 2-3 g reduce LDL-cholesterol concentrations about 10%. Since high LDL-cholesterol concentrations increase CHD risk, decreasing

LDL-cholesterol concentrations by means of products enriched with plant sterols may be used to prevent the development of atherosclerosis. Some data confirm this hypothesis, due to some reduction has been found in the atherosclerotic plaque after plant sterols and stanols intake.

Although plant sterols and stanol intake has been considered safe to date, the side effects reported in sitosterolemic and also in non-sitosterolemic subjects, claim for a special attention to the possible association between high blood phytosterol concentrations and the risk for cardiovascular diseases.

2.2 PECTIN

2.2.1 DIETARY FIBER

Dietary fiber is a concept that includes materials of diverse chemical and morphological structure, resistant to the action of intestinal human enzymes. Within the gastrointestinal tract, fiber forms a matrix with both fibrous and amorphous characteristics.

A variety of definitions of fiber exist worldwide (IOM, 2001). Some of them are based in one or more analytical methods for isolating it, while others are based in its physiological effects. The Food and Nutrition Board, under the oversight of the Standing Committee on the Scientific Evaluation of Dietary References Intakes, assembled a Panel on the Definition of dietary fiber that proposed two definitions of fiber (IOM, 2001). On one hand, "*Dietary Fiber* consists of nondigestible carbohydrates and lignin that are intrinsic and intact in plants". On the other hand, "*Functional Fiber* consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in humans". Finally, *Total Fiber* would be the sum of dietary fiber and functional fiber.

The main sources of dietary fiber are plants, vegetables, cereal grains, fruits, legumes, leguminous plants, etc. Based on their intestinal solubility, dietary fiber may be classified as soluble and insoluble. Insoluble fiber includes lignin, cellulose and hemicellulose, whereas soluble fiber includes pectin, β-glucan, galactomannan gums, and a large range of nondigestible oligosaccharides such as inulin.

According to well-documented studies (Kay, 1982; Truswell, 1995; King, 2005), it is now generally accepted that dietary fiber plays an important role in the prevention of several diseases; therefore diets with a high content of fiber, such as those rich in cereals, fruits and vegetables, have a positive effect on health. The physicochemical properties of fiber determine its homeostatic and therapeutic functions in human nutrition (Kay, 1982). Of particular interest are its susceptibility to colonic bacterial enzymes, the water holding capacity and the adsorptive and ion exchange capacity.

Burkitt and Trowell (1977) described important metabolic effects of dietary fiber such as reduction of risk for cardiovascular disease, diabetes and cancer. Since then, many studies have been undertaken and most of them support a positive role for fiber in the prevention of chronic diseases. Proposed mechanisms for the biological action of dietary fiber include increasing faecal excretion of cholesterol and decreasing hepatic cholesterol synthesis (Vahouny *et al.*, 1980; Jenkins *et al.*, 1993), increasing satiety (Raben *et al.*, 1994) and insulin sensitivity (Hallfrisch *et al.*, 1995) and lowering plasminogen type 1 (Sundell & Ranby, 1993) and factor VII coagulation activity (Marckmann *et al.*, 1990). Dietary fiber has also been shown to reduce glycaemic response and circulating insulin concentrations in healthy adults (Fukagawa *et al.*, 1990), as well as in patients with hypercholesterolemia (Hallfrisch *et al.*, 1995) or diabetes (Anderson *et al.*, 1991; Chandalia *et al.*, 2000). Delaying carbohydrates may be one mechanism for the improvement of insulin sensitivity associated with a greater intake of dietary fiber (Jenkins & Jenkins, 1985).

Next, we discuss the actual knowledge about the soluble fiber pectin, with the main objective to justify its use as a functional ingredient.

2.2.2 DEFINITION OF PECTIN

Pectin refers to a family of oligosaccharides and polysaccharides with several structural parts that suffer a great number of biosynthetic modifications (Willats *et al.*, 2001). It is the main component of cells walls, with a great variety of biological functions in plants. For instance, pectin has a great number of physiological effects in the control of cell growth and defence against microorganisms, playing an important role in the physical properties of ripe fruits and their characteristics while being processed. Pectins are very insoluble in the unripe fruit but become much more hydrosoluble in the ripe fruit.

Pectin is extracted by means of a complex physico-chemical process, including solubilization, extraction and depolimerization steps. Most of the commercial pectins are extracted from apple pomace and citrus peel, although it is also possible to extract them from other vegetal products.

In human diet, pectin occurs in different forms: as a constituent of plant cell walls (fruits, vegetables and products of them) or in isolated form (e.g. in jams, jellies or milk products). In contrast to most of other dietary fibers, all pectins are rich in galacturonic acid (GalA); the FAO and EU stipulate that pectin must consist of at least 65% GalA. Three major pectic polysaccharides are recognized, all containing GalA to a greater or lesser extent. Homogalacturonan (HG) is a linear polymer with 1,4-linked α-D-GalA, whilst rhamnogalacturonan I (RGI) consists of the repeating disaccharide [$\rightarrow$4)-α-D-Gal(1-2)-α-L-Rha-(1$\rightarrow$] to which different glycan chains (principally arabinan and galactan) are attached to the Rha residues. Rhamnogalacturonan II (RHII) has a backbone of homogalacturonan with complex residues of side chains attached to the GalA residues (Ridley *et al.*, 2001; Willats *et al.*, 2001). In addition, in homogalacturonan, GalA may be both methyl-esterified and acetylated.

The degree of methylation (DM) is defined as the degree of esterification of carboxyl groups with methanol (Dongowski *et al.*, 2004). Traditionally pectin are categorised as high-ester or low-ester with DM of >50% or <50% respectively (Voragen *et al.*, 1995)

Many functional properties of pectin (rheological behaviour, gelation or binding of metal ions) are dependent on its structural parameters like molecular weight, DM or distribution of free and methoxylated carboxyl groups within the galacturonan chains (Dongowski *et al.*, 2002).

In the small intestine, physiological effects of pectin (interactions with bile acids, lowering of serum cholesterol, effects on the postprandial lipemia, etc.) are closely related to its macromolecular status. Pectin is not depolymerised by intestinal enzymes; however, a partial degradation is possible under the physicochemical conditions of the stomach and small intestine. Pectin is fermented by the microflora in cecum and colon. This degradation is a multistep process that involves depolymerization under formation of oligomeric and monomeric GalA, fermentation of the monomers and formation of SCFA (acetic, propionic and butyric acids), as well as different gases (hydrogen and methane), as end products. As a result, the qualitative

(bacterial species) and quantitative (germ number) composition of the microflora may be modified (Vargo *et al.*, 1985). Low-methoxyl pectin is fermented faster than high-methoxyl pectin *in vivo* and *in vitro* (Dongowski *et al.*, 2002).

2.2.3 NUTRITIONAL AND PHYSIOLOGICAL EFFECTS

Pectin belongs to dietary fiber because it is not digested by intestinal enzymes and also to functional fiber since a wide range of physiological and nutritional effects has also been attributed to pectin (Klavons & Bennet, 1995; Lupton, 2000; Kim, 2005).

Although it is not digested, pectin is fermented by colon microflora, generating carbon dioxide, methane, hydrogen and SCFA, especially acetic, propionic and butyric acids. These FA are a potential energy source for mucose of the large intestine cells; moreover, some of them may be absorbed in the colon providing additional metabolic effects.

There are clear evidences that pectin may lower cholesterol levels, serum glucose concentrations and may also have some anti-cancer activity. According to several authors, nutritional and physiological properties of pectin depend, on changes in the degree of methylation, molecular weight and/or distribution of carboxyl groups along polymer chain (Atallah & Melnik, 1982; Van Soest, 1984; Kim & Atallah, 1993; Kim, 1998; Zanutto *et al.*, 2002; Aprikian *et al.*, 2003).

In the next sections, we discuss the role of pectin on lipid and carbohydrates metabolism. Furthermore, we also discuss the new data available about pectin effects on colon cancer. Finally, other health effects associated to pectin consumption are also discussed.

2.2.3.1 Pectin effects on lipid metabolism

In the 60s, Keys and co-workers described the hypocholesterolemic properties associated with pectin intake, both in rats and humans. Since then, this effect has been demonstrated in many short-term studies (Jenkins *et al.*, 1975; Judd & Truswell, 1982; Haskell *et al.*, 1992; Brown *et al.*, 1999). Most of them reported that consumptions of 10-50 g/d are enough to achieve reductions in total-, LDL- and VLDL-cholesterol, as

well as in the ratio LDL/HDL. HDL-cholesterol concentrations were not affected by pectin addition.

However, there are some conflicting data in long-term studies. Knopp *et al.* (1999) studied the hypocholesterolemic effects of a dietary supplement of water-soluble fibers (guar gum, pectin) and non-water soluble fibers (soy fiber, pea fiber, corn bran) in subjects with mild to moderate hypercholesterolemia after 12 weeks of a 51-week treatment period. They reported marked reductions in LDL-cholesterol (around 12%) without reductions in HDL-cholesterol or increases in triacylglycerols. However, Marett & Slavin (2004) conducted a 6-month randomised, double blind, parallel trial in which subjects consumed their usual diet plus arabinogalactan (soluble dietary fiber) and they did not find any effect on cholesterol levels.

It is suggested that the efficacy of pectin depends on the kind and form of pectin used. This efficacy is greater, as higher is the degree of methylation (Furda, 1990). With low pH values (characteristic of colon environment), high-methylated pectin forms gels easier than that with low degree of methylation (Gallaher *et al.*, 1999). Further, pectin with high degree of methylation ferments slower than that with low degree (Dongowski *et al.*, 2002).

Davidson *et al.* (1998) confirmed the importance of the viscosity of the soluble fiber to achieve hypocholesterolemic effects. This author gave low-viscosity mixture with arabic gum and pectin (4:1) with doses of 5, 9 and 15 g/d, without finding any effect on serum cholesterol concentrations. It has been reported that a diet with 3% macromolecular pectin was more effective in reducing plasma cholesterol and increasing the excretion of bile acids and neutral steroids in hamsters than pectin with a lower molecular weight (Terpstra *et al.*, 1998). Studies in rats demonstrated that citrus pectin reduced plasma cholesterol concentrations more effectively than that of carrots, due to a viscosity 8 times lower (Ebihara *et al.*, 1979). Dongowski & Lorenz (2004) suggested that the amount of bile acids and neutral steroids excreted, as well as their composition, are highly influenced by the molecular and structural properties of pectin, finding the highest excretion after high-methylated pectin (DM 92.6%) intake. According to these authors, pectin can disturb or influence the micelle formation, as well as the lipid digestion and absorption in the small intestine, which is correlated with the greater faecal steroid concentrations found in animals fed pectin.

Like other dietary fibers, pectin interacts with bile acids through mechanisms still unknown (Pfeffer *et al.*, 1981; Falk & Nagyvary, 1982; Pandolf & Clydesdale; 1992; Klavons & Bennett, 1995). According to Ahrens *et al*. (1986), the passage of pectin through the small intestine in minipigs, is necessary for lowering serum cholesterol, because the intracecal infusion of pectin has no effect on total serum cholesterol level. Because of the interactions between pectin and bile acids in the small intestine, less bile acids are re-absorbed and therefore, more bile acids are transformed into the colon, where they are deconjugated and partly dehydroxylated by enzymes of the microflora. The greater excretion of bile acids is connected with a decrease in bile acids pool, since micelle formation with bile acids is essential for cholesterol absorption. This effect causes probably an increase in hepatic synthesis of bile acids and liver depletion of cholesterol resulting in reduced blood cholesterol levels (Miettinen & Tarpila, 1977; Stasse-Wolthuis *et al.*, 1980; Garcia-Diez *et al.*, 1996). Also, the enhanced excretion of bile acids increases the hepatic uptake of LDL by increasing LDL receptors (Slater *et al.*, 1980).

Moreover, SCFA (especially propionic acid), produced during fermentation of pectin by the intestinal microflora, may be absorbed in the portal vein and inhibit FA and cholesterol synthesis in animals (Nishina & Freedland, 1990) and in hypercholesterolemic subjects (Veldman *et al.*, 1999). Additionally, the formation of FA causes a decrease in colonic pH values, with a decrease in the solubility of bile acids, depressing thus their passive re-absorption in the lower parts of the intestine. Thus, the direct effect of pectin in the intestine does not look as the only explanation of its hypocholesterolemic properties.

2.2.3.2 Pectin effects on carbohydrate metabolism

In the 70s, the group of Jenkins stated that after carbohydrate intake, pectin may attenuate the postprandrial increase of glucose and insulin concentrations in diabetic subjects dependent on insulin, as well as in those non-dependent (Jenkins *et al.*, 1976). Most of these results have been corroborated, showing that pectin decreases glucose levels and/or insulin secretion after a sugar load (Nuttall, 1993; Jenkins *et al.*, 2000; Kalkwarf *et al.*, 2001; Ou *et al.*, 2001; Giacco *et al.*, 2002). Furthermore, it has been shown that pectin improves glucose tolerance by decreasing the peak of postprandial glycaemia and/or by preventing late hypoglycaemia in normal (Jenkins *et al.*, 1977) and diabetic subjects (McIntosh & Miller, 2001). Soluble dietary

fiber has been shown to reduce the rate of starch digestion and alter the rate of glucose absorption (Wood *et al.*, 1994; Pereira *et al.*, 2005). The viscosity-altering action of these soluble dietary fibers within the small intestine must account for some of these effects. However, the dietary fibers also appear to alter the structure of the foods and hence the accessibility of the starch granules to the amylase enzymes (Brennan *et al.*, 1996; Turorica *et al.*, 2002).

Most of the studies have proved that soluble fiber, like pectin or guar gum, reduces blood glucose concentration and/or insulin secretion after sugar intake in healthy subjects (Jenkins *et al.*, 1977) and diabetics (McIntosh & Miller, 2001). In a recent study, Kim (2005) corroborated that the addition of pectin decreases intestinal glucose absorption, this effect being more important with high-methylated pectin.

Guar gum, another soluble fiber with hypoglycaemic effects, has shown its efficacy to control glycaemia in long-term studies (Groop *et al.*, 1993). It is reasonable to guess that pectin and other soluble fibers have similar effects. Studies conducted until now suggest that consumptions of 15-20 g/d of pectin together with foods with carbohydrates are necessary to achieve the desirable hypoglycaemic activity.

Mechanisms underlying the hypoglycaemic effects of pectin and other soluble fibers are mainly due to the ability to reduce the absorption of carbohydrates in the intestinal tube (Mann, 2001). These effects may be the result of a decreased rate of the emptying of the stomach, altered motility in the stomach, poorer mixing of dietary components in the small intestine and a decreased rate of absorption across the epithelial cell membrane. In addition, a slower rate of diffusion of nutrient from the gut lumen toward the epithelial surface and an increase in the thickness of the mucosal layer could contribute to the decreased nutrient absorption of soluble fibers (Johnson & Gee, 1981).

The magnitude of the postprandial hyperglycaemia after test meals-containing pectin is related to the viscosity of the mixture. If the accessibility of starch to amylases is reduced, the digestibility and the absorption of glucose in the small intestine is also reduced (Giacco *et al.*, 2002).

2.2.3.3 Pectin and colon cancer

Epidemiological and controlled studies have revealed a negative association between dietary fiber and colorectal cancer risk (Dongowski & Lorenz, 2004), although recent studies present some conflicting results. There are many evidences, pointing to a connection between a decrease of colon cancer risk and a diet rich in insoluble fiber (Bingham *et al.*, 2003; Ferguson & Harris, 2003; Peters *et al.*, 2003). However, this association is not so clear for soluble fibers like pectin.

Thornton (1981) and other authors (Bruce, 1987) suggested that the risk of colon cancer was lower in people with low colonic pH values. The hypothesis proposed by these authors is that pH values lower than 6.5 may have a protective effect against colon cancer, since 7α-hydrolase is inhibited at these pH levels. 7α-hydrolase is a bacterial enzyme, responsible for the conversion of primary into secondary bile acids, which are considered as promoters of colon cancer (more than their primary derivatives) (Velazquez *et al.*, 1996). Dongowski & Lorenz (2004) found a lower proportion of secondary bile acids in conventional rats fed pectin-containing diets, supporting the role of pectin as a chemoprotective agent.

However, other studies (Lupton, 2000) showed that the reduction in colonic pH is not associated with a reduction in secondary bile acid concentration. One possible explanation may be the mechanism underlying the pH decrease. When the fiber is fermented, SCFA are produced, causing a drop in colonic pH. If the fiber in the colon increases, there is more carbon available for colonic microflora, increasing the amount of 7α-dehydrolase. Thus, the final result would be that the concentrations of secondary bile acids would be unchanged due to, although the activity of 7α-hydrolase is lower with low pH, the total concentration of this enzyme is higher (Lupton, 2000). Moreover, *in vitro* experiments have demonstrated that butyrate may stimulate the development of colon cancer by promoting differentiation and apoptosis in cell lines of this type of cancer (Smith *et al.*, 1998; Mandal *et al.*, 2001). However there are some contradictory data about butyrate effects, as well as the effects of other SCFA and their relationship with the incidence of colon cancer.

2.2.3.4 Other biological effects of pectin

There is a number of studies on other biological effects of pectin. For example, pectin is known for its antidiarrheic properties and it has been used for this purpose for more than half century (Malyoth, 1934). This effect is often followed by an antivomitive effect, that in newborns improve the assimilation and tolerance of food products. The mechanisms that may explain these effects are related to the high content of amylase-resistant starch, which passes into the colon and undergoes bacterial fermentation into SCFA, stimulating the colonic absorption of salt and water (Binder & Mehta, 1989; Rabbani *et al.*, 1999) and reducing thus diarrhoeal fluid losses. However, an indirect mechanism has also been suggested. SCFA may be absorbed by colon mucose and induce metabolic and proliferative changes in the epithelium of the small intestine (Rabbani *et al.*, 2001; 2004).

Other pectin properties under study are related to the treatment of gastritis, ulcers and gastroesophagic reflux (Havelund & Aalykke, 1997), to help the antibacterial activity in colon (Sepehri *et al.*, 1998) and to treat haemorrhoids (Alonso-Coello *et al.*, 2006), among others. However, not all pectins are biologically active and, therefore, the physico-chemical properties of pectin should be taken into account to get the desirable effects.

2.2.4 PECTIN SAFETY AND SIDE EFFECTS

Another important subject of study is the effect of pectin on the bioavailability of other nutrients, different from carbohydrates and lipids.

Animal experiments point to a negative influence of pectin on digestibility and use of proteins (Mosenthin *et al.*, 1994). In patients with ileostomy who received 15 g/d of pectin, only a slight increase in nitrogen excretion was found, due to a decrease in the digestibility and absorption of proteins and an increase in the losses of endogen nitrogen (Sandberg *et al.*, 1983). This negative pectin effect might be due to a decrease in the accessibility of protein molecules to the digestive enzymes, but also to a decrease in the transport of digestion products to the absorption sites. Pectin may also inhibit enzymatic activities and increase the secretion of proteolitic enzymes (Eggum, 1992).

The effect of pectin in the absorption and use of minerals have also been examined, with conflicting results. Although most of the studies have proved that pectin, irrespective of the degree of methylation has no influence on zinc, magnesium or iron absorption (Rossander, 1987), other studies suggest that pectin may reduce the bioavailability of these minerals (Kelsay *et al.*, 1979a,b). Galibois *et al*. (1994) found a reduction in the apparent absorption of zinc and magnesium in rats fed with soluble fiber, although no differences were found for iron. In this study, iron absorption was modified according to the amount of fiber added to diets. With 10% of fiber, iron apparent absorption decreased more than 5%. Pectin may also link calcium, reducing its absorption in small intestine. Further, after pectin fermentation, calcium linked may be available to be absorbed in the lower parts of the intestine (Trinidad *et al.*, 1996).

A number of studies with animal models has demonstrated that dietary pectin reduces the absorption and bioavailability of β-carotene (Rock & Swendseid, 1992; Hoffmann *et al.*, 1999). Riedl and co-workers (1999) found an important reduction in relative absorption of β-carotene, lycopene and luteins in healthy women. Besides, results about apparent absorption of α-tocopherol after soluble fiber intake, are conflicting, and the magnitude of the reduction depends on the doses of pectin added to diets. For instance, De Lumen *et al.* (1982) reported a decrease of α-tocopherol when nonpurified diets were supplemented with 6% of pectin. Nevertheless, no reduction was found with doses of 3%. Riedl *et al.* (1999) found no reduction in α-tocopherol concentration, again with the 3% dose.

The possible effect of pectins, modifying physico-chemical characteristics of the micelle, may be an explanation for the differences found between the apparent absorption of β-carotene and that of α-tocopherol, since these compounds have different polarities then being found in different sites in the mixed micelles (Riedl *et al.*, 1999). Hydrocarbon carotenoids (β-carotene, lycopene) are non-polar compounds, located in the core of the mixed micelle, whereas xanthophylls and compounds like α-tocopherol are more hydrophilic and are present in the surface of the mixed micelles (Borel *et al.*, 1996).

Finally, although pectin intake may reduce the digestibility of some micro- and macronutrients as it does for lipids and glucose, it is possible to counteract these effects following a recommended diet (Zanutto *et al.*, 2003).

2.2.5 CONCLUSIONS

Most of the studies conducted until now have concluded that pectins are able to modify lipid and carbohydrates metabolism, but also other components of the diet. Pectins are able to reduce the absorption of neutral sterols and bile acids, as well as to reduce postprandial glycaemia and insulinemia. However, another important factor to be taken into account is the physical-chemical properties of pectin used. Pectins with high-methylated degree are needed to find the most relevant effects, since they are able to form gels and more resistant to colonic fermentation. However, besides reducing carbohydrate and lipid absorption, pectin may also reduce protein digestibility and the availability of liposoluble antioxidants (especially carotenes) and minerals. In addition, there are not many data about the association of pectin and colon cancer, and what it is known is conflicting.

Although the decrease in the carotenes bioavailability may be considered as an undesirable effect, the dietary recommendations encourage increasing the consumption of soluble fiber in developed countries, where the fat consumption, especially that of saturated fat and cholesterol, is high. Therefore, the introduction of functional foods enriched with fiber (especially soluble fiber like pectin), could have favourable effects for the general population, as well as in that part of population with high levels of cholesterol, and/or in diabetic subjects.

OBJECTIVES

Since both plant sterols and pectin may reduce plasma concentrations of total- and LDL-cholesterol, it may be interesting to study the effects of functional foods enriched with both compounds. Moreover, functional foods enriched with phytosterols or pectin are on the food market. Functional foods enriched with phytosterols and pectin might have a synergic cholesterol-lowering effect, allowing for a reduction of the concentrations needed to achieve these effects separately. The main objective of this thesis is **to study the potential synergic effects of a high-saturated fat diet enriched with phytosterols and pectin on the lipid profile in an animal model, the guinea pig. Results will be compared with the effects of phytosterols and pectin separately.**

The diet used was rich in SFA and cholesterol, due to many reasons. First of all, we tried to simulate the real situation in Western countries, since many people from these countries do not meet recommended nutritional intakes, and the intake of fat, especially saturated fat and cholesterol, is high (Table 2) (Kris-Etherton *et al.*, 2002; Simopoulos, 2002). Second, functional foods should be part of a healthy diet, but they should never be used to counterbalance the effect of an unhealthy diet. Nevertheless, functional foods are extensively used in societies where saturated fat and cholesterol intake is high. Finally, to our knowledge, no studies have been conducted to study the effects of these functional ingredients when they are added to such a diet.

The animal model we used was the guinea pig. This model has been proposed as one of the best models to study human cholesterol and lipoprotein metabolism (Fernandez & Volek, 2006), by many reasons. (i) They have high LDL-to-HDL ratios. (ii) They have higher concentrations of free cholesterol than of esterified in liver. (iii) They have cholesteryl ester transfer protein (CETP), lecithin:cholesterol acyltransferase (LCAT) and lipoprotein lipase (LPL) enzimes. (iv) They exhibit comparable rates of cholesterol synthesis and catabolism. (v) Similar to humans, the binding domain for the LDL receptor differentiates between normal and familial binding defective receptor apolipoprotein B-100. (vi) ApoB mRNA editing in the liver is negligible, compared with other species. (vii) They require dietary vitamin C. (viii) Females have higher HDL concentrations than males. (ix) Ovariectomized guinea pigs have plasma lipid profile similar to that of postmenopausal women. (x) During exercise in guinea pigs, plasma triacylglycerols decrease and plasma HDL-cholesterol increases. (xi) Guinea pigs respond to dietary interventions and drug treatments, by lowering LDL-cholesterol.

To achieve the main objective, several goals were arisen:

- Study the influence of a high-saturated fat diet enriched with phytosterols on lipid profile, in plasma, liver and faeces.
- Study the effects of a high-saturated fat diet enriched with pectin on lipid profile.
- Study the possible synergic effects of the consumption of a saturated diet enriched with phytosterols and pectin.

To carry out all these specific objectives, a complementary task was needed:

- Validate a new analytical method to determine plant sterols and cholesterol in liver samples.

EXPERIMENTAL SECTION

1 EXPERIMENTAL DESIGN

The design of the study was as follows.

Animals

We used the animal research facilities of the Faculty of Pharmacy (University of Barcelona). The care and use of Laboratory Animals were in compliance with the article 5 from the "Law for the protection of animals for experimental uses and other scientific purposes" ("Llei de protecció dels animals utilitzats per l'experimentació i per altres finalitats científiques") from the Generalitat of Catalunya (Llei 5/1995, June 21). All procedures were approved by the Animal Care and Use Committee of the University of Barcelona.

To study the effects of the treatments on the lipid profile, we used a 3x3 factorial design. 72 female Dunkin Hartley guinea pigs were randomly assigned to each treatment group (8 animals/group). The animals were housed two per cage in a light cycle room (light from 08:00 to 20:00), under control of humidity and temperature.

The duration of the whole study was 5 weeks. Prior to the start of the study, all animals received control feeding without addition of phytosterols (PH0) or pectin (PE0) for one week, in order to get used with the base diet of the study. After this week, they were assigned to a treatment group and kept for 4 weeks, having free access to water and food. During the whole study, body weight and water and food consumption were controlled.

Diets

Three doses of phytosterols (0, 1.37 and 2.45%, wt/wt) and three doses of pectin (0, 3.67 and 6.93%, wt/wt) were used (Table 4). These doses were chosen according to studies already published.

The phytosterols used were esterified with UFA, since the esterification with these compounds increases their efficacy and the ease of solving them in food matrix

(Weststrate & Meijer, 1998; Hendriks *et al.*, 1999). The pectin used was "*GENU-type Freeze pectin*", with more than 50% of carboxyl groups methylated. High-methylated pectins were chosen, since a high-methylation degree is needed to achieve physiological effects (Gallaher *et al.*, 1999; Dongowski *et al.*, 2002). These functional ingredients were added on basis of a diet rich in SFA, in order to simulate the diet consumed in most of the developed countries, high in saturated fat and cholesterol. With this aim, 0.33% (wt/wt) of cholesterol was added to all diets.

Table 4. Diets composition

	Ingredients[a] (g/100g)								
Diets	**PE0/PH0**	**PE0/PH1**	**PE0/PH2**	**PE1/PH0**	**PE1/PH1**	**PE1/PH2**	**PE2/PH0**	**PE2/PH1**	**PE2/PH2**
Pectin	0	0	0	3.67	3.67	3.67	6.93	6.93	6.93
Phytosterols[b]	0	1.27	2.45	0	1.27	2.45	0	1.27	2.45
Protein	18.3	18.5	17.7	18.6	18.9	18.0	18.7	18.9	18.1
Fat	15.9	17.6	19.6	15.9	17.4	19.7	15.6	17.4	19.5
SFA[c]	11.3	8.7	9.8	12.1	9.2	10.0	12.4	9.2	9.5
Carbohydrates	38.4	40.0	39.4	35.3	36.2	33.6	33.1	30.5	29.2
Total fiber	13.7	12.8	12.4	15.9	16.3	16.3	19.2	18.9	18.7
Minerals and Vitamins[d]	6.5	6.7	6.5	6.7	6.7	6.7	6.7	6.8	6.7

[a] All diets were supplemented with 0.33% of cholesterol. [b] Phytosterols were added to basal diets. Phytosterol mixture was composed by 6.4% of brassicasterol, 24.9% of campesterol, 1.0% of campestanol, 18.5% of stigmasterol, 45.6% of β-sitosterol, 1.8% of sitostanol, 0.9% of δ-5-avenasterol and 1.0% other sterols. [c] SFA means saturated fatty acids. [d] Vitamin and mineral mix were adjusted following the guinea pigs dietary requirements.

All percentages are expressed as wt/wt.

1.1 SAMPLE PREPARATION

After 4 weeks of treatment, the animals were sacrificed, by heart puncture after halothane anaesthesia. Blood samples were obtained by means of an intracardiac extraction. After the sacrifice, the liver was extracted and homogenised. Faeces were collected three times a week and freeze-dried immediately. All samples were kept at –80ºC until the moment of the analysis.

1.2 ANALYTICAL METHODS

The following analytical methods have been used:

- Qualitative and quantitative determination of the main FA in the feeds and in biological samples (plasma, liver and faeces), by gas-chromatography.
- Qualitative and quantitative determination of cholesterol and main phytosterols in liver and plasma, by gas-chromatography.
- Qualitative and quantitative determination of plant sterols, cholesterol and cholesterol metabolites (coprostan-3-ol and coprostan-3-one) and primary and secondary bile acids in faeces, by gas-chromatography.

2 RESULTS

In order to determine cholesterol and plant sterol concentrations in liver, the validation of a new analytical method was needed. This led to the following paper:

- Rapid and quantitative determination of total sterols of plant and animal origin in liver samples by gas-chromatography
  Gemma Brufau, Rafael Codony, Miguel Angel Canela & Magda Rafecas
  Chromatographia (*In press*)
  Impact factor (2005): 0,959

Four additional papers are based on the analysis of the experimental results:

- A high-saturated fat diet enriched with phytosterol and pectin affects the fatty acid profile in guinea pigs
  Gemma Brufau, Miguel Angel Canela & Magda Rafecas
  Lipids (2006) 41; 159-168
  Impact factor (2005): 1.905

- Phytosterols, but not pectin, added to a high-saturated fat diet, modify saturated fatty acid excretion in relation to chain length
  Gemma Brufau, Miguel Angel Canela & Magda Rafecas
  The Journal of Nutritional Biochemistry (*Accepted*)
  Impact factor (2005): 2.459

- Plant sterol and pectin, added to a high-saturated fat diet, do not show the hypocholesterolemic activity in guinea pigs
  Gemma Brufau, Miguel Angel Canela, Joan Quílez & Magda Rafecas
  Clinical Nutrition (*Submitted*)
  Impact factor (2005): 2.296

- Reduction in cholesterol output after plant sterol and pectin addition to a high-saturated diet
  Gemma Brufau, Miguel Angel Canela, Rafael Codony & Magda Rafecas
  Lipids (*Submitted*)
  Impact factor (2005): 1.905

2.1 A HIGH-SATURATED DIET ENRICHED WITH PHYTOSTEROL AND PECTIN AFFECTS THE FATTY ACID PROFILE IN GUINEA PIGS

Authors: Gemma Brufau, Miguel Ángel Canela & Magda Rafecas

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A High-Saturated Fat Diet Enriched with Phytosterol and Pectin Affects the Fatty Acid Profile in Guinea Pigs

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ABSTRACT: This paper presents the results of a study whose aim was to test the effects of several doses of pectin and phytosterols on the body weight gain and the FA content in female guinea pigs. The treatments resulted from supplementing with pectin and plant sterol a guinea pig diet (rich in saturated FA), following a 3 × 3 factorial design, with three levels of pectin (0, 3.67 and 6.93%) and three levels of phytosterols (0, 1.37, and 2.45%). Seventy-two female Dunkin Hartley guinea pigs were randomly assigned to the treatment groups (8 animals/group), the duration of the treatment being 4 wk. Pectin dietary intake led to a significant increase in body weight ($P < 0.001$), food consumption ($P = 0.025$), and feed efficiency ($P < 0.001$), but no influence of phytosterols on weight gain or food consumption was detected. We found a significant negative effect of the addition of phytosterols on lauric, myristic, and palmitic acid contents in feces, and a positive effect on their concentration in plasma and liver, but no significant effect on stearic acid content. Apparent FA absorption was assessed by calculating the ratio of FA in feces and diets that the absorption of the different FA could be compared, and the negative effect of phytosterol supplementation on these ratios, especially for lauric and myristic acids, was established.

Paper no. L9877 in *Lipids 41*, 159–168 (February 2006).

During the past several decades, reduction in fat intake has been the main focus of dietary recommendations. In particular, it has been recommended to "choose a diet low in saturated fat and cholesterol and moderate in total fat" to prevent coronary heart disease (CHD) (1,2). Thus, there is a shift going on in nutrition, from the elimination of nutrient deficiencies, toward the possibility of improving health and quality of life. This awareness has lead to the development of functional foods, claiming beneficial effects for consumers. Several studies have shown that the ingestion of bioactive substances, such as soluble fibers (guar gum, psyllium, pectin, and oat products) and phytosterols (stanols and sterols) may have a positive lipidemic effect. As a consequence, they have been incorporated into some foods to reduce LDL-cholesterol levels and therefore prevent the development of CHD (3,4).

The cholesterol-lowering efficacy of plant sterol and stanol esters in food products has been confirmed in a great number of studies (5–10). Several mechanisms have been suggested to explain the action of phytosterols on lipid metabolism. For instance, β-sitosterol and sitostanol reduce plasma total-cholesterol levels through several mechanisms, such as competitively blocking cholesterol absorption from the intestinal lumen (11,12), displacing cholesterol from bile salt micelles (13), increasing bile salt excretion (14), or hindering the cholesterol esterification rate in the intestinal mucosa (15,16). Daily consumption of moderate quantities of phytosterols also has been shown to reduce plasma total-cholesterol by 5 to 13% and LDL-cholesterol by 7 to 16%, in both hyper- (17,18) and normocholesterolemic (19) individuals, without affecting HDL-cholesterol or TAG concentrations.

On the other hand, it is well known that including in the diet soluble viscous fibers such as pectin or guar gum as well as nonfermentable viscous fibers such as psyllium helps to lower plasma cholesterol concentrations (20–24). The soluble fiber action is mainly effected by binding bile acids, thus provoking a reduction in bile acid reabsorption of the small intestine.

Nevertheless, a separate examination of the effect of both phytosterols and pectin on the different FA is needed because there are different pathways of absorption for short-chain and long-chain FA. As early as 1935, Hughes and Wimmer (25) suggested that FA may be absorbed by different routes, according to their chain length. Since then, a number of studies (26) have established the role of the lymph and portal blood absorption pathways.

Finally, it is important to note that the consumption of vegetable shortenings is increasing because of their use in ready-to-eat foods (27) and in deep-fat frying. This is an undesirable development, because the intake of *trans* FA and saturated FA (SFA), which are two of the most important components of Western diets (28,29), is related to an increased risk of cardiovascular disease (1,2).

Therefore, the aim of the present study was to examine the effects of several doses of pectin and phytosterols on FA content in female guinea pigs fed a diet rich in SFA and cholesterol. The comparison of fecal and dietary levels allows us to examine the effects of this type of supplementation to a diet enriched with SFA on the apparent absorption (30–33) of the different FA. It has been reported (34,35) that results regarding apparent absorption were similar to those obtained using stable isotopes. Moreover, it is also important to determine the FA content in some biological tissues, such as liver and plasma.

The guinea pig was chosen as the animal model owing to its similarities with humans (LDL/HDL ratio, distribution of

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Abbreviations: CHD, coronary heart disease; MUFA, monounsaturated FA; SFA, saturated FA.

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cholesterol pools, and activities of main enzymes regulating cholesterol metabolism), and because guinea pigs respond to dietary treatments by changing plasma cholesterol concentrations in the same way as humans do (36).

MATERIALS AND METHODS

Materials. All FAME standards (12:0, 14:0, 16:0, 18:0, 18:1n-9, 18:2n-6, 18:3n-3, 19:0, and 21:0), with purity greater than 99%, were purchased from Sigma Chemical Co. (St. Louis, MO). The identification of FAME was made with FAME Mix, C_4–C_{24} (Supelco, Bellefonte, PA).

Boron trifluoride in methanol (1.7 mol/L) and *n*-hexane were purchased from Merck (Darmstadt, Germany), and sodium chloride and anhydrous sodium sulfate from Panreac (Barcelona, Spain). Sodium methylate (0.5 mol/L) was prepared by dissolving 17 g of sodium in 1 L of dry methanol.

Diets. Nine isocaloric diets, designed to meet all the nutritional requirements for guinea pigs, were prepared by Mucedola SRL (Settimo Milanese, Italy). The composition of all diets was similar, except for the content of pectin and phytosterols. Pectin was purchased from CP Kelco (Atlanta, GA). We used GENU-type Freeze pectin, which is a high (methyl) ester pectin (containing more than 50% of the carboxylic acid as methyl esters). This kind of pectin is a mixture of high-ester pectin extracted from citrus peel and α-amylolytic enzyme complex, which is standardized by addition of sucrose. Plant sterols were esterified with unsaturated FA (Raisio, Helsinki, Finland).

The treatments resulted from the addition of pectin and plant sterols, following a 3 × 3 factorial design, with three levels of pectin, denoted by PE0 (0%), PE1 (3.67%), and PE2 (6.93%), and three levels of phytosterols, denoted by PH0 (0%), PH1 (1.37%), and PH2 (2.45%). The chemical composition of the diets is shown in Table 1. The FA content in each diet was determined by the method proposed by Lopez-Lopez *et al.* (37).

Table 2 shows the content (μg/mg) of seven FA (12:0, 14:0, 16:0, 18:0, 18:1n-9, 18:2n-6, and 18:3n-3) in the nine diets. The presentation of results in this article is restricted to these seven FA, selected by their accumulation pattern and the differences observed in feces. The addition of pectin did not affect the total FA content but, since phytosterols were esterified with unsaturated FA, the FA profile was modified.

The SFA fraction ranged from 84 to 96% of the total FA content of the diet. The monounsaturated FA (MUFA) fraction was much smaller, but the most variable, ranging from 2 to 11% owing to the variations in the oleic acid content (Table 2). The PUFA fraction fluctuated from 1 to 5% of the total FA content. The main SFA was lauric acid (40–47%), followed by myristic acid (about 16%) and palmitic and stearic acids, with similar levels (9–10%). Also relevant was the presence of short-chain SFA, essentially 8:0 and 10:0, which accounted for 10% of the total FA content; however these are not reported in Table *2*, since their content in feces was below 1%. Oleic acid was the main unsaturated FA, presenting also the highest variation across diets (2–10%).

Animals. Seventy-two female Dunkin Hartley guinea pigs, supplied by Harlan Interfauna Ibérica (Barcelona, Spain) and weighing 300–350 g, were randomly assigned to the treatment groups (8 animals/group). The duration of the treatment was 4 wk. The guinea pigs were housed two per cage in a light-cycle room (light from 0800 to 2000), with free access to feed and water. They were killed by heart puncture after halothane anesthesia. Blood samples were obtained by means of an intracardiac extraction, and liver was extracted after the sacrifice and homogenized. Feces were collected with a 2-d periodicity and freeze-dried immediately. All procedures were approved by the Animal Care and Use Committee of the University of Barcelona.

FA analysis. The FA content in feces and in diets was determined with the method of Lopez-Lopez *et al.* (37). To determine the FA concentration in liver and plasma, we used a

TABLE 1
Composition of Diets[a] Fed to Guinea Pigs

Treatment	Pectin	Phytosterols[b]	Protein[c]	Fat[c]	Sugars[c]	Insoluble fiber[c]	Mineral and vitamin mix[d]
PE0/PH0	0	0	18.3	15.9	38.4	12.7	6.5
PE0/PH1	0	1.37	18.5	17.6	40.0	11.9	6.7
PE0/PH2	0	2.45	17.7	19.6	39.4	11.5	6.5
PE1/PH0	3.7	0	18.6	15.9	35.3	12.5	6.7
PE1/PH1	3.7	1.37	18.9	17.4	36.2	12.5	6.7
PE1/PH2	3.7	2.45	18.0	19.7	33.6	12.5	6.7
PE2/PH0	6.9	0	18.7	15.6	33.1	12.4	6.7
PE2/PH1	6.9	1.37	18.9	17.4	30.5	11.9	6.8
PE2/PH2	6.9	2.45	18.1	19.5	29.2	11.8	6.7

[a]All diets had been enriched with 0.33% of cholesterol.
[b]Added on top of the basal diet. The plant sterol ester mixture was mixed into the feed and comprised 6.4% brassicasterol, 24.9% campesterol, 1.0% campestanol, 18.5% stigmasterol, 45.6% β-sitosterol, 1.8% sitostanol, 0.9% Δ5-avenasterol, and 1.0% other sterols. PE0, PE1, PE2, pectin levels of 0, 3.67, and 6.93%, respectively; PH0, PH1, and PH2, phytosterol levels of 0, 1.37, and 2.45%, respectively.
[c]Protein content was casein; fat was hydrogenated coconut oil; sugars were corn starch, dextrose, and sucrose; and insoluble fiber was cellulose.
[d]Vitamin and mineral mixes were adjusted to meet National Research Council requirements for guinea pigs. A detailed composition of the vitamin and mineral mix has been reported by Krause and Newton (36).

TABLE 2
Content of the Main FA in the Diets

		FA (µg/mg)						
Pectin	Phytosterol	12:0	14:0	16:0	18:0	18:1n-9	18:2n-6	18:3n-3
PE0	PH0	54.61	20.62	11.35	12.63	2.98	1.27	ND[a]
PE0	PH1	41.98	15.88	9.35	10.00	6.45	2.32	0.60
PE0	PH2	47.26	17.87	10.53	11.38	11.81	4.20	1.27
PE1	PH0	49.61	22.23	12.12	13.30	3.65	1.42	ND
PE1	PH1	44.82	16.73	9.66	10.13	6.92	2.48	0.61
PE1	PH2	48.04	18.12	11.04	11.31	12.14	4.26	1.23
PE2	PH0	60.76	22.52	12.41	13.46	3.53	1.37	ND
PE2	PH1	44.32	16.75	9.99	10.44	6.82	2.30	0.61
PE2	PH2	45.34	17.19	10.30	10.98	11.20	3.76	1.16

[a]ND, not detected. For other abbreviations see Table 1.

method described elsewhere (38) but with some modifications to adapt it to our samples, followed by a double methylation to obtain FAME.

(i) Determination of FA content in feces. First, 25 µL of internal standard (21:0, 466 µg/mL) was added to 50 mg of freeze-dried fecal homogenate. Then, a small magnetic stirring bar was added and the sample was saponified with 1 mL of sodium methylate (0.5 mol/L) and heated in a water bath at 90°C for 15 min. The tubes were removed from the water bath and cooled in another water bath at room temperature. Then, 1 mL of boron trifluoride/methanol (1.7 mol/L) was added. Next, the tubes were placed in the water bath at 90°C for 15 min and subsequently cooled as before; 400 µL of *n*-hexane was then added. The tubes were shaken, and 1 mL of a saturated solution of sodium chloride in distilled water was added, followed by centrifugation. The clear *n*-hexane top layer, containing the FAME, was transferred to another tube, and a small quantity of anhydrous sodium sulfate was added. The tubes were stored at −20°C until the contents were injected into the gas chromatograph.

(ii) Determination of FA content in liver. Hepatic homogenate (between 0.8 and 1.0 g) was weighed into a 32 × 210 mm tube, and 200 µL of internal standard (21:0, 466 µg/mL) was added. Just before homogenization, 20 mL of chloroform/methanol (2:1, vol/vol) was added. The tube contents were homogenized (30 s, 12,000 rpm) in a Polytron PT 3000 (Kinematica, Lucerne, Switzerland), keeping the tubes in an ice bath. The extract was filtered through a Whatman No. 1 filter paper into a 50 mL screw-capped tube, and the residue was re-extracted twice (10 mL) with the same solvent. Next, 10 mL of water was added to the tube and, after centrifugation (20 min, 750 × *g*), the chloroform phase was filtered through anhydrous sodium sulfate (Whatman No. 1 filter paper), which was then washed twice with 10 mL of chloroform. The lipid extract obtained was dried in a vacuum rotary evaporator at 30°C, and re-extracted five times with 2 mL of diethyl ether. The tubes were dried first by a slight nitrogen stream and then by keeping the flask in a vacuum desiccator at 10 mmHg overnight.

After lipid extraction, a small magnetic stirring bar was added, and the samples were saponified with 2.5 mL of sodium methylate (0.5 mol/L) and heated in a water bath (90°C, 20 min). After that, the tubes were cooled in a water bath at room temperature, and 3 mL of boron trifluoride/methanol (1.7 mol/L) was added. Next, they were placed in the water bath at 90°C for 20 min, subsequently cooled as before, and 2 mL of *n*-hexane was added. The tubes were shaken, and 3 mL of a saturated solution of sodium chloride in distilled water was added, followed by centrifugation. The clear *n*-hexane top layer, containing the FAME, was transferred to another tube, and a small quantity of anhydrous sodium sulfate was added. The tubes were stored at −20°C until the contents were injected into the gas chromatograph.

(iii) Determination of FA content in plasma. Plasma (100 µL) was added to a tube containing 25 µL of internal standard (19:0, 996 µg/mL). Then, 3 mL of chloroform/methanol (2:1, vol/vol) was added and mixed. After that, 0.75 mL of 0.8% aqueous NaCl was added, mixing again. After centrifugation (7 min, 1025 × *g*), the chloroform phase was transferred to another tube, and the aqueous one was washed with 3 mL of chloroform. The lipid extract obtained was dried with a slight nitrogen stream, keeping the flask in a vacuum desiccator at 10 mmHg overnight. The double methylation was done in the same way as in hepatic samples, but instead of using 2.5 mL of sodium methylate and 3 mL of boron trifluoride/methanol, 1 mL of each was used. FAME were obtained by adding 400 µL of *n*-hexane to tubes, followed by 1 mL of a saturated solution of sodium chloride in distilled water. The clear *n*-hexane top layer, containing FAME, was transferred, adding a small quantity of anhydrous sodium sulfate. The tubes were stored at −20°C until injection into the gas chromatograph.

GC conditions. FAME were analyzed with an Agilent 4890D gas chromatograph, equipped with an FID. The separation of FAME was done in a fused-silica column (60 m × 0.20 mm i.d., 0.2 µm). The split-splitless injector was used in split mode with a ratio of 1:30. Injector and detector temperatures were kept at 270 and 300°C, respectively. For feces analysis the oven temperature was programmed as follows: an initial period of 5 min at 100°C, followed by an increase of 4°C/min, up to 235°C, and a final period of 2 min at this temperature. In liver samples the oven program started with a period of 5 min at 140°C, followed by an increase of 1.8°C/min until 180°C, followed by another increase of 7.5°C/min until 235°C. Finally, for FAME determination in plasma samples, the initial temperature was 170°C (11 min), being for the rest the same as in liver samples.

TABLE 3
Food Consumption and Final Body Weight

Pectin	Phytosterol	Food consumption[a]	Body weight[b]
PE0	PH0	35.30 ± 7.84	374.55 ± 13.11
PE0	PH1	32.56 ± 1.33	364.75 ± 30.20
PE0	PH2	33.59 ± 5.78	368.41 ± 25.61
PE1	PH0	32.27 ± 4.55	400.97 ± 34.97
PE1	PH1	35.69 ± 7.18	400.04 ± 52.80
PE1	PH2	33.66 ± 14.47	397.09 ± 24.19
PE2	PH0	29.25 ± 1.49	388.85 ± 66.68
PE2	PH1	28.70 ± 3.35	412.44 ± 17.00
PE2	PH2	27.99 ± 2.75	412.70 ± 33.04

[a]Mean of consumption (g/d/animal) ± SD.
[b]Mean of body weight (g) ± SD. For abbreviations see Table 1.

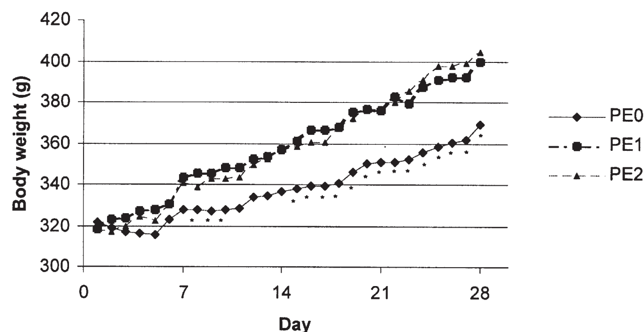

FIG. 1. Mean body weight curves for different pectin levels. PE0, PE1, and PE2, pectin levels of 0, 3.67, and 6.93%, respectively. An asterisk (*) means significant differences among diets ($P < 0.05$).

Helium was used as the carrier gas. Chromatographic peaks were identified by comparing the retention times with those of known standards and by cochromatography. FAME quantification was performed by the internal standard addition method.

Statistical analysis. The effects of the treatments on body weight gain and FA content were tested by two-way ANOVA with the amount of pectin and phytosterols as factors. Since an assumption of homogeneity of variance in the case of food consumption (Table 3), where coefficients of variance range from 4 to 43%, was unrealistic, we used the Kruskal–Wallis test to assign a significance level to the pectin supplementation effect.

On the other hand, since the FA profile of the diets changes with phytosterol supplementation, as a result of esterification, the phytosterol effect can be confounded with a diet effect, especially for the MUFA and PUFA. To account for these diet effects, we performed the statistical analysis, for the different FA considered, for both the content and the tissue/diet ratio. For the liver, dividing the numbers of Table 5 (see below) by those of Table 1 gives a set of percentages that can be interpreted as the percentages of the different FA that are incorporated into liver. For plasma, assuming unit density, the ratio comes in a 1/100 scale since the results in plasma are given in µg/mL whereas those in liver are given in µg/100 mg, but the interpretation is the same.

The significance of the differences in FA content and incorporation ratios was also tested by two-way ANOVA. We used SPSS 11.0 (Erkrath, Germany) in the calculations. As usual, $P < 0.05$ was considered significant.

RESULTS

Food intake and body weight. Guinea pigs were healthy throughout the feeding period except for four animals (6%) from groups PE0/PH2, PE1/PH0, PE1/PH2, and PE2/PH1 that died. No relation was found between the diets and the death of these animals. These deaths were taken into account in all measurements, such as body weight, food intake, and FA concentrations. Treatment means (±SD) of food consumption and body weight are shown in Table 3. Although no relevant effect of phytosterol supplementation was detected, we observed a decrease in food consumption from PE1 to PE2, and an increase in body weight from PE0 to PE1. Overall significance tests on pectin effect gave $P = 0.025$ for food consumption and $P < 0.001$ for body weight.

As shown by the curves of Figure 1, all groups gained weight during the study. The differences in weight increase in successive periods were tested. Whereas the pectin effect was significant ($P < 0.001$) from the second week, as shown in Figure 1, the phytosterol effect was not significant (data not shown). Feed efficiency was significantly higher ($P < 0.001$) in the animals fed with pectin than in those fed without, but the phytosterol effect again was nonsignificant.

Fecal FA. The mean content in feces of the seven FA of Table 2 can be seen in Table 4. Comparing the figures in Table 4 with those in Table 2 reveals that the total FA was lower in feces than in the diet, suggesting that fat was better absorbed than the other components of a guinea pig standard diet. Nevertheless, there was a great variability in absorption across the FA spectrum.

Using the ratio of the mean content in feces divided by the content in diet, for the different components of the FA spectrum, we have a rough assessment of apparent absorption. First, we found an overall ratio of 0.35 for total FA. The ratios of the SFA fraction were clearly related to chain length. Thus, the short-chain SFA (8:0 and 10:0) were almost completely absorbed. For the medium- and long-chain SFA, the ratios ranged from 0.12 in lauric acid to 1.07 in stearic acid, with intermediate values for myristic (0.36) and palmitic (0.74) acids. Palmitic and stearic acids were the only ones for which the fecal content was higher than the diet content. MUFA and PUFA ratios must be evaluated more cautiously, since we were dealing with a diet containing highly SFA. Anyway, the ratios for these three FA as reported in Tables 2 and 4 were similar (around 0.16).

Such a variability in fat absorption calls for a separate analysis of the supplementation effects on the different FA. The effect of phytosterol supplementation on the lauric acid content in feces is illustrated in Figure 2, which is a plot of lauric acid content as a function of the phytosterol level, for different levels of pectin. The curves corresponding to levels PE0 and PE1 are remarkably parallel and can be easily related to the variation of the lauric acid content of the diet. The curve corresponding to level PE2 is clearly different, suggesting that high

TABLE 4
Concentrations of the Main FA in Total Fecal Content

		FA[a] (µg/mg)						
Pectin	Phytosterols	12:0	14:0	16:0	18:0	18:1n-9	18:2n-6	18:3n-3
PE0	PH0	8.19 (0.38)	9.81 (0.90)	10.09 (1.64)	14.87 (3.01)	0.81 (0.01)	0.28 (0.01)	ND
PE0	PH1	5.60 (1.23)	6.34 (1.31)	8.13 (2.48)	12.85 (3.99)	1.79 (0.62)	0.49 (0.17)	0.12 (<0.01)
PE0	PH2	5.81 (1.33)	6.32 (1.36)	8.03 (1.93)	13.85 (4.29)	3.08 (0.61)	0.78 (0.14)	0.25 (0.01)
PE1	PH0	6.76 (2.59)	8.31 (2.52)	9.07 (2.24)	12.92 (4.45)	0.75 (0.14)	0.23 (0.01)	ND
PE1	PH1	4.48 (0.39)	5.45 (0.70)	6.83 (1.13)	10.54 (2.42)	1.49 (0.16)	0.42 (0.01)	0.01 (<0.01)
PE1	PH2	4.38 (1.10)	5.16 (0.95)	7.25 (0.84)	12.75 (2.16)	2.31 (0.44)	0.59 (0.19)	0.16 (<0.01)
PE2	PH0	6.53 (1.99)	7.72 (1.64)	7.96 (0.78)	11.01 (1.47)	0.68 (0.18)	0.18 (0.01)	ND
PE2	PH1	5.57 (1.25)	6.78 (1.21)	7.39 (1.35)	11.63 (2.65)	1.58 (0.57)	0.41 (0.16)	0.01 (<0.01)
PE2	PH2	3.97 (0.75)	5.12 (0.46)	7.22 (1.15)	10.98 (2.02)	1.96 (0.41)	0.50 (0.01)	0.14 (<0.01)

[a]FA content expressed as µg/mg of total fecal content (SD in parenthesis). For abbreviations see Tables 1 and 2.

levels of pectin supplementation modulated the effect of phytosterol supplementation, although the small sample size did not allow us clarify this point. The ANOVA test yielded $P <$ 0.001 for the phytosterol effect and indicated no significance for the pectin and the interaction effect. Since part of the phytosterol effect could be attributed to the increase of lauric acid content in the diet, one could choose to test the ratio of apparent absorption mentioned above. The result was then $P = 0.044$, which is still significant.

The results of myristic acid, not plotted, were very similar, giving again $P < 0.001$ for the feces content and $P = 0.017$ for the ratio of apparent absorption. Looking at the variation in these ratios, one could see that the phytosterol supplementation decreased the amount of lauric and myristic acid that was excreted by 20%, thus increasing the apparent absorption.

The effect of phytosterol supplementation on palmitic acid content is illustrated in Figure 3. The comparison of the curves shows again the combined effect of phytosterol and pectin supplementation. Here, the decrease in the second part of the curve that is related to pectin level PE2 contrasts with the increase in the palmitic acid content of the diet (Table 2). The test of significance for the phytosterol effect was $P = 0.014$. Although there was a decrease in the feces/diet ratio, from 0.89 in the control diet to 0.70 in the PH2/PE2 diet, the test for this ratio indicated no significant difference, so the conclusions about the phytosterol effect cannot be so firm as for palmitic acid. The results for stearic acid, not plotted here, presented a similar pattern, although the supplementation effects were not significant.

For oleic acid in feces, we found no effect of the addition of pectin, but a significant positive phytosterol effect ($P < 0.001$) correlated with the variation in the content of this FA in the diet. Nevertheless, when looking at the feces/diet ratio, the effects were opposite, so the combined phytosterol and pectin supplementation produced a decrease of 20% with respect to the control diet (PE0/PH0). The results for linoleic acid were similar, with a decrease in the feces/diet ratio of 30%.

Finally, the results of linolenic acid were somewhat different. For the content in feces, we found the same highly significant positive phytosterol effect but a significant negative pectin effect ($P = 0.021$). For the feces/diet ratio, we found negative effects. The pectin effect, in particular, was almost significant ($P = 0.083$).

FA content in liver. Table 5 is a summary of the FA content in liver (µg/100 mg) for the different treatment groups. The SFA fraction, which dominated the FA profile, was quite stable (54–58%). Second came the PUFA fraction, which was more variable (19–29%), and then the MUFA fraction (14–23%).

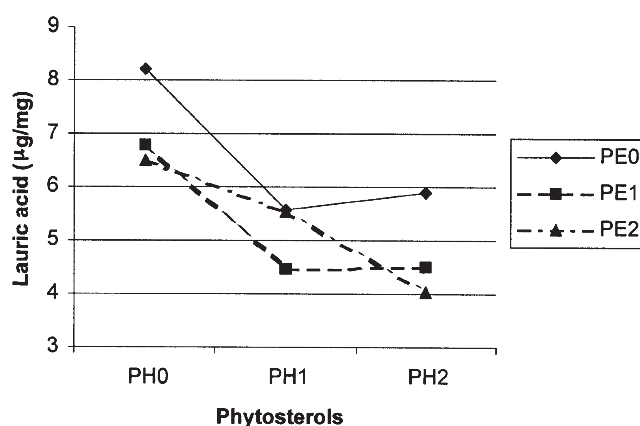

FIG. 2. Concentration (µg/mg) of lauric acid in total feces. PH0, PH1, and PH2, phytosterol levels of 0, 1.37, and 2.45%, respectively; for other abbreviations see Figure 1.

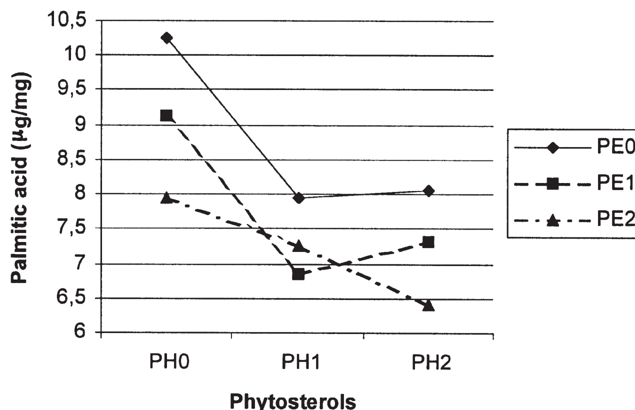

FIG. 3. Concentration (µg/mg) of palmitic acid in total feces. For abbreviations see Figures 1 and 2.

We consistently found, across the different components of the SFA fraction, a nonlinear effect of supplementation, an increase from PH0 to PH1, and a slight decrease from PH1 to PH2. The same effect, but generally weaker, was found for the pectin supplementation. In particular, the highest SFA levels were found when the intermediate doses were combined (PE1/PH1), showing an increase in the SFA liver incorporation ratio from 1.3% in group PE0/PH0 to 2% in group PE1/PH1. The effect of phytosterol on the incorporation ratio was significant ($P < 0.001$), but the pectin effect was not.

Although lauric acid (12:0) was the main component of the FA profile of the diet, it was a minor component in the liver (3–6%), whereas myristic acid (14:0) was more abundant (10–14%). The phytosterol effect on the incorporation ratio was significant in both cases ($P < 0.001$ and $P = 0.002$, respectively), and the pectin effect was not significant. Except for higher doses of pectin (PE2), the phytosterol effect was approximately linear.

Palmitic acid (16:0) was one of the major components of the FA profile in the liver (16–20%). The results for palmitic acid were not significant, although the pectin/phytosterol interaction was almost significant ($P = 0.089$). The content of stearic acid (18:0) was a bit higher (18–23%). Both pectin ($P = 0.002$) and phytosterol ($P = 0.003$) had significant positive effects on this ratio.

The increase in the relative content of oleic acid (18:1n-9) in the diet (2.5–10%) was correlated with the increase in the liver samples (12–16%), but this effect was not significant. The results of linoleic acid (18:2n-6), the last of the major components of the FA profile (12–21%), had a similar pattern, but they were very significant ($P < 0.001$). Finally, for the n-3 PUFA fraction, only linolenic acid was reported, since others were not detected in the diet. The percentage of the n-3 PUFA fraction remained quite stable (11–13%) and was unaffected by changes in these FA in diet.

FA content in plasma. Table 6 is a summary of the FA content in plasma (µg/mL). The percentage of SFA (53–63%) was more variable than in liver, but the levels were similar. Here, palmitic acid was the major component (20–23%), followed by stearic acid (12–14%), myristic acid (10–15%), and lauric acid (4–7%). The percentages of MUFA (19–26%) and PUFA (14–28%) were correlated with the contents of oleic acid and of linoleic and linolenic acids, respectively, in the diet.

The supplementation effects on the total FA content were very clear. As in liver, both pectin and phytosterol supplementation had a nonlinear effect, and the FA content was higher at intermediate doses. As a consequence, the maximal FA content was found when combining intermediate doses (PE1/PH1). For the total FA content, the pectin effect was significant ($P = 0.002$),

TABLE 5
Content of the Main FA in Liver

		FA[a] (µg/mg)						
Pectin	Phytosterols	12:0	14:0	16:0	18:0	18:1n-9	18:2n-6	18:3n-3
PE0	PH0	9.67	29.78	53.77	55.23	36.65	44.39	3.30
		(7.51)	(15.31)	(14.78)	(3.97)	(10.54)	(9.87)	(1.65)
PE0	PH1	9.76	34.20	55.97	56.05	37.69	48.31	4.50
		(3.96)	(11.21)	(13.91)	(4.21)	(9.26)	(7.52)	(1.08)
PE0	PH2	16.70	39.39	51.47	52.27	37.41	57.71	5.78
		(8.71)	(11.76)	(12.82)	(4.78)	(12.19)	(8.09)	(2.45)
PE1	PH0	7.67	23.15	40.26	55.12	30.71	46.04	3.18
		(4.34)	(10.21)	(9.25)	(4.57)	(11.87)	(8.63)	(1.06)
PE1	PH1	16.27	42.23	58.65	59.28	38.37	58.22	5.85
		(10.32)	(17.99)	(20.71)	(6.35)	(11.33)	(8.62)	(2.43)
PE1	PH2	19.96	46.27	56.85	58.93	43.08	67.88	6.35
		(9.20)	(13.21)	(14.50)	(6.18)	(11.92)	(11.09)	(2.08)
PE2	PH0	10.20	33.16	62.98	58.69	48.05	36.56	2.35
		(3.12)	(8.17)	(17.79)	(3.89)	(18.62)	(9.46)	(0.86)
PE2	PH1	17.55	40.53	54.32	56.09	35.11	53.43	5.05
		(14.40)	(30.11)	(32.67)	(6.78)	(18.33)	(8.05)	(3.40)
PE2	PH2	16.81	38.42	46.88	58.70	34.50	61.97	5.33
		(9.75)	(14.46)	(8.72)	(3.26)	(5.81)	(6.39)	(1.51)

[a]FA content expressed as µg/100 mg of liver (SD in parenthesis). For abbreviations see Table 1.

TABLE 6
Content of the Main FA in Plasma

		FA[a] (μg/mL)						
Pectin	Phytosterols	12:0	14:0	16:0	18:0	18:1n-9	18:2n-6	18:3n-3
PE0	PH0	93.16	187.40	316.06	172.33	268.57	195.21	14.36
		(46.79)	(53.17)	(68.12)	(51.68)	(103.83)	(137.09)	(14.94)
PE0	PH1	84.55	206.69	335.52	210.04	275.82	235.70	20.66
		(37.66)	(83.37)	(96.70)	(68.63)	(95.03)	(178.95)	(13.10)
PE0	PH2	78.92	178.01	318.14	189.89	320.84	352.68	19.58
		(30.49)	(32.22)	(96.26)	(46.67)	(150.49)	(109.88)	(14.69)
PE1	PH0	133.33	277.18	357.33	219.49	336.66	315.18	16.68
		(57.83)	(115.90)	(84.65)	(68.73)	(103.44)	(135.30)	(9.83)
PE1	PH1	143.91	266.02	433.06	274.82	389.87	460.62	31.05
		(73.74)	(66.47)	(124.95)	(50.08)	(72.92)	(100.56)	(15.54)
PE1	PH2	65.95	190.34	367.89	234.65	332.49	472.58	35.34
		(15.10)	(47.35)	(124.67)	(84.58)	(104.80)	(136.15)	(28.58)
PE2	PH0	92.59	199.85	344.72	168.04	330.62	171.35	11.03
		(27.46)	(64.58)	(98.62)	(40.04)	(126.80)	(30.03)	(6.71)
PE2	PH1	127.15	212.55	380.97	210.90	344.44	350.63	20.13
		(65.03)	(44.99)	(135.98)	(36.63)	(182.73)	(139.21)	(9.80)
PE2	PH2	70.75	174.45	319.45	211.15	295.22	351.14	23.50
		(20.21)	(25.66)	(55.10)	(54.80)	(37.61)	(72.82)	(8.37)

[a]FA content expressed as μg/mL of plasma (SD in parenthesis). For abbreviations see Table 1.

whereas the phytosterol effect was not, but both effects were significant for the total SFA content ($P = 0.012$ and $P = 0.050$, respectively). Much more significance was found in the plasma/diet ratio, especially for the phytosterol effect ($P < 0.001$). In absolute terms, the type of supplementation used in this study could increase by 50% the total FA content (from 1409 μg/mL in group PE0/PH0 to 2182 μg/mL in group PE1/PH1), without increasing the content in the diet. A similar increase was found in the total SFA content. This pattern was consistently found in all SFA fraction. The effects were stronger for stearic acid and weaker (in decreasing order) for myristic, lauric, and palmitic acids.

The results for oleic acid were again nonsignificant. For linoleic acid, the pectin effect showed the same pattern as in the SFA fraction and was significant ($P = 0.007$). The phytosterol effect was confounded with a diet effect, being positive when the plasma content was used for the comparison, but negative for the plasma/diet ratio. The levels of n-3 PUFA in plasma were very low.

DISCUSSION

Although many research groups in the last 50 years have studied the role of phytosterols and pectin as cholesterol-lowering compounds (7,9,10,20,39–42) few studies have been conducted to determine how these compounds may affect FA metabolism. In the present study, we examined the effect of phytosterol supplementation, added to a diet rich in SFA, on the content of certain FA in plasma and liver and how this effect can be changed when pectin is also supplemented. We decided to add these compounds to a SFA-enriched diet since most of the studies used low-fat products as carriers (43,44). However, to our knowledge, no data are available about the effects of the addition of phytosterols and/or pectin to a Western diet (rich in SFA and cholesterol) (28,29).

In our study, we found a negative effect of pectin supplementation on food consumption, and no effect of phytosterol supplementation. Dietary pectin intake also resulted in a significant body weight increase, which agreed with some results described previously (23), although others (21,45,46) did not find such an effect. We found an increase in feed efficiency at higher doses of pectin, contrary to the results of other studies (45,46). The unexpected increase in feed efficiency and body weight may be due to an early breakdown of this fiber in the gut, although we used high-esterified pectin, which is less fermentable than low-esterified (47,48). This might be explained by taking into account the possible interaction between pectin and the source of added fat, since it has been reported that pectin may cause mucosal proliferation in the lower intestinal tract (49), and this effect can be modulated by the source of fat administered (50,51). Further studies are necessary to clarify the interactions between sources of fat and doses of pectin with different degrees of esterification.

On the other hand, our results are consistent with a previous study (52), in which no effect of different doses of phytosterols on body weight was observed. Despite the high amount of fat in diets enriched with phytosterols (Table 1), our results indicate that slight differences in composition of diets had no effect on growth.

Since the supplementation, whose effects are examined in this paper, was made to a standard guinea pig diet, we deal here with a highly SFA profile. This allows us to emphasize our conclusions on SFA profile but calls for some caution when extending the conclusions about MUFA and PUFA absorption to diets with a completely different FA profile.

Whereas the absorption rates of the MUFA and PUFA examined in this paper look quite uniform, with a feces/diet ratio

of about 0.16, those of SFA are clearly related to the chain length, showing big differences from the short-chain FA (8:0 and 10:0) to long-chain FA. Such a variety suggests that any approach to fat absorption must account for the specificities of the different FA, especially of those related to chain length.

The results for lauric and myristic acid are similar and consistent with the fact that both are medium-chain SFA, so that they should have a similar absorption pathway. The concentration of lauric acid in feces is shown in Figure 2, where the interpretation of the curves corresponding to levels PE0 and PE1 is straightforward: Although the decrease in the first segment can be partly attributed to a decrease in the dietary supply of these FA, the second segment shows that at least for the highest doses, phytosterol supplementation has a negative effect on the fecal content of lauric and myristic acids. When relating the feces content to the diet content, we find a significant positive effect on apparent absorption, suggesting that phytosterols added to a diet enriched with SFA increase the absorption of these FA. Although the conclusions about absorption rates for palmitic acid cannot be so firm, the results point in the same direction, that of a negative effect of phytosterol supplementation. Moreover, the results in plasma and liver agreed with their apparent absorption, in which a significant association was also found between SFA and phytosterol intake.

Unsaturated FA are much less abundant in the diets used in this study, and the results for them are not so clear but point in the same direction, toward a positive effect on FA absorption.

On the other hand, the influence of the dietary fiber intake on SFA is not clear. Whereas some authors (53) reported that the effects of dietary fiber intake might counteract the effects of saturated fat intake and thus reduce CHD events, others (39,54,55) did not find any differences in SFA content in biological samples after fiber ingestion. A recent study (56) reported a liver weight gain in rats fed with a fiber mixture. However, in our study, we did not find a direct effect on FA concentrations after fiber feeding, although pectin modulated the phytosterol effect on FA absorption (results not statistically significant).

The main animal studies (3,57–60) show that dietary phytosterols inhibit the atherosclerotic process, although others (61–65) report an association between increased plasma levels of phytosterols and increased risk of CHD in phytosterolemic and nonphytosterolemic subjects. It remains to be shown whether the high serum plant sterol levels caused by long-term plant sterol ester consumption are harmful. However, taking into account that SFA as lauric, myristic, and palmitic acids have been associated with cardiovascular risk (54,66,67), our results may agree with those who found a potential atherosclerotic effect of plant sterols in the way that they are capable to increase biological SFA concentrations.

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2.2 PHYTOSTEROLS, BUT NOT PECTIN, ADDED TO A HIGH-SATURATED DIET, MODIFY SATURATED FATTY ACID EXCRETION IN RELATION TO CHAIN LENGTH

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Date: Oct 06, 2006
To: "Magda Rafecas" magdarafecas@ub.edu
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Subject: JNB-06-255R1: Final Decision

Ms. No.: JNB-06-255R1
Title: Phytosterols, but not pectin, added to a high-saturated fat diet, modify saturated fatty acid excretion related to chain length
Corresponding Author: Dr Magda Rafecas
Authors: Gemma Brufau; Miguel Angel Canela;

Dear Dr Rafecas,

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Close

Phytosterols, but not pectin, added to a high-saturated fat diet, modify saturated fatty acid excretion related to chain length

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Keywords: fatty acid chain length, phytosterol, pectin, SFA excretion, saturated diet

Running title: PHY, not PE, modify SFA excretion related to chain length

Abstract

The main objective of this paper was to study how the excretion of saturated fatty acids (SFA) is modified after the consumption of a high-saturated fat diet, supplemented with phytosterol and pectin. We present the results of a longitudinal 4-week study on in guinea pigs. Diets were supplemented with 0,33% of cholesterol and differed in the content of pectin (three levels) and of phytosterols (three levels). Seventy-two female Dunkin Hartley guinea pigs were randomly assigned to the treatment groups (8 animals/group). Addition of phytosterol resulted in a decrease of lauric (12:0) and myristic (14:0) excretions and an increase of arachidic (20:0) and behenic (22:0) excretions. Palmitic (16:0) and stearic (18:0) acids did not show a clear change after phytosterol supplementation. Addition of pectin resulted in a decreased excretion of all SFA, although this was not significant. These results suggest that phytosterols added to a high-saturated-fat diet enhance the absorption of the most atherogenic fatty acids (lauric and myristic) after 1 week of treatment, as compared with the high-saturated-fat diet alone.

Introduction

The marked hypercholesterolemic effect of saturated fatty acids (SFA) has long been known [1, 2]. Recent investigations have shown that fatty acids may be classified into three metabolic groups. The first group is mainly made up of short-chain fatty acids such as butanoic (4:0), hexanoic (6:0), octanoic (8:0) and the medium-chain fatty acid decanoic (10:0), which are rapidly oxidized to acetyl CoA in the liver. These fatty acids do not alter the composition of the lipid pool in the liver, the concentration of free or esterifed cholesterol in the hepatocyte, or hepatic LDL receptor activity. Therefore, they are biologically neutral with respect to the regulation of the LDL cholesterol concentration. Interestingly, the long-chain saturated fatty acid, stearic acid, also appears to belong to this biologically neutral group [3].

The second group includes lauric, myristic and palmitic acids, which inhibit LDL receptor activity [4], enhance LDL cholesterol production and increase the concentration of LDL cholesterol in the serum [3, 5].

The third group includes the unsaturated fatty acids, which may be divided in monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) *n*-6 and *n*-3. It has been reported that both MUFA and PUFA, when replaced by SFA, reduce total and LDL cholesterol concentrations [6], although the mechanisms underlying these effects are different. It has been suggested that MUFA, especially oleic acid, increase hepatic uptake of LDL particles as well as enhance the activity of cholesterol ester transfer protein [7]. *n*-6 PUFA reduce cholesterol levels by increasing the expression of hepatic LDL receptors and increasing the activity of 7α-hidrolase [8]. Finally, *n*-3 PUFA reduce blood cholesterol and triglyceride concentrations by reducing lipogenesis and secretion of VLDL, increasing lipoprotein lipase activity and enhancing cholesterol reverse transport [8, 9].

The finding that SFA (lauric, myristic and palmitic acid) may have different metabolic effects on cholesterol levels or coronary heart disease (CHD) is relevant for practical dietary recommendations since those fatty acids that increase cholesterol levels contribute to the major portion of SFA intake in developed countries. In addition, there are epidemiological evidences that support the assumption that there is a positive correlation between SFA consumption and the incidence of CHD [10, 11]. Therefore, general recommendations advise to limit the consumption of SFA to less than 10% of

total daily energy supply [12], especially in Western countries where SFA consumption is very high [13, 14].

Although a diet in accordance with the dietary reference intake should always be recommended, functional foods could be useful to further improve a favourable lipoprotein profile. During the past years, many studies have demonstrated the cholesterol-lowering effects of plant sterols and stanols [15-17] and soluble fibers [18-20].

Plant sterols and stanols may displace cholesterol in the micelles since there is a competition between phytosterols/phytostanols and intestinal cholesterol with regard to incorporation into micelles. This effect will result in a decreased incorporation of cholesterol into micelles, a consequent reduced availability of cholesterol for absorption and lower serum LDL cholesterol concentrations [15-17].

Another food component with possible cholesterol-lowering properties is soluble fiber such as pectin. Pectin is able to bind bile acids, thus provoking a reduction in bile acids reabsorption by the small intestine [18-20].

Since plant sterols and pectin may both reduce plasma concentrations of total and LDL cholesterol, it may be interesting to study the effects of functional foods enriched with both compounds, especially since a great number of products enriched with them are in the food market. Functional foods enriched with phytosterols and pectin might have a synergic cholesterol-lowering effect, allowing for a reduction of the concentrations needed to achieve these effects separately.

Functional foods should be part of a healthy diet but should never be used to counterbalance the effects of an unhealthy diet. Thus, since the diet of many people in developed countries does not meet recommended intakes and since functional foods are extensively used, we consider that the effect of a high-saturated-fat diet supplemented with phytosterols and pectin should be studied.

Our group [21] has already reported that plant sterols may enhance lauric and myristic apparent absorption. In our opinion, however, it would be important to elucidate how this effect takes place. Thus, the aim of this article is to study how SFA excretion is modified during the consumption of a high-saturated-fat diet enriched with

phytosterols and pectin for 4 weeks. For this study, guinea pigs were the animal of choice because their lipoprotein profile is similar to that of humans [22].

Materials and methods

Materials

All fatty acid methyl ester (FAME) standards with purity greater than 99% were purchased from Sigma Chemical Co. (St. Louis, MO). The identification of FAME was made with FAME Mix, C_4-C_{24} (Supelco, Bellefonte, PA).

Boron trifluoride in methanol (1.7 mol/L) and *n*-hexane were purchased from Merk (Darmstadt, Germany), and sodium chloride and anhydrous sodium sulphate from Panreac (Barcelona, Spain).

Diets

Nine isocaloric diets, designed to meet all the nutritional requirements for guinea pigs, were prepared by Mucedola SRL (Settimo Milanese, Italy), as it has been previously described [21]. Briefly, diets were supplemented with 0.33% of cholesterol and their composition differed in the addition of pectin [three levels, PE0 (0%), PE1 (3.67%) and PE2 (6.93%)] and phytosterols [three levels, PH0 (0%), PH1 (1.37%) and PH2 (2.45%)] following a 3x3 factorial design. The chemical composition of the diets is shown in Table 1. Table 2 shows the content in diets (in micrograms per milligram) of the most important SFA found in diets and feces ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{20:0}$ and $C_{22:0}$).

Animals and analysis

Seventy-two female Dunkin Hartley guinea pigs, supplied by Harlan Interfauna Ibérica (Barcelona, Spain), weighing 300-350 g, were randomly assigned to the treatment groups (8 animals/group). The guinea pigs were housed two per cage in a light cycle room (lights on from 8:00 to 20:00), with free access to feed and water. They were killed via heart puncture after administering halothane anaesthesia. All procedures were approved by the Animal Care and Use Committee of the University of

Barcelona. The duration of the treatments was 4 weeks, and the feces were collected three times every week and freeze-dried immediately.

The fatty acid content in feces and in diets was determined with the method of Lopez-Lopez *et al.* [23]. First, 25 μL of internal standard ($C_{21:0}$, 466 μg/mL) were added to 50 mg of freeze-dried fecal homogenate. Then, a small magnetic stirring bar was added and the sample was saponified with 1 mL of sodium methylate (0.5 mol/L) and heated in a water bath at 90ºC for 15 min. The tubes were removed from the water bath and cooled in a water bath at room temperature. Then, 1 mL of boron trifluoride-methanol (1.7 mol/L) was added. Next, they were placed in the water bath at 90ºC for 15 min and subsequently cooled in the same manner as above, after which 400 μL of *n*-hexane was added. The tubes were shaken, and 1 mL of saturated solution of sodium chloride in distilled water was added, following centrifugation. The clear *n*-hexane top layer, containing the FAMEs, was transferred to another tube, adding a small quantity of anhydrous sodium sulphate. The tubes were stored at -20ºC until injection into the gas chromatograph.

FAMEs were analyzed with an Agilent 4890D gas chromatograph, equipped with a flame ionization detector. The separation of FAME was done in a fused silica column (60 m x 0.20 mm i.d., 0.2 μm). The split-splitless injector was used in split mode with a ratio of 1:30. Injector and detector temperatures were kept at 270ºC and 300ºC, respectively. Oven temperature was programmed as follows: an initial period of 5 min at 100ºC, followed by an increase of 4ºC/min up to 235ºC and a final period of 2 min at this temperature.

Helium was used as the carrier gas. Chromatographic peaks were identified by comparing the retention times with those of known standards and by cochromatography. FAME quantification was performed by the internal standard addition method.

Statistical analysis

This article used a longitudinal analysis approach with five time points: Weeks 0, 1, 2, 3 and 4. The unit of analysis was the cage since feces were collected at this level. We have used the results at week 0 as the baseline, subtracting them from the rest. This leaves us with four time points but allows us to control for the initial differences among the sample animals and for the fact that they received the same diet

until Week 0, so that phytosterol and pectin effects cannot be estimated at this time. We have used the *xtreg* command of STATA 9.0 [24].

The analysis was based on a linear mixed-effects model, with the fecal content of the different SFA minus the baseline level as response variables and phytosterol (3 levels) and pectin (3 levels) as fixed effects. Since the SFA content of feces increases with time, we also included time effects in the model. Finally, we used food consumption per cage as a control variable to account for the possible influence of consumption on SFA fecal content. $P<0.05$ was considered significant.

Results

Table 3 gives a summary of the variation of fecal SFA content along the study, without distinction between treatments, which shows how the excretion varies depending on the week of treatment. It can be easily seen that there is an increase in fatty acid concentrations in the first week, a stabilization of these concentrations in the second week and a new increase in the last 2 weeks. We report lauric, myristic, palmitic, stearic, arachidic and behenic acids here.

Table 3 also includes food consumption (in grams) per cage, clearly showing linear time dependence (an increase of about 2 g/week). Due to a common time effect, SFA content and food consumption are positively correlated, but distinguishing the potential consumption effect on SFA excretion from the time effect seems difficult. Nevertheless, we have included food consumption (also subtracting the baseline level at Week 0) as a control variable. We did not find a significant effect of phytosterol or pectin supplementation on consumption.

Table 4 shows the results of SFA at the beginning and at the end of the study. It can be easily seen that the supplementation effects are different depending on the chain length of SFA.

Fig. 1 shows the lauric acid content in feces during the 4 weeks of phytosterol (left panel) and pectin (right panel) treatments. In Fig. 2, the concentrations of behenic acid in feces during 4 weeks of treatment with phytosterol (left) and pectin (right) are shown.

Differences between PH0 group and any of the other two phytosterol groups were highly significant (*P*<0.01 in all cases) for lauric, myristic, palmitic and stearic acids. Differences between PH2 group and the other two phytosterol groups (PH1 and PH0) were significant for arachidic (*P*=0.029 and *P*=0.004, respectively) and behenic (*P*=0.013 and *P*=0.021) acids. Comparing the data at the beginning of the study to those at the end, lauric acid levels increase almost 78% in the control group but only 20% in PH2 group. Behenic acid level increased by 31% in the control group and around 55% in PH2 group. According to the statistical analysis, the pectin effect was not significant for any of the SFA considered, although lauric and behenic acids had the same pattern of excretion, with the highest excretion found in the control group.

Discussion

Many studies have shown that a wide variety of functional foods enriched with plant sterols/stanols [15-17] and pectin [18-20] reduce cholesterol absorption. Daily intakes of 2 to 3 g of plant sterols and 6 to 12 g of pectin reduce serum LDL cholesterol concentrations by 10-15% and 5-16%, respectively. Nevertheless, most of these studies were performed using low-fat foods [15, 25]. Furthermore, to our knowledge, no studies have been addressed to clarify how these compounds may counteract with the absorption of SFA, when added to a high-saturated-fat diet.

Although most of these studies were performed using low-fat diets, in Western countries, high-fat diets are commonly consumed [13, 14]. Thus, to mimic the eating behaviour of the Westerners, we decided to use high-saturated-fat diets for supplementations with pectin and plant sterols.

We have found differences in SFA excretion after phytosterol feeding, depending on the fatty acid chain length. Plant sterols enhanced the excretion of behenic and arachidic acids, but decreased that of medium SFA such as lauric and myristic acid. Another interesting result of our study was the way in which the fatty acids were excreted throughout the whole study. We found a similar pattern of excretion of all fatty acids in the control group (Figs. 1 and 2). The excretion increased after the first week, remained stable for a week and increased again in the last 2 weeks. However, phytosterol effect varies across SFA. The excretion of medium-chain

SFA (lauric and myristic) increased about a 10% during the course of the study (Fig. 1), but that of long-chain SFA (behenic and arachidic) increased by more than 31%.

These differences in SFA excretion may be explained by the distribution of the lipid compounds in the mixed micelle. It is well known [26-30] that phytosterols can displace the compounds of the hydrophobic core of the micelles (such as hydrocarbon carotenoids), but no effect has been found on the compounds of the surface (such as tocopherols). Since lauric acid has 12 atoms of carbon, it is less fat-soluble and, thus, closer to the surface of the micelle than behenic acid (which has 22 atoms of carbon). Therefore, as it happens to carotenes, phytosterols may be more efficient in displacing behenic acid from the micelle than in displacing lauric acid.

Palmitic and stearic acids did not show a clear phytosterol effect. With chains from 16 to 18 atoms of carbon, they have a solubility between those of lauric and behenic acids. Thus, they may be situated between the core and the surface of the micelle.

Our results agree with those from a study of Normen et al. [30], who found that a saturated diet supplemented with phytosterols reduced SFA fecal excretion, this effect being more important for lauric and myristic than for palmitic and stearic acids. In addition, in the stanol group, they did not find such differences.

Pectin belongs to the family of soluble fibers, which are known to reduce cholesterol absorption in animal models [20, 31] and in humans [19, 32]. Since it is has already been proven in many studies that pectin with high degree of methylation is needed to find hypolipidemic effects [20, 33], we used pectin with a degree of methylation higher than 50% in our study. Nevertheless, we did not find differences in SFA excretion, although animals fed pectin showed lower concentrations in feces (nonsignificant). Moreover, excretion during the course of the study was between 30% and 40% in all groups. Several authors [34-36] have found higher concentrations of triglycerides in liver after pectin supplementation. These results might be in agreement with ours since one of the possible explanations is an enhanced fatty acid absorption. This hypothesis was strongly supported in our previous paper [21], wherein we described that animals fed pectin had higher body weight and feed efficiency than those from the control group (Table 5). However, fatty acid excretion was not studied in any of the mentioned studies.

In conclusion, we suggest that plant sterols and pectin may have different effects on SFA excretion depending on the composition of the fat used in diets. Furthermore, the excretion of the most atherogenic fatty acids (lauric, myristic and palmitic) was higher after consumption of only high-saturated-fat diet than after consumption of this diet supplemented with phytosterols and pectin. Finally, we hypothesize that the mechanisms underlying these effects are different for pectin and phytosterols since the phytosterol effect is different depending on the chain length of the fatty acids, whereas the pectin effect is not.

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Table 1. Composition of diets fed to guinea pigs

Ingredients[a] (g/100g)						
Pectin	Phytosterols[b]	Protein[c]	Fat[d]	Sugars[e]	Insoluble fiber[f]	Mineral and vitamin mix[g]
0	0	18.3	15.9	38.4	12.7	6.5
0	1.37	18.5	17.6	40.0	11.9	6.7
0	2.45	17.7	19.6	39.4	11.5	6.5
3.7	0	18.6	15.9	35.3	12.5	6.7
3.7	1.37	18.9	17.4	36.2	12.5	6.7
3.7	2.45	18.0	19.7	33.6	12.5	6.7
6.9	0	18.7	15.6	33.1	12.4	6.7
6.9	1.37	18.9	17.4	30.5	11.9	6.8
6.9	2.45	18.1	19.5	29.2	11.8	6.7

[a] All diets had been enriched with 0.33% of cholesterol.

[b] Added on top of the basal diet. The plant sterol ester mixture was mixed into the feeding and was composed of the following: 6.4 % of brassicasterol, 24.9 % of campesterol, 1.0 % of campestanol, 18.5 % stigmasterol, 45.6 % β-sitosterol, 1.8% sitostanol, 0.9 % delta-5-avenasterol and 1.0% other sterols.

[c] Protein content comprised casein.

[d] Fat content was composed of hydrogenated coconut oil.

[e] Sugar content was made up of corn starch, dextrose and sucrose.

[f] Insoluble fiber was made up of cellulose.

[g] Vitamin and mineral mix was adjusted to meet National Research Council requirements for guinea pigs. Detailed composition of the vitamin and mineral mix has been reported by Krause & Newton [37].

Table 2. Content (in micrograms per milligrams) of the main SFA in the diets

Pectin	Phytosterols	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$
PE0	PH0	54.61	20.62	11.35	12.63	0,18	0,05
PE0	PH1	41.98	15.88	9.35	10.00	0,18	0,06
PE0	PH2	47.26	17.87	10.53	11.38	0,26	0,10
PE1	PH0	59.61	22.23	12.12	13.30	0,19	0,07
PE1	PH1	44.82	16.73	9.66	10.13	0,19	0,07
PE1	PH2	48.04	18.12	11.04	11.31	0,26	0,11
PE2	PH0	60.76	22.52	12.41	13.46	0,19	0,05
PE2	PH1	44.32	16.75	9.99	10.44	0,19	0,07
PE2	PH2	45.34	17.19	10.30	10.98	0,25	0,10

Table 3. Variation of fecal SFA content (in micrograms per milligram) and food consumption (in grams) during the course of the study

Week	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$	Consumption[a]
0	4,03	5,74	6,98	10,54	0,189	0,090	24,12
1	4,51	6,02	7,43	11,40	0,230	0,108	26,01
2	4,52	5,89	7,03	10,58	0,212	0,101	28,70
3	4,89	6,48	7,74	12,07	0,247	0,114	30,46
4	5,70	6,79	7,91	12,42	0,254	0,124	32,36

[a] More details about food consumption, feed efficacy and body weight of animals can be found in Ref. [21]

Table 4. Mean SFA content (in μg/100 mg ± S.D.) in feces

Pectin	Phy		$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$
PE0	PH0	Start	4,22 ± 0,64	5,81 ± 0,68	6,79 ± 0,50	10,11 ± 0,92	0,18 ± 0,03	0,08 ± 0,02
		End	8,20 ± 1,44	9,91 ± 2,51	1,24 ± 3,19	15,10 ± 5,16	0,24 ± 0,08	0,12 ± 0,03
PE0	PH1	Start	4,67 ± 1,80	6,49 ± 2,49	7,89 ± 2,66	12,24 ± 3,92	0,22 ± 0,07	0,10 ± 0,03
		End	5,56 ± 1,37	6,28 ± 1,33	7,94 ± 1,94	12,55 ± 3,23	0,26 ± 0,06	0,13 ± 0,03
PE0	PH2	Start	4,58 ± 2,00	6,60 ± 2,60	8,33 ± 2,95	12,86 ± 4,21	0,23 ± 0,09	0,10 ± 0,03
		End	5,88 ± 1,86	6,37 ± 1,91	8,07 ± 2,57	13,99 ± 5,36	0,34 ± 0,13	0,16 ± 0,05
PE1	PH0	Start	4,44 ± 0,94	5,84 ± 0,96	6,42 ± 0,59	9,58 ± 0,72	0,16 ± 0,02	0,08 ± 0,02
		End	6,80 ± 2,82	8,38 ± 2,89	9,14 ± 2,63	13,05 ± 4,98	0,22 ± 0,07	0,11 ± 0,04
PE1	PH1	Start	3,87 ± 0,71	5,62 ± 0,99	6,76 ± 1,05	10,02 ± 1,13	0,19 ± 0,03	0,08 ± 0,01
		End	4,48 ± 0,44	5,46 ± 0,84	6,86 ± 1,34	10,61 ± 2,79	0,22 ± 0,05	0,11 ± 0,02
PE1	PH2	Start	3,57 ± 0,89	5,61 ± 1,26	7,33 ± 1,39	11,18 ± 2,30	0,20 ± 0,04	0,10 ± 0,03
		End	4,49 ± 1,73	5,27 ± 1,78	7,32 ± 1,80	12,88 ± 3,51	0,34 ± 0,08	0,17 ± 0,04
PE2	PH0	Start	3,50 ± 0,69	4,87 ± 1,25	5,85 ± 1,51	8,59 ± 2,29	0,15 ± 0,03	0,07 ± 0,01
		End	3,64 ± 1,84	7,59 ± 0,84	7,94 ± 0,84	11,03 ± 1,50	0,18 ± 0,03	0,09 ± 0,02
PE2	PH1	Start	3,90 ± 1,90	5,57 ± 2,80	5,54 ± 2,75	10,02 ± 3,63	0,18 ± 0,07	0,09 ± 0,03
		End	5,52 ± 1,72	6,69 ± 1,75	7,26 ± 1,79	11,36 ± 1,83	0,22 ± 0,05	0,10 ± 0,03
PE2	PH2	Start	3,79 ± 0,85	5,50 ± 1,46	6,89 ± 1,68	1,09 ± 2,37	0,18 ± 0,03	0,10 ± 0,01
		End	4,05 ± 1,36	5,17 ± 1,21	6,41 ± 1,72	11,19 ± 3,73	0,27 ± 0,07	0,13 ± 0,04

The number of cages in each group was four, with two animals per cage

Table 5. Food consumption and final body weight (data were extracted from a Ref. [21])

Pectin	Phytosterol	Food consumption (g/day/animal), mean ± S.D.	Body weight (g), mean ± S.D.
PE0	PH0	35.30 ± 7.84	374.55 ± 13.11
PE0	PH1	32.56 ± 1.33	364.75 ± 30.20
PE0	PH2	33.59 ± 5.78	368.41 ± 25.61
PE1	PH0	32.27 ± 4.55	400.97 ± 34.97
PE1	PH1	35.69 ± 7.18	400.04 ± 52.80
PE1	PH2	33.66 ± 14.47	397.09 ± 24.19
PE2	PH0	29.25 ± 1.49	388.85 ± 66.68
PE2	PH1	28.70 ± 3.35	412.44 ± 17.00
PE2	PH2	27.99 ± 2.75	412.70 ± 33.04

Fig. 1. Lauric acid content (μg/100 mg) in feces during 4 weeks of treatment after phytosterol (left) and pectin (right) intake

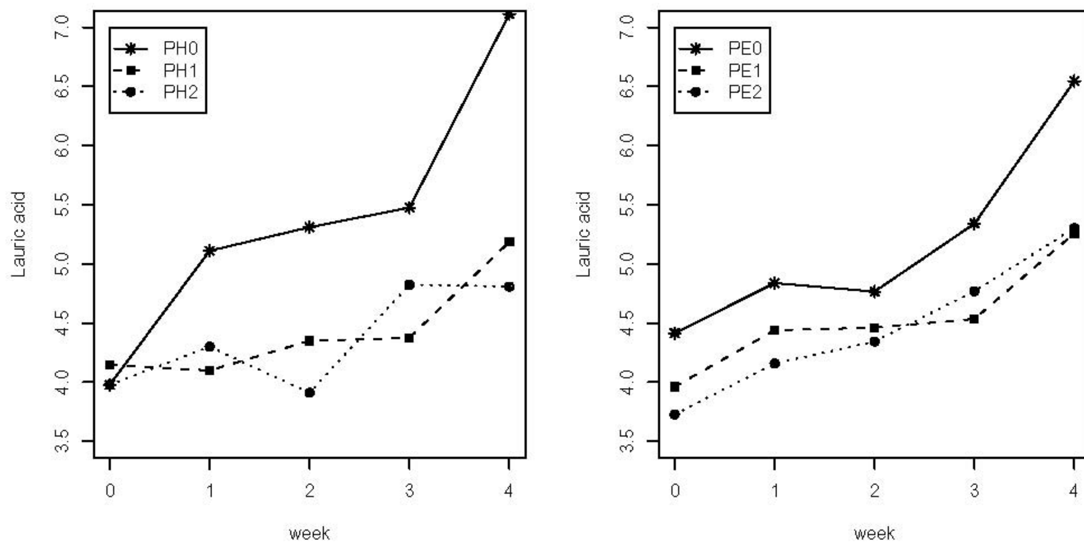
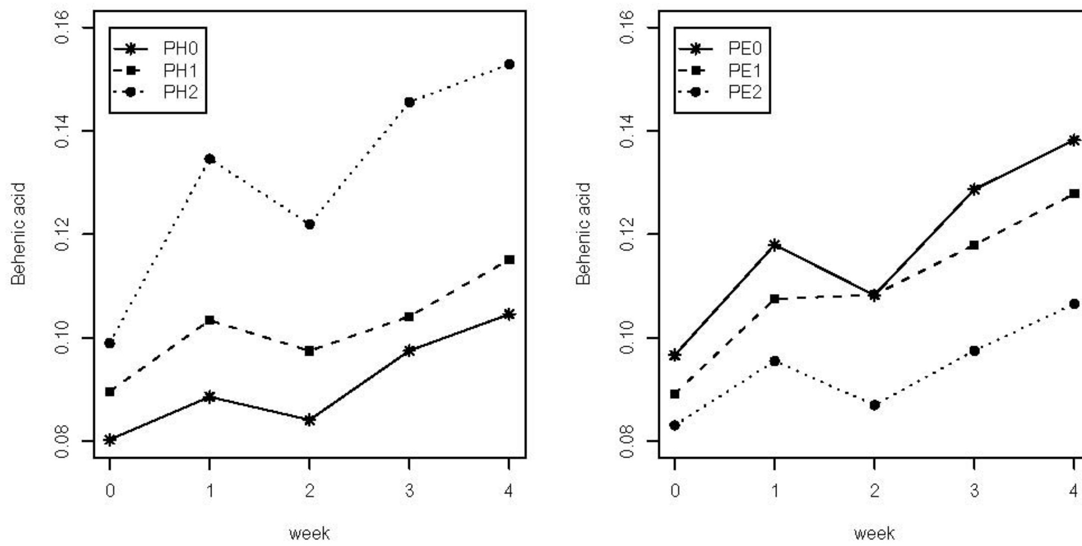


Fig. 2. Behenic acid (μg/100 mg) in feces during 4 weeks of treatment after phytosterol (left) and pectin (right) intake



2.3 PLANT STEROL AND PECTIN, ADDED TO A HIGH-SATURATED FAT DIET, DO NOT SHOW THE HYPOCHOLESTEROLEMIC ACTIVITY IN GUINEA PIGS

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Plant sterols and pectin, added to a high-saturated fat diet, do not show the hypocholesterolemic activity in guinea pigs

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Short title: Impaired PE/PH effect in a high-saturated fat diet

Abstract

Background & Aims: This paper presents the results of a study whose aim was to test the effects of several doses of pectin and phytosterols on the sterol content in plasma and in liver from guinea pigs when they were added to a "Western-like" diet, rich in saturated fat and cholesterol. **Methods**: The treatments followed a 3×3 factorial design, with three levels of pectin (0, 3,67 and 6,93%) and three levels of phytosterols (0, 1,37 and 2,45%). 72 female Dunkin Hartley guinea pigs were randomly assigned to the treatment groups (8 animals/group). The duration of the treatment was 4 weeks. **Results**: No differences were found in plasma cholesterol concentrations, while in liver we saw a reduction in cholesterol concentration after phytosterol feeding. Besides, no differences were found after pectin intake. Plant sterol concentration increased in plasma and liver after phytosterol ingestion, and the highest concentrations were found in those animals fed at intermediate pectin doses. **Conclusions**: Our results suggest that a saturated diet may impair cholesterol-lowering properties of plant sterols and pectin.

Keywords: high-saturated fat diet, phytosterol, pectin, animal model, atherosclerosis

Introduction

Cardiovascular disease (CVD) due to advanced atherosclerosis, is the leading cause of death and disability in western countries. Numerous risk factors, including dietary pattern, physical inactivity, serum lipids, diabetes, smoking, obesity and physiological stress, have been proposed as contributors to the initiation and development of atherosclerosis and its clinical manifestations (1). High intakes of saturated fat and cholesterol increase serum LDL cholesterol, probably by decreasing the amount and/or activity of LDL receptors in liver (2). Although CVD mortality was trending downward for almost 50 years, a global resurgence has occurred. The epidemic of obesity, decreasing physical activity, and persistent cigarette smoking are major behavioural factors underlying this change. Western-like diet and sedentary lifestyle increase CVD risk, both directly and indirectly. Direct effects include biological, molecular, and physiological alterations, including inflammatory stimuli and oxidative stresses. Indirect effects include diabetes, dyslipemias and hypertension. Consequently, some efforts have been addressed to achieve an effective lowering of serum cholesterol level, to get a marked reduction of different clinical manifestations of coronary heart disease (CHD) events and mortality for CHD (3). In addition to medication, plasma cholesterol concentration can also be reduced by naturally occurring dietary components, e.g. plant sterols and different kind of fibers like pectins (4, 5). Some evidences of association between plant sterols/dietary fiber and atherosclerosis have been accumulated from epidemiologic observations (6) and some clinical trials (4, 7, 8). However, it is unclear whether the combination of plant sterols and pectins would have an additive, synergistic or negative effect on their individual cholesterol-lowering abilities. These data are crucial in guiding the treatment of hypercholesterolemia via dietary approaches.

Plant sterols have molecular structures similar to that of cholesterol. Compared with cholesterol, plant sterols have an extra methyl or ethyl group and a double bond in the side chain. It is hypothesized that plant sterols inhibit intestinal cholesterol absorption through competition with cholesterol for incorporation into micelles formed in the intestinal lumen (9) and interference with the uptake of cholesterol into enterocytes, although the exact mechanisms of action remain still unclear (10, 11). Compared with plant sterols, pectins may lower cholesterol via other mechanisms, although these are not completely elucidated yet. Some possible mechanisms proposed for pectins include a decrease in cholesterol absorption, or interruption of enterohepatic circulation

of bile acids (12-15). Because plant sterols and pectins have different biological actions, the combination of these two dietary ingredients may lead to a more powerful cholesterol-lowering effect.

The present study examined this possible hypocholesterolemic effect of the combination of plant sterols and pectins added to a high-saturated fat diet. Compared with unsterified plant sterols, plant sterols ester (PSE) are more fat soluble and thus easier to incorporate into food products (16, 17). However, most of the foods where plant sterols were added, were low-fat products and this fat was mainly unsaturated. Therefore, it would be interesting to elucidate the effects of PSE on lipid profile in a “Western-like” diet rich in saturated fatty acids and cholesterol (18, 19). Additionally, it was shown that macromolecular and high-ethoxyl pectins were the most effective in lowering plasma cholesterol in rats (20). Tersptra *et al.* (21) found that 3% macromolecular pectin was more effective in reducing plasma cholesterol and excretion of BA and neutral sterols in hamsters than pectin with a lower molecular weight. Thus, in this study we used high methylated pectin added to a high-saturated fat diet.

This study was conducted with guinea pigs, an established animal model based on their similarities to humans, in plasma lipoprotein distribution and responses to dietary treatments to cholesterol concentrations (22).

Materials and methods

Reagents and standards

5α-cholestane, cholesterol, lathosterol, β-sitosterol, pyrogallol, BHT and Sylon BTZ (BSA (N,O-bis(trimethylsilyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI ((N-trimethylsilyimidazole); 3:2:3) kit were purchased from Sigma Chemical (St. Louis, MO, USA). Absolute ethanol, cyclohexane, hexane, chloroform and isopropanol were obtained from Panreac (Barcelona, Spain) and dry pyridine from Merck (Darmstadt, Germany). Distilled deionized water was used throughout.

Diets

In this study, nine isocaloric diets were used as it was described previously (23). Briefly, the diets differed in the levels of pectin (three doses; PE0, PE1 and PE2) and plant sterols (three doses; PH0, PH1 and PH2), following a 3x3 factorial design (Table 1). Pectin was purchased from CP Kelco (Atlanta, GA; USA). We used GENU-type Freeze pectin which is a high (methyl) ester pectin. Plant sterols were esterified with unsaturated fatty acids (Raisio, Helsinki, Finland).

The sterol content in each diet was determined by a method described previously (24). Table 2 shows the content (%) of the main plant sterols and cholesterol in the nine diets.

Animals

72 female Dunkin Hartley guinea pigs, supplied by Harlan Interfauna Ibérica (Barcelona, Spain), weighting 300-350 g, were randomly assigned to the treatment groups (8 animals/group). The duration of the treatment was 4 weeks. The guinea pigs were housed two per cage in a light cycle room (light from 0800-2000 h), with free access to feed and water, and sacrificed by heart puncture after halothane anaesthesia. Blood samples were obtained by means of an intracardiac extraction and liver was extracted after the sacrifice and homogenated. Samples were kept at -80ºC until the moment of the analysis (23). All procedures were approved by the Animal Care and Use Committee of the University of Barcelona.

Biochemical analysis

Triglycerides and glucose were measured on a Spotchem EZ analyser (Menarini Diagnostics, Germany) using reagent strips for quantitative measurement.

Sterol determination

To determine the sterol content, we used the method described for Phillips *et al.* (25) for plasma samples and that one described for Brufau *et al.* (26) for liver samples.

300 mg of liver or 500 μL of plasma were added into a 50 ml centrifuge tube containing 50 μL of internal standard (5α-cholestane (560 μg/ml)). Then, 8 mL of a mixture of pyrogallol 3% (w/v) and BHT 0,06% (w/v) in ethanol were added. Liver

samples needed a homogenisation, carried out for 30 s at 19.000 rpm using a Polytron PT 3000, maintaining samples in an ice bath. After that, in both samples (plasma and liver), 2 mL of aqueous potassium hydroxide (28,0 %, w/v) were added, and tubes were gently shaken.

Saponification was done by incubation of the reaction mixture at 80ºC for 30 min. Afterwards, tubes were cooled in an ice bath for 5 min. To extract non-saponfiable compounds, 10 mL of distilled water and 20 mL of cyclohexane were added and mixture was shaken, and then centrifuged at 3000 rpm for 10 min. The upper phase was transferred to a round-bottom flask and the aqueous layer was reextracted twice with 8 mL of cyclohexane. Finally, the solvent was evaporated to dryness with a vacuum rotatory evaporator at 30ºC.

After the saponification step, we used a Bond Elute LRC® aminopropyl solid phase extraction (SPE) cartridge (Varian Associates, Harbor City, CA, USA) because it could reliable quantify sterols and stanols. Therefore, cartridges were coupled to an elution vacuum system and conditioned with 4 mL of hexane, which was discarded. Each sample was dissolved with five consecutive 1 mL portions of trichlorometane/isopropanol (2:1; v/v), which were poured then into the cartridge. Finally, cartridges were drawn increasing the vacuum until no solvent was eluted. Solvent was dried under nitrogen stream at controlled temperature (30ºC). Samples were maintained overnight under vacuum conditions, in order to remove all residual solvent.

Gas-chromatography determination

Dry extracts were derivatized by addition of 50 μl of anhydrous pyridine and 50 μl of Sylon BTZ, and maintained for 30 min at room temperature before the GC analysis.

The gas chromatography analysis was performed with a Perkin Elmer GC Autosystem™ equipped with ZB1 (100% methylpolisiloxane) capillary column (30 m x 0,25 mm i.d.; 0,25 μm) and a flame ionisation detector. Helium was the carrier gas. Operation conditions were: injector temperature 290ºC; detector temperature 300ºC; and the oven temperature was kept at 245ºC for 0,5 min, then programmed at a rate of 2ºC/min until 265ºC, and finally at 3,5ºC/min to 290ºC (21 min). Split ratio was 12,5:1

and injection volume was 1 μL. Analytes were identified by their relative retention time and by co-chromatography with standards.

Statistics

Significance of the differences in plasma and liver sterol content was tested by two-way analysis of variance (ANOVA) with the amount of pectin and phytosterols as factors. We used SPSS 11.0 in the calculations. As usual, P<0,05 was considered significant.

Results

Results of feed efficiency, body weight and food consumption are described elsewhere (23).

No differences were found in triglycerides content in any of the groups compared with the control **(Table 3)**. Glucose concentrations showed a slight increase at higher levels of pectin and phytosterols (data not statistically significant).

Table 4 shows the results for cholesterol, lathosterol and plant sterol analysis in liver and cholesterol, lathosterol, desmosterol and plant sterols in plasma. Like plant sterols, we only determined the amount of campesterol and β-sitosterol, due to the concentration of the others was below the limit of detection. Moreover, these two plant sterols were the most representative in the mixture of phytosterols added to diets.

Liver cholesterol concentrations were reduced after phytosterol intake (P=0,013). However, this reduction was modified by the dose of pectin added (not significant differences).

No statistically significant differences were found in plasma cholesterol levels after phytosterol intake, although the highest values were found in PH2. Looking at the effect of the combination of pectin and phytosterol, some differences were found between groups but they were again not statistically significant. Animals fed PE0 and PE2 did not show differences in cholesterol concentration after plant sterol supplements. However, animals fed PE1 showed an inverse relation between cholesterol concentrations and plant sterol addition (significant effect of pectin;

P=0,001). Cholesterol concentration in plasma showed a strong correlation with cholesterol levels in diets (*P*<0,05).

Liver lathosterol concentrations and plasma lathosterol and desmosterol concentrations did not show any modification plant sterol addition nor after pectin supplementation.

Phytosterol concentrations in liver and plasma increased at higher doses of these compounds in diets. Whereas liver campesterol levels increased around 20/30 times in those animals fed PH1 or PH2 compared with PH0 (*P*<0,001), liver β-sitosterol concentrations only showed a 2-fold increase when PH2 was compared with PH0 (*P*<0,001). In plasma, campesterol and β-sitosterol also increased their concentrations after phytosterol addition (*P*=0,013 and *P*<0,001, respectively).

As what was found for cholesterol, the dose of pectin added modified the absorption of these compounds, although the way of this modification was different for each plant sterol. Fig. 1 shows the plasma campesterol concentrations in diets. Animals fed PE0 showed a positive lineal relation between phytosterol addition and concentrations in plasma; but in animals fed PE1 and PE2, although higher concentrations were found after phytosterol supplementation, the relation was not so clear. Whereas the increase found comparing the PH1 to PH0 was similar to those observed in PE0, a negative relation was found between pectin addition and increase of campesterol concentration when PH2 is compared PH1, suggesting that pectin reduced campesterol absorption at high doses of this plant sterol. The effect of pectin on campesterol levels was statistically significant in liver (*P*=0,029) and plasma (*P*=0,013).

On the other hand, β-sitosterol concentrations showed a pattern of absorption similar to that observed for campesterol, but differences were not statistically significant. Animals fed PE0 showed a positive linear relation between doses of phytosterols and β-sitosterol concentration.

Discussion

The aim of this study was to investigate whether phytosterols and pectin show the same hypocholesterolemic effects as it was described previously (4, 5), but when they were added to an atherosclerotic diet rich in saturated fatty acids and cholesterol. It is known that Western diets (rich in saturated and *trans* fatty acids and poor in n–3 PUFAs and n–6 PUFAs) (18, 19) increase the risk of CVD by rising blood concentrations of total- and LDL-cholesterol (27). Furthermore, it is also known that a relevant reduction in CVD events is achieved at least in part by lowering blood total and LDL-cholesterol levels, lowering TAG and increasing HDL-C. Dietary phytosterols incorporated into foods have been shown to be effective in lowering TC and LDL-C, but do not affect TAG or HDL-C (28). However, most of the foods supplemented with plant sterols used in these studies, were low fat-content (17, 29). Besides, intake of soluble fiber lowers plasma cholesterol concentrations by preventing the absorption of bile acids from the intestinal tract. Therefore, it would be beneficial to produce a dietary supplement that combines the cholesterol-lowering properties of phytosterols and pectin in a “Western-like” diet.

Normally, plant sterols are poorly absorbed in the intestine (0,4-3,5%) (30), and added to a solid food diet, they are quite resistant to destructive action of intestinal bacteria (31). Thus, our results in control group (PE0/PH0) are in agreement with those concentrations found in normocholesterolemic subjects (below 1%) (30). However, an increase in phytosterol concentrations (liver and plasma) was found in animals fed these compounds, campesterol being better absorbed than β-sitosterol (32). For instance, campesterol concentration was 70, and 50 times higher in PH2 and PH1 respectively, than in control group. β-sitosterol concentrations were 2 times higher in both PH1 and PH2 compared with PH0. Therefore, these results point to an increase in cholesterol absorption as it was proposed in previous studies (33). In addition, we did not observe any modification in liver lathosterol concentrations (precursor of cholesterol synthesis), neither in plasma lathosterol and desmosterol concentrations, associated with phytosterol consumption.

Plasma cholesterol concentrations were not modified, and liver cholesterol concentrations decreased after phytosterol intake. Our results disagree with most of the studies published (34) because they described a reduction in cholesterol values after phytosterol feeding. Nevertheless, these studies used low-fat products as carriers.

As mentioned before, we chose a saturated diet enriched with cholesterol because they are the main components in “Western-like” diets (18, 19). Nevertheless, our results are in agreement with a previous study of our group (23) in which an increase in saturated fatty acids (SFA) was found after phytosterol addition to a saturated diet. Therefore, it might seem reasonable that plant sterol supplementation in a saturated diet could enhance cholesterol absorption in a similar way as what happened for SFA (23). This effect is confirmed by the increase in phytosterol absorption (33) and by the positive correlation found between cholesterol levels in plasma and diets. Our hypothesis is that the increase in saturated fatty acid concentration found in liver (23) may reduce the transformation from free cholesterol to esterified cholesterol, as well as reduce the expression of LDL receptors (35). These modifications in liver would provoke a decrease in liver cholesterol levels, although this was not reflected in plasma cholesterol levels, since no differences were found. Thus, we consider that the decrease found in liver cholesterol concentrations was mainly due to a decrease in cholesterol uptake, and not due to a reduction in cholesterol synthesis since no differences were found in lathosterol concentrations (precursor of cholesterol).

Looking at the effects of pectin supplementation, in liver and plasma we found a significant increase in cholesterol and campesterol concentrations in PE1. No differences were found in lathosterol neither in desmosterol content. After our results, it may be hypothesized that pectin suffered an early breakdown since animals fed pectin showed an increase in body weight and feed efficiency compared with those from control group (23). However, although the dose of pectin from PE2 was almost double than that one of PE1, no differences were found in body weight between these groups. These results let us hypothesise that animals from PE2 had some pectin left which reached the intestine where it may show its hypocholesterolemic effects. This explanation would explain the higher concentrations found in PE1 group, but more studies are needed since we did not analyse the intestinal content. Our results seem to contradict those reported by some authors where a decrease in cholesterol levels was found after pectin ingestion (14), but they used low-fat diets. However, we cannot prove if the hypothesised breakdown was due to some abnormalities in the quality of pectin or some interactions with the fat content of diets.

Finally, some interactions between the dose of pectin and phytosterol used were found, although these are not significant. The only interaction found was in campesterol (Fig. 1) and β-sitosterol levels which were modified at high doses of both

compounds, but no conclusions can be made since pectin may have suffered an early hydrolysis.

In conclusion, the results of the present study show a lack of hypocholesterolemic properties of plant sterol and pectin when they are added to a “Western-like diet”, rich in saturated fat and cholesterol. However, more studies are needed to fully clarify the role of these compounds when they are added to diets with different fat profiles.

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Table 1. Composition of diets fed to guinea pigs

Diets	Ingredients[a] (g/100g)								
	PE0/PH0	PE0/PH1	PE0/PH2	PE1/PH0	PE1/PH1	PE1/PH2	PE2/PH0	PE2/PH1	PE2/PH2
Pectin	0	0	0	3,7	3,7	3,7	6,9	6,9	6,9
Phytosterols[b]	0	1,27	2,45	0	1,27	2,45	0	1,27	2,45
Protein	18,3	18,5	17,7	18,6	18,9	18,0	18,7	18,9	18,1
Fat	15,9	17,6	19,6	15,9	17,4	19,7	15,6	17,4	19,5
SFA	11,3	8,7	9,8	12,1	9,2	10,0	12,4	9,2	9,5
Sugars	38,4	40,0	39,4	35,3	36,2	33,6	33,1	30,5	29,2
Insoluble fiber	12,7	11,9	11,5	12,5	12,5	12,5	12,4	11,9	11,8
Mineral and Vitamin mix[c]	6,5	6,7	6,5	6,7	6,7	6,7	6,7	6,8	6,7

[a] All diets had been enriched with 0,33% of cholesterol

[b] Added on top of the basal diet. The plant sterol ester mixture was mixed into the feeding and comprised 6,4% of brassicasterol, 24,9% of campesterol, 1,0% of campestanol, 18,5% stigmasterol, 45,6% β-sitosterol, 1,8% sitostanol, 0,9% delta-5-avenasterol and 1,0% other sterols (such as 7-campesterol, δ523-stigmastadienol, clerosterol, 7-stigmasterol, cicloartenol, δ-7-avenasterol, 24-methylcicloartenol, citrostadienol).

[c] Vitamin and mineral mix were adjusted to meet National Research Council requirements for guinea pigs. Detailed composition of the vitamin and mineral mix has been reported elsewhere (21).

Table 2. Cholesterol and plant sterol amount (wt%) in diets

Pectin (%)	PE0			PE1			PE2		
Phytosterol (%)	PH0	PH1	PH2	PH0	PH1	PH2	PH0	PH1	PH2
Cholesterol (%)	0,26	0,25	0,27	0,29	0,28	0,29	0,27	0,27	0,26
Phytosterols (%)	0,025	1,38	2,41	0,025	1,37	2,56	0,048	1,36	2,39
Brassicasterol (%)	0,06	0,088	0,157	0,005	0,085	0,164	0,006	0,086	0,153
Campesterol (%)	0,003	0,341	0,595	0,003	0,338	0,632	0,004	0,337	0,595
Campestanol (%)	nd	0,014	0,024	nd	0,012	0,003	nd	0,014	0,026
Stigmasterol (%)	0,001	0,255	0,453	0,002	0,251	0,488	0,002	0,249	0,447
β-sitosterol (%)	0,011	0,631	1,089	0,010	0,634	1,162	0,012	0,624	1,078
Sitostanol (%)	0,003	0,025	0,043	0,004	0,025	0,046	0,003	0,026	0,043
Δ-5-avenasterol (%)	nd	0,110	0,022	Nd	0,012	0,023	nd	0,012	0,022
Others[a] (%)	0,002	0,015	0,027	0,002	0,012	0,028	0,001	0,012	0,026

[a] Others mean those compounds found in concentrations lower than 0,001%. They are 7-campesterol, δ523-stigmastadienol, clerosterol, 7-stigmasterol, cicloartenol, δ-7-avenasterol, 24-methylcicloartenol, citrostadienol and some others with a retention time of 21,07 min and with a clear sterol mass spectra, but with no clear identification.

nd means not detected

Table 3. Glucose and TG concentration in plasma

	Glucose	TG
PE0/PH0	168,71	38,57
	(66,51)	(14,50)
PE0/PH1	121,25	37,25
	(44,05)	(15,37)
PE0/PH2	172,14	40,57
	(40,24)	(11,89)
PE1/PH0	148,86	59,57
	(32,19)	(28,38)
PE1/PH1	161,33	51,00
	(78,03)	(19,98)
PE1/PH2	169,86	40,57
	(48,61)	(10,16)
PE2/PH0	127,50	42,63
	(29,92)	(17,53)
PE2/PH1	202,71	50,14
	(51,29)	(15,21)
PE2/PH2	174,38	42,25
	(26,81)	(12,13)

Results are means (SD in parenthesis). No statistically significant differences were found between groups ($p \geq 0,05$).

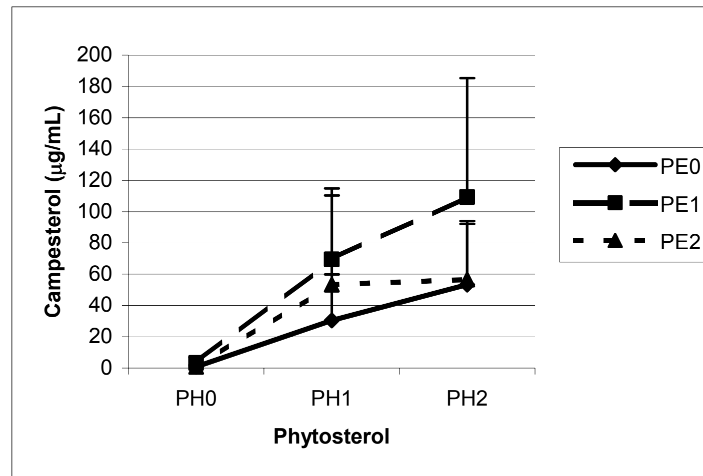
Table 4. Cholesterol and plant sterol concentration

	Liver (μg/100g)				Plasma (μg/mL)				
	Chol	Lath	Camp	β-sitost	Chol	Lath	Desm	Camp	β-sit
PE0/PH0	321,00	0,56	0,71^{ab}	1,40	464,61	0,53	2,39	0,87^{a}	2,36
	(110,27)	(0,21)	(0,54)	(1,23)	(246,80)	(0,84)	(3,70)	(1,22)	(2,63)
PE0/PH1	300,41	0,56	8,00^{abc}	1,94	627,20	0,51	2,89	30,35^{ab}	5,08
	(34,17)	(0,11)	(6,46)	(1,33)	(305,25)	(1,01)	(3,70)	(29,50)	(2,95)
PE0/PH2	271,30	0,73	15,70^{cd}	3,13	636,51	0,37	1,32	53,40^{ab}	7,54
	(46,37)	(0,16)	(5,78)	(0,77)	(133,24)	(0,98)	(2,32)	(40,49)	(5,37)
PE1/PH0	358,34	0,69	0,67^{a}	1,60	1056,53	2,48	11,08	3,21^{a}	2,95
	(110,37)	(0,42)	(0,20)	(1,40)	(687,24)	(3,51)	(15,39)	(3,49)	(2,97)
PE1/PH1	260,70	0,76	12,35^{abcd}	2,70	861,69	0,31	1,48	69,47^{ab}	9,54
	(26,68)	(0,25)	(5,91)	(0,76)	(211,68)	(0,83)	(2,52)	(45,40)	(3,33)
PE1/PH2	288,14	0,59	22,71^{d}	2,63	755,46	0,99	2,32	109,20^{b}	9,74
	(34,40)	(0,27)	(8,86)	(0,75)	(256,44)	(1,40)	(2,50)	(76,02)	(5,10)
PE2/PH0	310,44	0,67	0,69^{ab}	1,47	456,77	0,26	1,41	0,89^{a}	3,70
	(63,33)	(0,28)	(0,24)	(1,18)	(142,40)	(0,48)	(2,11)	(0,97)	(8,15)
PE2/PH1	289,85	0,69	12,55^{bcd}	2,28	593,64	0,52	1,50	53,36^{ab}	8,84
	(29,86)	(0,26)	(6,64)	(0,55)	(189,37)	(0,90)	(2,57)	(57,03)	(5,80)
PE2/PH2	270,02	0,77	17,73^{cd}	2,48	683,52	0,46	0,95	56,50^{ab}	6,48
	(26,78)	(0,38)	(7,03)	(0,42)	(235,49)	(0,85)	(1,76)	(35,65)	(2,01)

Chol means cholesterol, lath means lathosterol, desm means desmosterol, camp means campesterol and β-sit means β-sitosterol.

Results are means (SD in parenthesis). Values in a column with the same superscript are not significantly different ($p \geq 0,05$).

Figure 1. Concentration of campesterol (μg/mL) in plasma



PH0, PH1 and PH2 are the different concentrations of phytosterols supplemented to diets and PE0, PE1 and PE2 are the concentrations of pectin.

2.4 REDUCTION IN CHOLESTEROL OUTPUT AFTER PLANT STEROL AND PECTIN ADDITION TO A HIGH-SATURATED DIET

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Reduction in cholesterol output after plant sterol and pectin addition to a high-saturated diet

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Abstract

The main objective of this paper was to study whether plant sterol and cholesterol apparent absorption is modified after adding plant sterol and pectin in a saturated diet. 72 female Dunkin Harley guinea pigs were used. Three levels of pectin (0, 3.67 and 6.93%) and three of phytosterols (0, 1.37 and 2.45%) were added to a high-saturated guinea pig diet for 4 wk. Pectin did not modify plant sterol absorption but increased that of cholesterol ($p<0.05$). Furthermore, phytosterol supplementation decreased the apparent absorption (defined as the ratio faeces/diet) of β-sitosterol and stigmasterol, but enhanced the absorption of campesterol. Plant sterol also reduced the total cholesterol output. Plant sterols added to a high-saturated diet may enhance the absorption of campesterol and, although the absorption of cholesterol is decreased, the total cholesterol output decreases. These results suggest that a Western-like diet may counteract the hypocholesterolemic effects of plant sterols and pectin.

Key words: saturated diet, phytosterol, pectin, cholesterol output

Introduction

During the last decade, the prognosis of patients with coronary heart disease (CHD) has improved, because of a better understanding of the pathology. It is known that high serum concentrations of cholesterol are related to the risk of CHD in a progressive and continuous way *(1)*. Consequently, prevention and treatment strategies for CHD often involve changes in lifestyle and dietary habits, indicating that individuals may benefit from dietary approaches to further lower cardiovascular risk *(2)*. This is one of the main reasons why functional foods aiming to lower serum low-density lipoprotein (LDL) cholesterol concentrations can be beneficial for a large segment of the population. Some compounds such as plant sterols and stanols, and soluble fiber (pectins) have been supplemented to some foods due to their LDL-cholesterol-lowering efficacy *(3-7)*.

Phytosterols are non-nutritive compounds, which are effective in reducing cholesterol absorption because they displace cholesterol from micelles, making unsterified cholesterol less available for absorption. Doses of phytosterols or stanols ranging from 0.8 *(8-9)* to 4.0 g/d *(9-10)* were used to reduce LDL-cholesterol concentrations by 10-15%, although most of the studies *(11-12)* consider that 2 g/d of phytosterol or stanols are enough to achieve a reduction of 10% in LDL-cholesterol concentrations.

Pectin, as a dietary fiber, is not degraded by intestinal enzymes during the passage by the stomach and the small intestine. Therefore, it is present in the upper part of the intestinal tract in macromolecular form *(13)*. Physicochemical and functional properties of pectin, and in consequence several physiological effects, are influenced by structural parameters like the degree of methylation (DM).

Like other soluble dietary fibers, pectin is capable of reducing plasma LDL-cholesterol levels by increasing bile acids extraction *(2, 14-17)*. This effect probably causes the increased hepatic synthesis of bile acid and liver depletion of cholesterol, resulting in reduced serum cholesterol levels *(4, 14, 18)*. Furthermore, the short-chain fatty acids (SCFA), especially propionate, produced during the fermentation of dietary fiber by the intestinal microflora, may inhibit the hepatic cholesterol synthesis. Additionally, formation of the SCFA causes a drop in colonic pH. This effect may lower the solubility of the bile acids and depress their passive re-absorption in the lower parts of the intestinal tract.

Most of the studies reported, used low-fat diets (whose profile was low in saturated fatty acids) as a carriers for these compounds *(5-7, 9, 19)*. However, it should be taken into account that in most of the developed countries where functional foods (such as phytosterols and soluble fibbers) are extensively used, the consumption of vegetable shortenings is increasing because they are one of the main ingredients of the foods ready-to use. This is an undesirable development, because the intake of *trans* FA and saturated fatty acids (SFA), which are two of the most important components of Western diets *(20-21)*, is related to an increased risk of cardiovascular disease *(22-23)*. To our knowledge, no data are available on the effects of these functional components added to a Western-like diet (with high fat content rich in SFA). Therefore, the aim of this study was to determine whether the cholesterol and bile acid apparent absorption is modified after ingestion of plant sterols and pectin supplemented to a diet rich in saturated fatty acids.

To examine the effects on apparent absorption *(24-27)* we used the comparison between faecal and dietary levels, since it has been reported *(28-29)* that results regarding apparent absorption were similar to those obtained using stable isotopes.

Guinea pigs were used in this study, because their response to dietary treatment to modulate the cholesterol concentrations is similar to that of humans *(30)*.

Materials and methods

Materials

5α-cholestane, cholesterol, stigmasterol, β-sitosterol, cholic acid, lithocholic colic, chenodeoxycholic acid, pyrogallol, BHT and Sylon BTZ kit were purchased from Sigma Chemical (St. Louis, MO, USA). Nor-cholic, coprostan-3-ol and coprostan-3-one were supplied by Steraloids (Newport, Rhode Island, USA). Absolute ethanol, cyclohexane, hexane, chloroform and isopropanol were obtained from Panreac (Barcelona, Spain) and dry pyridine from Merck (Darmstadt, Germany). Distilled deionized water was used throughout.

Diets

In this study, nine isocaloric diets were used as it has been described previously *(31)*. Briefly, the diets differed in the levels of pectin (three doses; PE0, PE1 and PE2) and plant sterols (three doses; PH0, PH1 and PH2), following a 3x3 factorial design (Table *1*). All diets were added with a 0.33% (w/w) of cholesterol.

The sterol content in each diet was determined by a method described previously *(32)*. Table *2* shows the content (μg/100g) of the main plant sterols and cholesterol in the nine diets.

Animals

72 female Dunkin Hartley guinea pigs, supplied by Harlan Interfauna Ibérica (Barcelona, Spain), weighting 300-350 g, were randomly assigned to the treatment groups (8 animals/group). The duration of the treatment was 4 weeks. The guinea pigs were housed two per cage in a light cycle room (light from 0800-2000 h), with free access to food and water, and sacrificed by heart puncture after halothane anaesthesia. Faeces were collected with a 2-day periodicity and freeze-dried immediately. Samples were kept at -80ºC until the moment of the analysis. All procedures were approved by the Animal Care and Use Committee of the University of Barcelona. Further details about food intake, body weight and feed efficiency are reported elsewhere *(31)*.

Sample preparation

Neutral and acidic sterols were determined according to Phillips *et al. (32)* and Keller & Jahreis (*33)* with minor modifications.

For duplicate analysis, two aliquots of 100 mg of freeze-dried faeces were weighed into a 50 mL centrifuge tube containing 280 μg of 5α-cholestane and 134 μg of nor-cholic acid. Then, 8 mL of a mixture of pyrogallol 3% (w/v) and BHT 0,06% (w/v) in ethanol were added. After a half an hour of mild hydrolysis with freshly prepared 2 mL of ethanolic potassium hydroxide (28.0 %, w/v) in a water bath at 80ºC, the samples were cooled to room temperature. 10 mL of distilled water were added and neutral sterols were extracted exhaustively with four portions of 8 mL of cyclohexane. The solvent of the combined extracts was evaporated to dryness with a vacuum

rotatory evaporator at 30ºC. After that, Bond Elute LRC® aminopropyl solid phase extraction (SPE) cartridge (Varian Associates, Harbor City, CA, USA) was conditioned with 4 mL of hexane. Each sample was dissolved with five consecutive 1 mL portions of trichlorometane/isopropanol (2:1; v/v), which were poured then into the cartridge. Finally, cartridges were drawn, increasing the vacuum until no solvent was eluted. Solvent was dried under nitrogen stream at controlled temperature (30ºC). The samples were maintained overnight under vacuum conditions, in order to remove all the residual solvent.

In order to quantify the bile acid content, 200 μL of ethanolic sodium hydroxide (10 mol/L) were added to the aqueous layer, and samples saponified at 120ºC for 120 min. After that, samples were acidified to pH<1 with hydrochloric acid and bile acids extracted with diethyl ether (4 x 1 mL). The organic layer was then washed three times with 3 mL of aqueous sodium chloride 1%. Finally, solvent was evaporated under a stream of nitrogen.

Neutral and acidic sterols extracts were derivatized by addition of 50 μL of anhydrous pyridine and 50 μL of Sylon BTZ, and maintained for 30 min at room temperature before the GC analysis.

Gas-chromatography determination

The gas chromatography analysis was performed with a Perkin Elmer GC Autosystem™ equipped with a ZB1 (100% methylpolisiloxane) capillary column (30 m x 0.25 mm i.d.; 0.25 μm) and a flame ionisation detector. Helium was the carrier gas. Operation conditions were: injector temperature 290ºC; detector temperature 300ºC. For neutral sterols, oven temperature was kept at 245ºC for 0,5 min, then programmed at a rate of 2ºC/min until 265ºC, and finally at 3.5ºC/min to 290ºC (21 min). For acidic sterols, oven temperature started at 210ºC (1min), and after that a ratio of 5ºC/min was applied until 290ºC (14 min). Split ratio was 12.5:1 and injection volume was 1 μL. Analytes were identified by their relative retention time and by co-chromatography with standards.

Statistical analysis

Significance of the differences in total faecal content was tested by two-way analysis of variance (ANOVA) with the amount of pectin and phytosterols as factors.

Post hoc comparisons of means were performed using Bonferroni's honest significant difference test. We used SPSS 11.0 in the calculations. As usual, $p<0.05$ was considered significant.

Results

Phytosterol results

Table *3* shows the mean content and SD of the most important plant sterols (campesterol, stigmasterol, β-sitosterol) in total faecal content. A decrease in faecal plant sterols values was found in PE2 group compared to PE0, although only for β-sitosterol concentrations, this decrease was statistically significant (p=0.039) (for stigmasterol and campesterol were almost significant). Animals fed phytosterols showed an increase in plant sterol concentrations (p<0.001). When the combined effect of both compounds was studied, pectin addition modulated the concentration of plant sterols in faeces, since although there was a positive relationship between addition of plant sterols in diets and amount in faeces, the faecal plant sterol concentrations were lower in PE2 group than in PE1, and the same was found between PE1 and PE0.

If the ratio faeces/diet for each phytosterol is taken as a measure of apparent absorption (Table *5*), big differences are found in campesterol, compared to the rest of phytosterols. β-sitosterol and stigmasterol showed the highest ratio faeces/diet at high phytosterol supplementations. However, campesterol ratio was lower in animals fed phytosterols (p<0.001) than in control group (PH0). Another difference was that campesterol did not show any difference in the ratio after pectin feeding, whereas stigmasterol and β-sitosterol showed the highest ratio at PE0 group (p<0.001).

Cholesterol, cholesterol metabolites and bile acids results

Table *4* shows the mean content and SD of cholesterol and its metabolites (coprostan-3-ol and coprostran-3-ona) in total faecal content.

Pectin and phytosterol intake had opposite effects on cholesterol concentrations in faeces. The lowest cholesterol level in faeces was found for PE2 diets (p=0.008, compared with control (PE0)) and the highest in animals fed phytosterols (p<0.001)

compared with control group (PE0/PH0). Nevertheless, the increase in cholesterol levels found in PH1 group was around 87% compared with those fed PH0, whereas it was around 23% when the PH2 group was compared with PH1.

The ratio cholesterol in faecal content/cholesterol in diet (Table *5*) increased after phytosterol intake, finding the highest absorption in animals fed with no phytosterols. Moreover, animals from group PE2 showed a significant higher absorption than those fed PE0. Therefore, animals with the highest ratio (less absorption) were those from PE0/PH2, and the lowest was found in PE2/PH0 animals.

Coprostan-3-ol values decreased after phytosterol intake ($p<0.001$) compared with control group (PH0). Animals fed different doses of pectin did not show statistically significant differences, in spite of showing a slight decrease in their concentrations at higher levels of fiber. Looking at the combined effect of plant sterols and pectin supplementation, some differences were found, but none of them were significant. In PE0 and PE2, the reduction in coprostan-3-ol levels was almost the same (around 40%) when PH1 was compared with PH2, as when PH0 was compared with PH1. However, for animals from the PE1 group, the showed a reduction of 70% PH1 and, while the reduction between PH2 and PH1 was only 7%. Coprostan-3-one concentrations had the same pattern as coprostan-3-ol, but without showing statistically significant differences. The fecal neutral steroids (referred to the sum of cholesterol, coprostan-3-ol and coprostan-one) also decreased their amount in animals fed phytosterols ($p<0.001$), whereas no statistically significant differences were found in animals fed pectin, although a slight decrease appeared at higher doses of pectin.

No differences were found in primary nor in secondary bile acids when the combined effect of pectin and phytosterols were studied. Total bile acid content showed the highest value in those animals fed PE0 ($p<0.01$) and at the highest supplementation of phytosterols (PH2) ($p<0.01$). Addition of pectin to the diets reduced the primary and secondary bile acids (both $p<0.01$). After supplementation of plant sterols to saturated diets, primary bile acids were increased ($p<0.001$). However, secondary bile acids did not show any difference.

The global cholesterol output as the sum of faecal bile acids and faecal neutral sterols was reduced after phytosterol intake ($p<0.001$), showing no differences in animals fed pectin.

Discussion

It has been shown that diets with low-fat content and whose profile was high in PUFA *(34)*, or in MUFA *(35)* enriched with some compounds such as phytosterols *(7, 36)* or viscous fibers *(37)* may increase sterol excretion from the small bowel, either as bile acids or as cholesterol. However, to our knowledge, no studies have been conducted to investigate the role of plant sterols and pectin added to a high-fat diet rich in SFA. Therefore, the aim of this study was to find out how neutral and acidic sterols excretion may be modified after supplementing phytosterol and pectin to a Western-like diet (high in SFA and cholesterol).

In our study, we found that both the β-sitosterol and stigmasterol faecal amount of and the ratio faeces/diet increased at high levels of plant sterols. These results agree with the poor absorption of plant sterols in intestine (0.4-3.5%) described previously *(38)*. However, although faecal campesterol levels also increased at high levels of plant sterols, the ratio faeces/diet is lower, that is agreement to what was reported by other authors *(39)* since campesterol is better absorbed than the rest.

After phytosterol intake, we found that the excretion of cholesterol was increased, as well as the ratio faeces/diet, according to some authors *(40-42)* who considered the decrease in cholesterol absorption as one of the main mechanisms for the efficacy of plant sterols as hypocholesterolemic agents. However, better than looking at the faecal cholesterol concentrations, we should pay attention to the value of faecal neutral sterols, defined as the sum of cholesterol, and its metabolites (coprostan-3-ol and coprostan-3-one), or to the cholesterol output (faecal bile acids plus faecal neutral steroids). In both cases, we found a reduction after phytosterol addition, suggesting an increase in cholesterol absorption. These results are in agreement with the increase in the apparent absorption of campesterol, since it is known that increases in plant sterol absorption are surrogate markers of cholesterol absorption *(43-44)*. Moreover, it has already shown that plant sterols added to a saturated diet can enhance the absorption of several compounds, such as saturated fatty acids, both in guinea pigs *(31)* and in ileostomy subjects (although there was not tested statistically) *(45)*.

In our study, we also found that cholesterol output did not show any differences after pectin feeding, although cholesterol values, its apparent absorption and the faecal

bile acid content were lower in pectin group. Our findings disagree with most of the studies, which showed a decrease in cholesterol absorption as well as an interruption of enterohepatic of bile acids after pectin supplementation *(37, 46-48)*. However, in all these studies, pectin was added to a low fat-diet (and mainly unsaturated), whereas the diet used in our study was mainly saturated.

Animals fed intermediate doses of pectin (PE1) showed the highest phytosterol apparent absorption which is in agreement with some results previously reported *(31)*, according to which, at doses of 3.7% of pectin, plasma and liver saturated fatty acid concentration was higher than for animals fed diets supplemented with 6.9% of pectin. One possible explanation would be an early breakdown of pectin although high-methylated pectin (higher than 50%) was used. The main support for this hypothesis is that animals fed pectin had higher body weight and feed efficiency that those from control group. However, since the PE2 pectin doses was almost double than the PE1 dose, it should be expected a higher body weight in the PE2 group but not difference was found. Therefore, it may be hypothesised that the animals from PE2 group had some remains of intact pectin in intestine that may show the hypocholesterolemic effect. This would also explain concentrations in PE1 being higher than in PE2.

In conclusion, our results give some evidences that a high-fat diet rich in SFA can modify the hypocholesterolemic effects of some functional compounds such as phytosterol and pectin, since they may enhance cholesterol absorption and reduce cholesterol output in guinea pigs. Therefore, the consumption of low-saturated fat content diets should be encouraged when phytosterol are consumed. Nevertheless, some studies in humans are warranted to confirm our preliminary results, restricted to one animal model.

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Table 1. Composition of diets fed to guinea pigs

Ingredients[a] (g/100g)									
Diets	**PE0/PH0**	**PE0/PH1**	**PE0/PH2**	**PE1/PH0**	**PE1/PH1**	**PE1/PH2**	**PE2/PH0**	**PE2/PH1**	**PE2/PH2**
Pectin	0	0	0	3.7	3.7	3.7	6.9	6.9	6.9
Phytosterols	0	1.27	2.45	0	1.27	2.45	0	1.27	2.45
Protein	18.3	18.5	17.7	18.6	18.9	18.0	18.7	18.9	18.1
Fat	15.9	17.6	19.6	15.9	17.4	19.7	15.6	17.4	19.5
SFA	11.3	8.7	9.8	12.1	9.2	10.0	12.4	9.2	9.5
Sugars	38.4	40.0	39.4	35.3	36.2	33.6	33.1	30.5	29.2
Insoluble fiber	12.7	11.9	11.5	12.5	12.5	12.5	12.4	11.9	11.8
Mineral and vitamin mix[b]	6.5	6.7	6.5	6.7	6.7	6.7	6.7	6.8	6.7

[a] All diets had been enriched with 0.33% of cholesterol

[b] Vitamin and mineral mix were adjusted to meet National Research Council requirements for guinea pigs. Detailed composition of the vitamin and mineral mix has been reported by Krause *et al. (30)*.

Table 2. Cholesterol and plant sterol amount in diets (μg/100 mg)

Pectin	**PE0**			**PE1**			**PE2**		
Phytosterol	**PH0**	**PH1**	**PH2**	**PH0**	**PH1**	**PH2**	**PH0**	**PH1**	**PH2**
Cholesterol	341.53	347.37	342.55	319.97	358.29	353.03	326.82	342.76	354.53
Sterols	23.69	1269.51	2880.12	47.76	1526.55	3231.18	29.33	1506.68	3210.82
Campesterol	4.86	370.91	860.86	5.37	448.05	939.23	3.84	441.13	925.66
Stigmasterol	2.70	236.43	540.28	2.29	287.45	604.98	3.71	286.35	602.87
β-sitosterol	16.14	661.16	1478.99	33.10	791.14	1686.97	21.78	779.20	1682.28

Table 3. Plant sterols content in total fecal content

	Campesterol	Stigmasterol	β-sitosterol	Total phytosterol content
PE0/PH0	26.05^{ab} (11.21)	Nd	10.55^{a} (3.13)	36.71^{a} (13.80)
PE0/PH1	514.98^{cde} (87.49)	251.39^{ab} (35.62)	587.71^{b} (85.67)	1354.08^{bc} (185.07)
PE0/PH2	997.16^{f} (281.72)	548.25^{c} (136.71)	1334.17^{c} (305.67)	2879.58^{d} (708.26)
PE1/PH0	21.26^{a} (2.91)	Nd	8.55^{a} (3.34)	29.81^{a} (4.01)
PE1/PH1	411.97^{bcd} (54.75)	203.56^{a} (26.94)	489.77^{b} (57.88)	1105.29^{b} (133.26)
PE1/PH2	897.49^{ef} (143.05)	494.56^{c} (75.40)	1201.90^{c} (179.37)	2593.96^{d} (397.43)
PE2/PH0	19.26^{a} (2.68)	Nd	9.54^{a} (2.68)	28.81^{a} (5.13)
PE2/PH1	376.65^{abc} (60.86)	176.40^{a} (36.20)	409.67^{ab} (93.54)	962.72^{ab} (174.62)
PE2/PH2	786.95^{def} (181.68)	434.28^{bc} (82.77)	1041.64^{c} (176.53)	2262.89^{cd} (440.45)

Results are means (μg/100 mg) (SD in parenthesis); values in a column with the same superscript are not significantly different ($p \geq 0.05$).

Nd means not detected

Table 4. Cholesterol, cholesterol metabolites and bile acids content in total fecal content

	Cholesterol	Coprostan-3-ol	Coprostan-3one	Fecal neutral steroils[1]	Primary bile acids	Secondary bile acids	Total bile acids	Cholesterol output[2]
PE0/PH0	138.83^{abc}	509.67	10.89	659.38	12.47	37.90	50.38	709.76
	(28.31)	(244.08)	(6.44)	(269.92)	(3.62)	(14.58)	(17.58)	(276.89)
PE0/PH1	255.19^{cd}	236.23	10.71	502.13	20.66	34.60	55.26	557.38
	(46.02)	(125.66)	(5.11)	(131.11)	(10.25)	(14.51)	(24.59)	(140.73)
PE0/PH2	308.69^{d}	124.59^{a}	7.22	440.50	27.32^{b}	45.47	72.79^{b}	513.29
	(65.11)	(36.07)	(5.91)	(65.15)	(10.24)	(10.70)	(18.53)	(81.06)
PE1/PH0	109.29^{ab}	553.48^{b}	10.31	673.09	6.64^{a}	24.87	31.51	704.60
	(40.75)	(211.18)	(3.23)	(228.64)	(1.83)	(2.70)	(1.19)	(229.28)
PE1/PH1	210.42^{abcd}	172.66	7.45	390.54	11.90	31.88	43.78	434.32
	(24.39)	(48.07)	(3.73)	(63.19)	(2.13)	(9.00)	(7.89)	(59.80)
PE1/PH2	272.54^{d}	166.91	8.28	447.73	19.05	32.95	52.00	499.73
	(50.94)	(31.20)	(2.75)	(76.96)	(8.00)	(9.03)	(15.43)	(86.32)
PE2/PH0	97.82^{a}	480.45	7.58	585.84	7.29^{a}	20.53	27.52^{a}	613.67
	(18.18)	(70.00)	(3.29)	(83.98)	(3.22)	(6.96)	(10.17)	(79.08)
PE2/PH1	183.85^{abcd}	219.87	8.08	411.81	12.24	25.48	37.72	449.52
	(41.71)	(106.48)	(4.70)	(89.69)	(4.11)	(3.68)	(6.44)	(90.58)
PE2/PH2	230.41^{bcd}	140.77^{a}	7.81	378.98	15.06	24.87	39.93	418.90
	(40.41)	(46.25)	(7.14)	(93.08)	(0.16)	(9.31)	(9.43)	(102.51)

Results are means (μg/100 mg) (SD in parenthesis); values in a row with the same superscript are not significantly different ($p \geq 0.05$). Nd means not detected

Faecal neutral steroids[1] Refers to the sum of cholesterol, coprostan-3-ol and copoprostan-3-one and Cholesterol output[2] refers to the sum of faecal neutral steroids and faecal bile.

Table 5. Ratio of plant sterols in total faecal content between content in diet

	Ratio Campesterol	Ratio Stigmasterol	Ratio β-sitosterol	Ratio Cholesterol
PE0/PH0	5.36^{b} (2.31)	Nd	0.65^{abc} (0.19)	0.41^{ab} (0.08)
PE0/PH1	1.39^{a} (0.24)	1.06^{b} (0.15)	0.89^{b} (0.13)	0.73^{bc} (0.13)
PE0/PH2	1.16^{a} (0.33)	1.01^{b} (0.25)	0.90^{b} (0.21)	0.90^{c} (0.19)
PE1/PH0	3.96^{b} (0.54)	Nd	0.26^{a} (0.10)	0.34^{a} (0.13)
PE1/PH1	0.92^{a} (0.13)	0.71^{ab} (0.09)	0.62^{abc} (0.07)	0.59^{abc} (0.07)
PE1/PH2	0.96^{a} (0.15)	0.82^{ab} (0.12)	0.71^{bc} (0.11)	0.77^{bc} (0.14)
PE2/PH0	5.02^{b} (0.70)	Nd	0.44^{ab} (0.12)	0.30^{a} (0.06)
PE2/PH1	0.85^{a} (0.14)	0.62^{a} (0.13)	0.53^{abc} (0.12)	0.54^{abc} (0.12)
PE2/PH2	0.80^{a} (0.19)	0.68^{ab} (0.14)	0.59^{abc} (0.11)	0.61^{abc} (0.12)

Results are means (μg/100 mg) (SD in parenthesis); values in a column with the same superscript are not significantly different ($p \geq 0.05$).

Nd means not detected

2.5 RAPID AND QUANTITATIVE DETERMINATION OF TOTAL STEROLS OF PLANT AND ANIMAL ORIGIN IN LIVER SAMPLES BY GAS-CHROMATOGRAPHY

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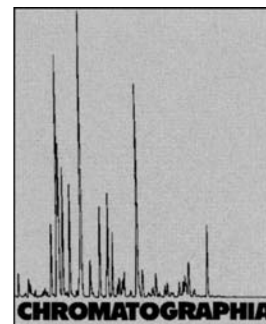
Rapid and Quantitative Determination of Total Sterols of Plant and Animal Origin in Liver Samples by Gas Chromatography



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Abstract

A simple and rapid method to quantify sterols by gas- chromatography (GC) in liver samples is described. The most usual methods to determine sterol content by GC in biological samples involve lipid extraction, saponification, extraction of non-saponifiables and their fractionation. This paper proposes a direct saponification of biological samples (liver) in order to save time and solvents. Samples were homogenised with ethanol, which contained a mixture of antioxidants, followed by a saponification step using KOH 1 N, extraction of non-saponifiables and, finally, purification step using a SPE cartridge. Gas-chromatography analysis was performed using a ZB-1 (100% methylpolisiloxane) capillary column and an oven temperature program, which involves two rates from 245 to 290 °C. Analytes were identified by their relative retention time and by co-chromatography. Quantitative analysis was carried out by using 5α-cholestane as internal standard (IS). Repeatability, recovery and linearity were determined. Values of repeatability (n = 8) in liver were [mean µg/100 mg (RSD)]: for squalene [0.68 (12.99%)], for cholesterol [190.82 (2.34%)], for lathosterol [0.48 (3.05%)], for campesterol [3.90 (2.55%)] and for β-sitosterol [0.27 (19.69%)]. In conclusion, this method offers interesting perspectives for the quantitative analysis of these sterols not only in liver, but also in other biological samples.

Keywords

Gas chromatography
Saponification of biological samples
Plant sterols
Liver samples

Introduction

It was recognized in the 1950s that plant sterols may lower total and LDL cholesterol [1] and the risk of cardiovascular disease [2–5].

Phytosterols' body levels are derived from the diet since they are not synthesized by animal tissues [6]. Their absorption is very low as Jones et al. [7] described in a previous report (for instance, about 5% of β-sitosterol, 15% of campesterol and less that 1% of dietary stanols are absorbed).

Since plasma phytosterols concentrations are around 1% of plasma cholesterol concentration, sensitive analytical methods are necessary to detect phytosterols in animal biological samples. Moreover, these methods should be able to analyse total sterols of plant and animal origin simultaneously.

There are many methods for the analysis of the non-cholesterol sterols in biological samples. Most of them have several difficulties if they have to be applied to determine all compounds [8]. There are many compounds (such as sterols and stanols), which are present in very low concentrations in relation to cholesterol levels [9] and many compounds in complex matrices such as liver that may interfere with separation/quantification. These methods show different performance characteristics, which should be taken into account in order to choose the most adequate method to consistently determine sterols, depending on the analytical situation (e.g. composition of sample, number of samples to be analysed, method quality parameters required, concentration of sterols).

Most of the reported methods used lipid extraction before saponification. Nonpolar solvents, such as hexane [10], chloroform–methanol–water [11, 12], dichloromethane–methanol–water [13] and chloroform–isopropanol–water [14] quantitatively extract free phytosterols and phytosteryl fatty-acid esters. However, in some reports [15–17] the first solvent extraction was avoided to reduce the analysis time. After the saponification step, others found it to be useful to further clean-up the samples by thin layer chromatography (TLC) [18, 19], column chromatography [20, 21] or solid phase

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extraction (SPE) [9, 22]. The main advantages of SPE methods are (1) the short preparation time, partly due to the possibility of the simultaneous handling of several samples, (2) the reduced use of solvents as compared to column chromatography clean-up procedures and (3) SPE is more precise and less tedious to recover samples.

Since levels of several plant sterols, plant stanols, cholesterol and its metabolites (e.g. lathosterol) in liver provides relevant information on the regulation of cholesterol and bile acid synthesis under various experimental conditions [23], it is necessary to have a method to determine these compounds in liver, showing high selectivity and sensitivity, since methods described [15, 24–26] do not include validation data of the separation and quantification of these compounds.

This work reports the development of a rapid and suitable method for the simultaneous analysis of total sterols of plant and animal origin in liver, based on a modification of the method proposed by Phillips et al. [9].

Experimental

Reagents and Standards

5α-cholestane, cholestanol, cholesterol, squalene, β-sitosterol, pyrogallol, BHT (butylated hydroxytoluene) and Sylon BTZ (BSA (*N*,*O*-bis(trimethylsilyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI [(*N*-trimethylsilyimidazole); 3:2:3] kit were purchased from Sigma Chemical (St. Louis, MO, USA). Absolute ethanol, cyclohexane, hexane, chloroform and isopropanol were obtained from Panreac (ACS certified grade) (Barcelona, Spain) and dry pyridine from Merck (Darmstadt, Germany). Distilled deionized water was used throughout.

Sample Preparation

The study used samples of 72 female Harley guinea pigs and included 9 treatments. Further details about the design of the study as well as composition of diets are described elsewhere [27]. All procedures were approved by the University of Barcelona Institution Animal Care and Use Committee.

After the sacrifice, liver was homogenized and maintained at −80 °C until the moment of the hydrolysis, followed by analysis. The liver samples used to validate the method were those from animals of the control group, fed with a standard diet for guinea pigs.

Sterol Determination

Non-Saponfiable Extraction

Three hundred milligram of liver were exactly weighed in a 50 mL centrifuge tube containing 50 μL of internal standard [5α-cholestane (560 μg mL^{-1})]. Then, 8 mL of a mixture of pyrogallol 3% (*w*/*v*) and BHT 0.06% (*w*/*v*) in ethanol was added. Homogenisation was carried out for 30 s at 20.000 g using a Polytron PT 3000, maintaining samples in an ice bath. Afterwards, 2 mL of aqueous potassium hydroxide (28.0%, *w*/*v*) were added, and tubes were gently shaken.

Saponification was done by incubation of the reaction mixture at 80 °C for 30 min. Afterwards, tubes were cooled in an ice bath for 5 min. To extract non-saponfiable compounds, we used cyclohexane, a non-polar organic solvent, because it is less toxic, does not form peroxides as does diethyl ether and, above all, because it does not form stable emulsions during the procedure [19]. Therefore, 10 mL of distilled water and 20 mL of cyclohexane were added and mixture was shaken, and then centrifuged at 1,000*g* for 10 min.

The upper phase was transferred to a round-bottom flask and the aqueous layer was reextracted twice with 8 mL of cyclohexane. Finally, the solvent was evaporated to dryness with a vacuum rotatory evaporator at 30 °C.

Solid-Phase Purification

After the saponification step, we used a Bond Elute LRC® aminopropyl solid phase extraction (SPE) cartridge (Varian Associates, Harbor City, CA, USA). Therefore, cartridges were coupled to an elution vacuum system and conditioned with 4 mL of hexane, which was discarded. Each sample was dissolved with five consecutive 1 mL portions of trichlorometane/isopropanol (2:1; *v*/*v*), which were then poured into the cartridge. Finally, cartridges were drawn increasing the vacuum until no solvent was eluted. Solvent was dried under nitrogen stream at controlled temperature (30 °C). Samples were maintained overnight under vacuum conditions, in order to remove all residual solvent.

Gas Chromatography Determination

Dry extracts were derivatized by addition of 50 μL of anhydrous pyridine and 50 μL of Sylon BTZ, and maintained for 30 min at room temperature before injection into the gas chromatograph.

The GC analysis was performed with a Perkin Elmer GC Autosystem™ equipped with ZB1 (100% methylpolisiloxane) capillary column (30 m × 0.25 mm i.d.; 0.25 μm) and a flame ionisation detector. Helium was the carrier gas. Operation conditions were: injector temperature 290°C; detector temperature 300 °C; and oven temperature was kept at 245 °C for 0.5 min, then programmed at a rate of 2 °C min^{-1} until 265 °C, and finally at 3.5 °C min^{-1} to 290 °C (21 min). Split ratio was 12.5:1 and injection volume was 1 μL. Analytes were identified by their relative retention time and by co-chromatography with standards. The co-chromatography was conducted as follows: Briefly, the purified test solution prior to the chromatographic injection was divided in two parts. One of them was injected into the GC as such, and the other one was first enriched with the standard analyte that has to be identified, and afterwards, injected into the GC, as well. The amount of added standard analyte was similar to the estimated amount of the analyte in the test solution.

Validation

The following parameters were determined: linearity, repeatability and recovery rate.

Linearity

Concentrations' range that we expected to find in our samples was tested for linearity. Standard solutions were prepared at five different concentrations between 0.10 and 700.00 μg mL^{-1} for each compound studied (cholesterol, stigmasterol, β-sitosterol and sitostanol). Each concentration level was determined in quadruplicate. A linear regression was performed between the component/IS area ratio and the component/IS mass ratio. Campesterol linearity had not been

studied because no standard were available. Moreover, since campesterol is eluted between some compounds which linearity were determined, we considered that it has a similar linearity. Therefore, in order to determine its concentration in the samples, the response factor was determined for all compounds tested. Response factors were calculated according to:

$$RF_i = \frac{\mathrm{area_{IS}} \times \mathrm{conc_{sterol_i}}}{\mathrm{area_{sterol_i}} \times \mathrm{conc_{IS}}},$$

where RF_i is the response factor of sterol$_i$, area$_{IS}$ is the peak area of the internal standard compound, area$_{sterol}$ is the peak of area of sterol$_i$, conc$_{IS}$ is the concentration of the internal standard compound in the calibration mix and conc$_{sterol}$ is the concentration of sterol$_i$ in the calibration mix. However, because no standard of campesterol and lathosterol is available, we calculated a relative response factor for these compounds according to their retention time. Retention time and response factor of cholesterol and stigmasterol eluted before and after that of lathosterol and campesterol. The relative response factor was calculated according to:

$$RF_x = RF_{\mathrm{chol}} + \frac{RT_x - RT_{\mathrm{chol}}}{RT_{\mathrm{stig}} - RT_{\mathrm{chol}}} \times \left(RF_{\mathrm{stig}} - RF_{\mathrm{chol}}\right),$$

where RF_x is the response factor of sterol which does not have any available standard, RF_{chol} is the response factor of sterol cholesterol, RT_x is the retention time (min) of sterol studied, RT_{chol} is the retention time (min) of cholesterol, RF_{stig} is the retention time of stigmasterol (min) and RF_{stig} is the response factor of stigmasterol.

The limit of detection and quantification was determined as was previously reported [28].

Repeatibility

Repeatibility had been assayed for squalene, cholesterol, cholestanol, lathosterol, campesterol and β-sitosterol because it was determined by measuring eight sample replicates, without the need a adding any standard.

Recovery Rates

The investigation of potential interfering effect of liver and the determination of the analytical recovery was made by addition of three different concentrations to the samples, according to:

$$\%R = \frac{M_{fc} - M_c}{M_f} \times 100,$$

where $\%R$ is percent recovery, M_{fc} is the raw amount in µg of component determined in the spiked sample, M_c is the raw amount in µg of component in the unfortified material, and M_f is the fortification amount in µg. Standards were solved with cyclohexane and added in the same way as was done for the internal standard.

Three different compounds were assayed in each sample, according to the amount expected. Although stigmasterol was not detectable in our liver samples, we chose to spike the samples with stigmasterol because it elutes close to campesterol, and campesterol standard was not available to study its recovery. Thus, we assumed they had a similar pattern of elution. We studied only cholesterol and stigmasterol because to the other compounds are only found in slight concentrations.

We also studied the recovery rate for squalene but, although it is not sterol, it is found in high amount in relation to other compounds. The lowest and the highest concentration of all compounds studied corresponded to the limits expected in samples. The third concentration was the intermediate between these limits. Each spiked sample was prepared in triplicate.

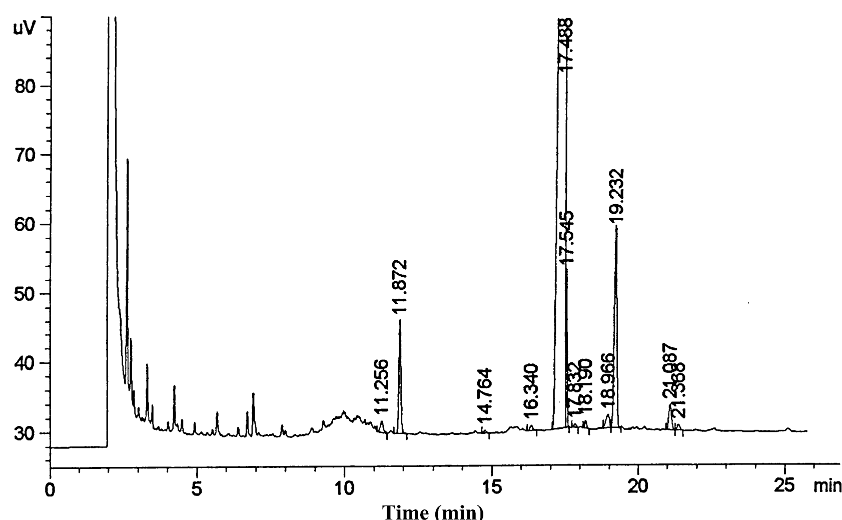

Fig. 1. GC chromatogram of the liver samples Peaks: (11,256) squalene, (11,872) 5α-cholestane (IS), (17,488) cholesterol, (17,545) cholestanol, (18,190) lathosterol, (19,232) campesterol, (21,087) β-sitosterol. Column and GC conditions are given in the gas chromatography analysis section

Results and Discussion

Most common methods for the extraction of lipids, also extract phytosterols. Many authors used a lipid extraction before saponification step [10–14] but Toivo et al. [22] compared several analytical conditions to determine total plant sterol in wheat flour and concluded that total lipid extraction did not significantly affect sterol values. Therefore, on the basis on previous results, we decided to use a direct saponification instead of doing a prior lipid extraction.

Identification of different phytosterols (Fig. 1) was performed using their relative retention times and by co-chromatography.

Table 2 summarises the linear concentrations range, the calibration curve, the response factor, the retention time for each standard assayed, as well as the limit of detection and quantification. A plot of the ratio peak area of the sterols analysed/peak area of IS versus the ratio amount of sterols/amount of IS gave a linear response in a wide range, with their determination coefficient (r^2) ranged between 0.996 and 1.000 (Table 1). One of the main problems in the analysis of plant sterols and cholesterol is that they are found in a wide range of concentrations in biological samples, so it is often necessary determine them in different analysis [9]. However, our method offers the possibility to determine these compounds simultaneously because of the linear response in a wide range of concentrations (Table 1).

Table 1. Linearity of phytosterols and cholesterol metabolites using the present method

Component	Linear range (μg mL^{-1})	Calibration curve	R^2	Response factor	Retention times (min)	LOD[c] (μg mL^{-1})	LOQ[c] (μg mL^{-1})
5α-cholestane (IS)	–	–	–	1.0	12.022	–	–
Cholesterol	6.94–693.95	$Y = 1.045x - 0.078$	1.000	0.991	17.376	0.22	0.75
Stigmasterol	4.07–406.61	$Y = 1.005x - 0.035$	0.999	0.991	20.174	0.10	0.35
β-sitosterol	0.41–616.92	$Y = 0.881x + 0.066$	0.997	1.066	21.836	0.22	0.75
Sitostanol	3.89–194.33	$Y = 0.980x + 0.046$	0.996	1.000	22.051	0.14	0.47
Lathosterol[a]	–	–	–	0.991	17.784	–	–
Campesterol[a]	–	–	–	0.991	19.573	–	–
Squalene[b]	–	–	–	1.000	11.890	–	–

[a]Relative response factor of these compounds was determined according their retention times, and retention times and response factor of cholesterol and stigmasterol (see Experimental)
[b]Relative response factor of squalene has been considered 1.000, because it was eluted close to internal standard
[c]The limit of detection (LOD) and the limit of quantification (LOQ) have been calculated as has been described previously [28]

Table 2. Summary of assay precision for liver samples ($n = 8$)

Component	Mean μg/100 mg (SD)	RSD (%)
Squalene	0.69 (0.089)	12.99
Cholesterol	190.82 (4.465)	2.34
Cholestanol	3.20 (0.159)	4.98
Lathosterol	0.48 (0.015)	3.05
Campesterol	3.90 (0.099)	2.55
β-sitosterol	0.27 (0.054)	19.69

SD Standard deviation, *RSD* relative standard deviation, expressed in % of the mean

Table 3. Recovery of sterols from liver samples

Compound	Amount spiked (μg)	Number of replicates	Average recovery[a] (%)	Average[b] (%)
Squalene	3.3	3	112.52 (6.67)	
	33.0	3	110.20 (0.59)	
	66.0	3	109.04 (1.49)	
				110.59 (1.60)
Cholesterol	721.5	3	100.09 (2.21)	
	1442.9	3	102.13 (1.11)	
	2885.8	3	98.45 (2.59)	
				100.22 (1.84)
Stigmasterol	0.33	3	ND	
	40.98	3	103.49 (1.38)	
	101.6	3	99.97 (0.62)	
				101.73 (2.45)

ND Not detected
[a]Standard deviation of replicates is shown in parentheses
[b]Mean of recovery expressed in %

The interassay precision was assessed by the coefficient of variation, which was determined by analysing eight aliquots of liver. Table 2 shows values of the mean, standard deviation and relative standard deviation for squalene, cholesterol, cholestanol, lathosterol, campesterol and β-sitosterol. Validation data showed good precision in liver samples. In liver samples, the RSD of the main sterols were within the limits of acceptable variability proposed by Hubert [29]; however, squalene and β-sitosterol have a higher RSD due to the low concentration present in this tissue (0.69 μg/100 mg of squalene and 0.27 μg/100 mg of β-sitosterol). Our results are similar to those described by others who used more purification steps [9, 30] and offer the possibility to determine several compounds present in a wide range of concentrations; for instance, cholesterol and campesterol showed RSD lower than 5% (Table 2), although cholesterol concentrations were 50 times higher than those for campesterol.

The standard addition method was used to test the recovery of the analytes. Three levels of standard concentrations (made by triplicate) were added to a known sample mass, and then they were carried through the entire procedure. The sterol recovery rates determined are presented in Table 3. The average sterols recovery rates ranged between 101.73 and 110.59%, which were satisfactory [29]. Moreover, the precision of the recoveries ranged between 1.60 and 2.45%.

Conclusion

Our method allows the analysis of sterols in liver without the necessity of a lipid extraction step before saponification. Furthermore, it gives us the possibility to determine the amount of several compounds in a wide range of concentrations. Therefore, this method offers interesting perspectives for the quantitative analysis of these compounds not only in this matrix, but also in other biological samples.

Acknowledgments

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3 GENERAL DISCUSSION

As already mentioned in the Introduction, Western diets (with higher amounts in SFA and tFA and lower in n-6 and n-3 PUFA than what it is desirable according to nutritional recommendations) increase cardiovascular risk by rising blood total- and LDL-cholesterol concentrations (Spady *et al.*, 1993; Mustad *et al.*, 1997; Stamler *et al.*, 2000; Hu *et al.*, 2001). Moreover, it is also known that a relevant reduction in CHD events is achieved by lowering blood total- and LDL-cholesterol and triacylglycerols levels, and by increasing HDL-cholesterol. Since SFA intake is too high in Western countries (Kris-Etherton *et al.*, 2002; Simopoulos, 2002), actual recommendations claim to limit SFA intake to less than 10% of total daily caloric content (Krauss *et al.*, 2000; Serra-Majem *et al.*, 2001).

Although a diet in accordance with the dietary reference intakes should always be recommended, functional foods are useful to further improve a favourable lipoprotein profile. During last years, many studies have demonstrated the hypocholesterolemic efficacy of plant sterols and stanols (Mensink *et al.*, 2002; Katan *et al.*, 2003; Miettinen & Gylling, 2004) and soluble fibers (Galibois *et al.*, 1994; Brown *et al.*, 1999; Dongowski & Lorenz, 2004). However, to our knowledge, no data are available about the effects of the addition of phytosterol and pectin to a diet with a high content in saturated fatty acids and cholesterol.

In the first section of this chapter, we discuss our proposal of a new analytical method to determine sterols of animal and plant origin on liver samples. Next, we discuss the effects of the addition of plant sterols (section 2) and pectin (section 3) to a saturated diet on the lipid profile in guinea pigs. The last section deals with the influence of the simultaneous intake of pectin and plant sterols.

3.1 VALIDATION OF AN ANALYTICAL METHOD TO DETERMINE STEROLS FROM ANIMAL AND PLANT ORIGIN IN LIVER SAMPLES

To be able to accomplish our objectives, one of the first problems we had to face was the lack of a suitable analytical method to determine sterols in liver samples. Most of the methods reported show several difficulties when applied to plant sterols/stanols and cholesterol, found in a wide range of concentrations in animal tissues. Moreover, there are many compounds in complex matrices, such as liver, that may interfere with the analysis.

The levels of plant sterols/stanols, cholesterol and its precursor lathosterol in liver provide relevant information on the regulation of cholesterol metabolism under various experimental conditions (Miettinen & Kesaniemi, 1989). Since the available methods (Ntanios & Jones, 1998; 1999; Tatematsu *et al*., 2004) do not include validation data of the separation and quantification, we developed a method with high selectivity and sensitivity.

Basically, samples were homogenised with ethanol, followed by a saponification step using KOH 1N, extraction of non-saponifiable compounds and purification using a solid phase extraction (SPE) cartridge. Finally, samples were injected to a gas-chromatograph (GC), using flame-ionization detector (FID) as a detector.

We decided to avoid the lipid extraction before saponification, since Toivo *et al*. (2000) reported that an additional total lipid extraction does not significantly affect sterol values. We determined the repeatability, recovery and linearity and values were within the limits of acceptable variability proposed by Hubert (1993).

To conclude, our method allows the analysis of sterols in liver without lipid extraction before the saponification. Furthermore, it gives the possibility to determine the amount of several compounds in a wide range of concentrations. Thus, this method offers interesting perspectives for the quantitative analysis of these compounds, not only in liver matrix, but also in other biological samples.

3.2 EFFECT OF THE ADDITION OF PHYTOSTEROLS TO A HIGH-SATURATED FAT DIET ON LIPID PROFILE IN GUINEA PIGS

3.2.1 BODY WEIGHT

The first data obtained in our study were those related to the growth of animals. Our results showed that the addition of phytosterols in a saturated diet did not modify the growth rate or the food consumption (Fig. 6), which is in agreement with many studies (Ewart *et al.*, 2002; Hayes *et al.*, 2002) although they did not use a diet rich in saturated fat. Moreover, animals fed diet with phytosterols showed no difference in plasma triacylglycerol or in glucose concentrations.

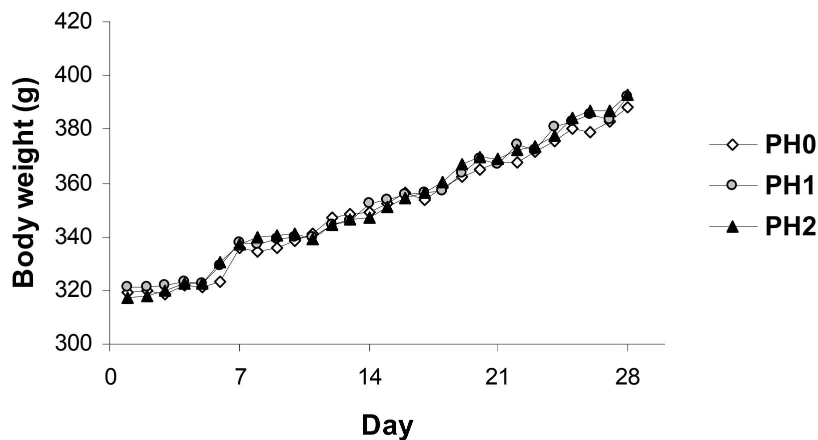

Fig. 6. Mean body weight curves for different phytosterol levels

3.2.2 FAECAL PLANT STEROL AND CHOLESTEROL EXCRETION

After phytosterol intake, concentrations of β-sitosterol, stigmasterol and campesterol in faeces increased (Fig. 7).

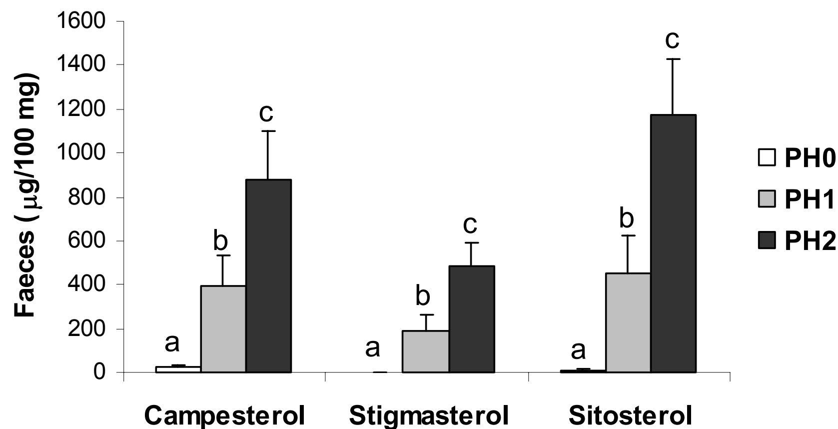

Fig. 7. Faecal concentrations (mg/100g) of the main plant sterols.
Different letter in superscript means $p<0.05$

Plant sterols in faeces were highly correlated with the amount in diets, since their absorption is very low. Therefore, more important than the total concentration in faeces is the absorption of these compounds. To determine this, we used the ratio of faecal content by diet content, that proxies apparent absorption (Bonanome & Grundy, 1988; Dougherty *et al.*, 1995; Baer *et al.*, 2003). This ratio has already been used in many studies, where similar values were found when the use of the ratio was compared with stable isotopes analytical methods (Emken, 1994; Jones *et al.*, 1999a).

Since faecal concentrations of phytosterol in the control group were very low, the ratio cannot be used in this group. By this reason, the comparison was restricted to PH1 and PH2 groups. As it can be seen in Fig. 8, all phytosterols analysed (campesterol, stigmasterol and β-sitosterol) had similar apparent absorption values, though campesterol was better absorbed than the rest, in agreement what was described previously (Sanders *et al.*, 2000). However, it is interesting to notice that, while the ratios of stigmasterol and β-sitosterol were higher with higher concentrations

in diets, suggesting lower absorption, campesterol ratio decreased with higher concentrations of plant sterols (nonsignificant results).

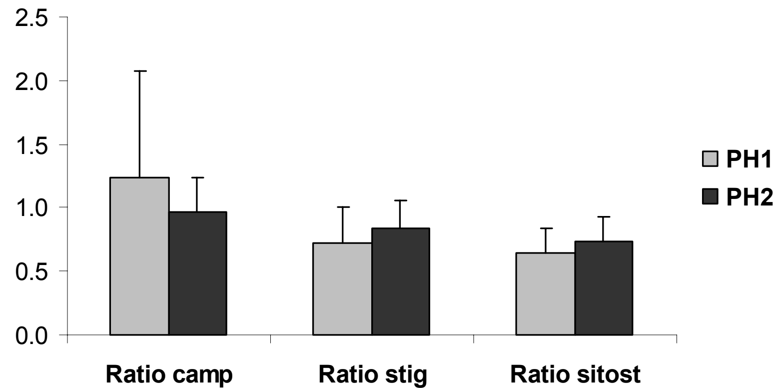

Fig. 8. Faecal phytosterol results

Camp means campesterol; *stig* means stigmasterol; *sitost* means β-sitosterol.

Furthermore, we found that cholesterol excretion and the ratio faeces/diet were higher after the intake of phytosterol. This finding is in agreement with several authors (Ntanios & Jones, 1999; Ramjiganesh *et al.*, 2001; Normen *et al.*, 2006), who considered the decrease in cholesterol absorption as one of the main mechanisms of plant sterols. However, we think that better than studying faecal cholesterol concentrations, is to study faecal neutral steroids (defined as the sum of cholesterol and its metabolites, coprostan-3-ol and coprostan-3-ona), since, when cholesterol reaches the lower intestinal tract, it is metabolised by colonic flora. Also interesting is the cholesterol output, defined as faecal bile acids plus faecal neutral steroids, because this value gives an idea about cholesterol metabolism.

We measured a decrease in faecal neutral steroids and cholesterol output after phytosterol intake (Fig 9). These results are in agreement with the increase found in the apparent absorption of campesterol, since it is known that phytosterol absorption is a surrogate marker of cholesterol absorption (Tilvis & Miettinen, 1986; Assmann *et al.*, 2006).

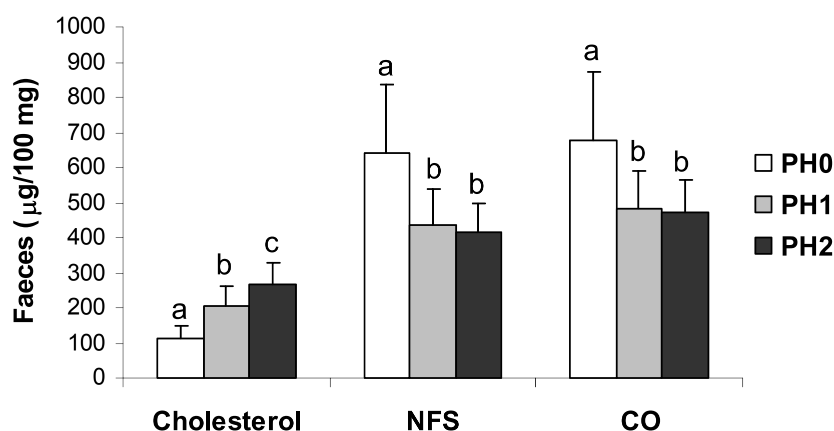

Fig 9. Cholesterol metabolism (μg/100mg)

NFS means neutral faecal sterols; *CO* means cholesterol output.

Different letter in superscript means $p<0.05$

3.2.3 SATURATED FATTY ACID CONCENTRATIONS

The changes in SFA excretion after phytosterol supplementation were different for different chain lengths. While plant sterols enhanced the excretion of long chain SFA (such as behenic and arachidic), the excretion of medium chain SFA (lauric and myristic) was reduced (Fig. 10).

Furthermore, the pattern of change in SFA excretion during the phytosterol treatments was also different depending on different chain length. We found a similar pattern of excretion of all SFA in control group (Fig. 10). The excretion increased after the first week, then remained stable for a week and increasing again in the last two weeks. However, the phytosterol effect varied across SFA. The excretion of lauric acid in control group increased around 78%, in PH1 group was 29% and the excretion in PH2 group was only increased in 20%, in the four weeks of the study. However, the highest excretion of behenic was found in PH2 group (around 55%), whereas control and PH1 group showed the same excretion (around 30%).

These differences may be explained by the distribution of the lipid compounds in the mixed micelle. It is well known (Borel *et al.*, 1996; Plat & Mensink, 2001) that plant sterols may displace compounds present in the core of the mixed micelle (such

as hydrocarbonated carotenoids), but have no effect on compounds present on the surface (such as tocopherols). Lauric acid, with 12 atoms of carbon, is less fat-soluble and may be situated closer to the surface of the micelle than behenic acid (with 22 atoms of carbon). Thus, as it happens for carotenes, phytosterols may be more efficient in displacing behenic acid from the mixed micelle than in displacing lauric acid. Moreover, palmitic and stearic acids did not show a clear phytosterol effect. With chains from 16 to 18 atoms of carbon, they have intermediate solubility. Thus, they may be situated between the core and the surface of the mixed micelle.

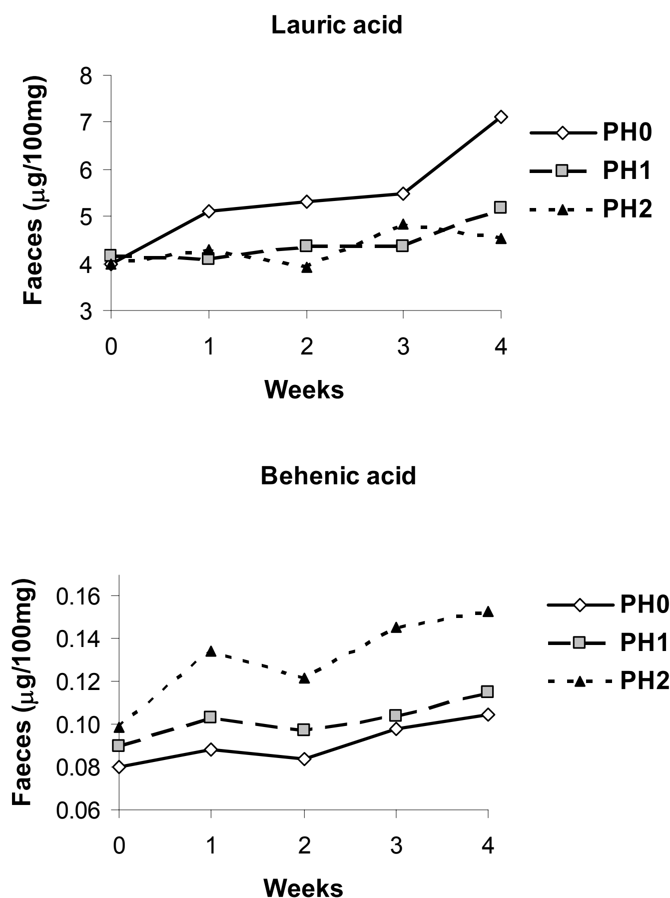

Fig. 10. Faecal concentrations of lauric and behenic acids during the study for the three different phytosterol supplementations

Our findings agree with those of Normen *et al.* (2006), who found that a saturated diet enriched with plant sterol decreases more the faecal excretion of lauric

and myristic than that of palmitic and stearic acids. However, if plant stanols were added, this effect did not appear. Also, we found an increase in lauric acid and myristic concentrations in liver with the highest concentrations of phytosterols added, suggesting an accumulation of these fatty acids in this tissue (Fig. 11), whereas no differences were found in plasma.

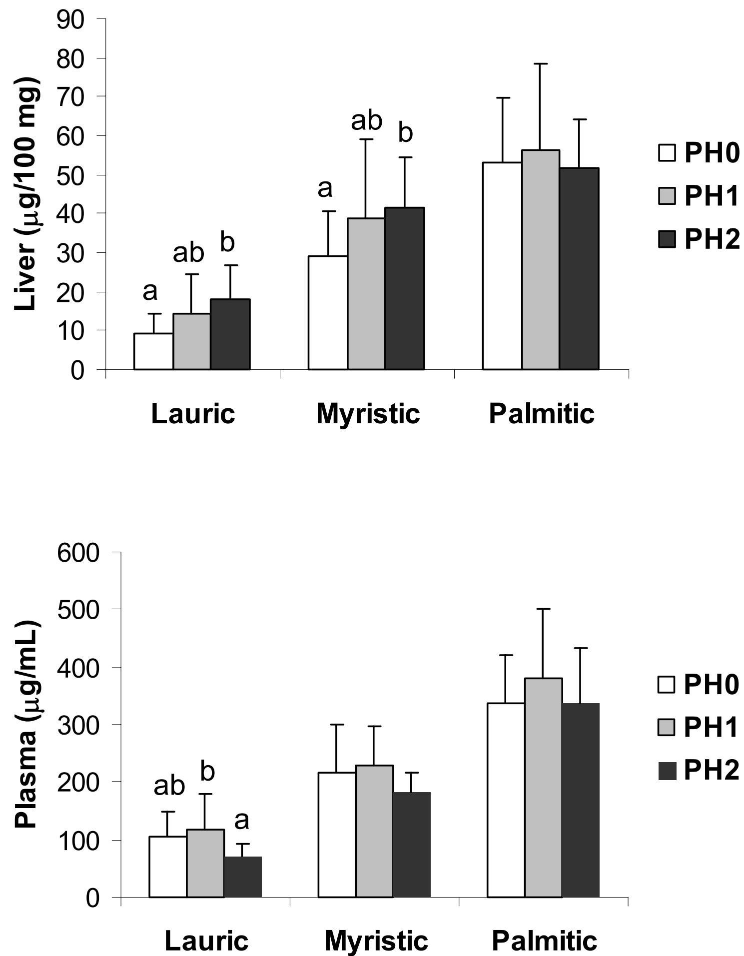

Fig. 11. Fatty acid contents in liver and plasma
Different letter in superscript means $p<0.05$

3.2.4 PHYTOSTEROL AND CHOLESTEROL CONCENTRATIONS IN PLASMA AND LIVER

Plasma phytosterol concentration in the control group (no phytosterol or pectin addition) was very low (under 1%), since phytosterols are poorly absorbed in the intestine (between 0.4 and 3.5%) (Ostlund, 2002), and cannot be synthesized by animals (Salen *et al.*, 1970). We also found a higher concentration of plant sterols in the liver from animals fed with the diet containing phytosterols (Fig 12). Plasma campesterol concentration was much higher than that of β-sitosterol, which is in agreement with the enhanced absorption of campesterol found in faeces. The reason for these results may be that hepatic ACAT has less affinity for β-sitosterol than for campesterol, thus being faster excreted in the bile (Tavani *et al.*, 1982), and campesterol is better absorbed in the intestine. For instance, in the PH1 and PH2 group, the plasma campesterol concentration was respectively 70 and 50 times higher, than in control group, while for β-sitosterol there was only a two-fold increase (Fig 12).

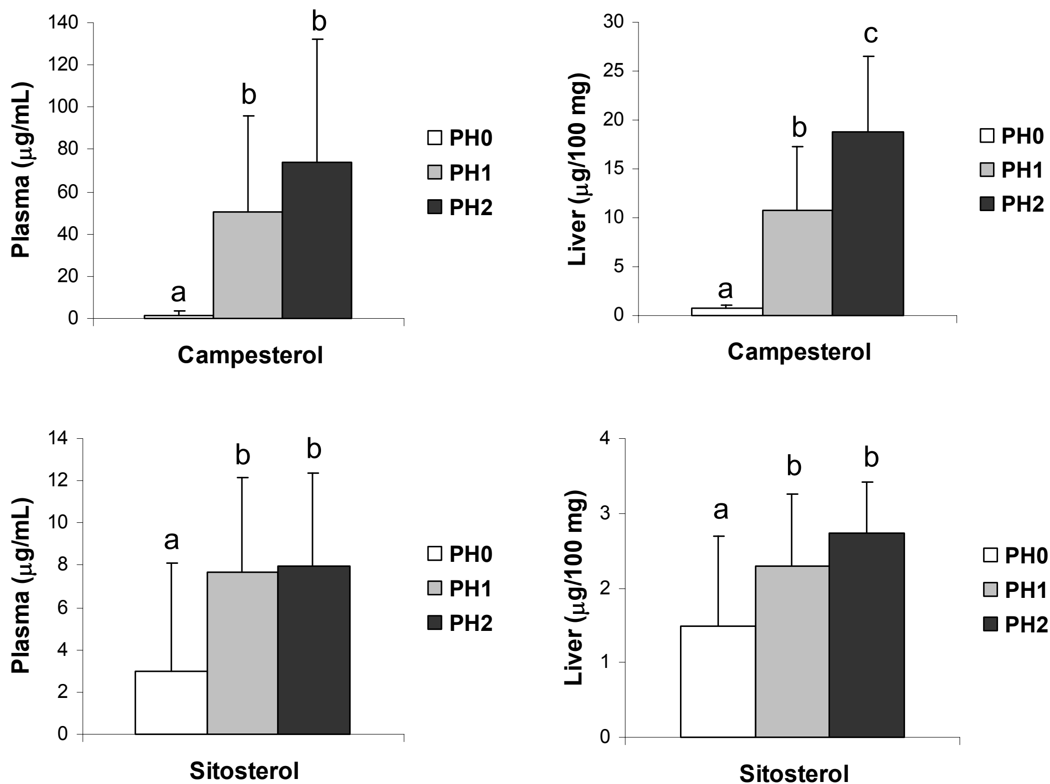

Fig 12. Plant sterol concentrations in liver and plasma
Different letter in the superscript means $p<0.05$.

The high plasma phytosterol concentrations found suggest enhanced cholesterol absorption, since plasma phytosterol concentration is considered as an indicator of cholesterol absorption (Tilvis & Miettinen, 1986; Miettinen & Kesaniemi, 1989; Miettinen *et al.*, 1990; Assmann *et al.*, 2006). However, we did not find differences in plasma cholesterol concentrations, although animals fed with phytosterol-diets showed concentrations slightly higher (no significant) (Fig 13). In contrast, hepatic cholesterol concentration was lower ($p<0.05$), although the reduction observed cannot be attributed to a decrease in cholesterol synthesis, since no difference was found in precursors of cholesterol (lathosterol and desmosterol).

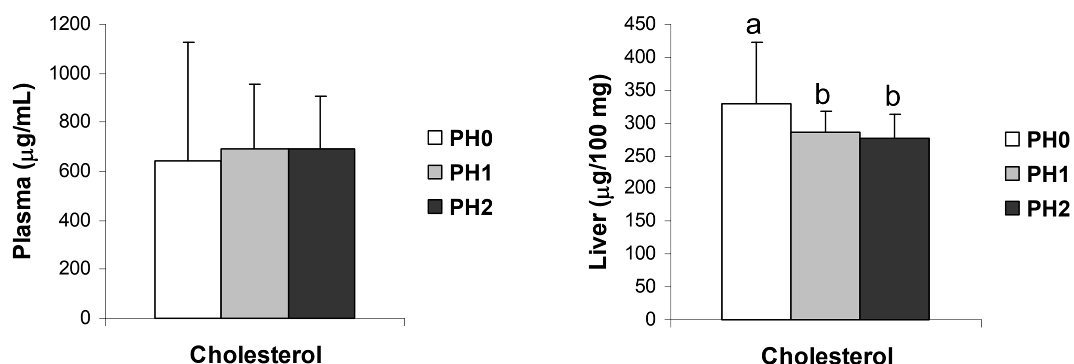

Fig 13. Cholesterol concentrations in plasma and liver

Different letter in superscript means different results ($p<0.05$).

Our results might seem to disagree with most of the studies published (Katan *et al.*, 2003; Korpela *et al.*, 2006) that report plasma cholesterol reductions after phytosterol intake. However, these studies used foods with low content in fat and as it has already been mentioned, we chose a diet enriched with cholesterol and saturated fat, since they are the main compounds of “Western-like” diets (Kris-Etherton *et al.*, 2002; Simopoulos, 2002).

Cholesterol concentrations were highly correlated with the increase in medium chain SFA (especially lauric and myristic) found in our study. An explanation for this may be that plant sterols, when added to this kind of diet, enhance SFA absorption.

Indeed, we have found an increase in the apparent absorption of these FA, positively correlated with their content in liver. This increase in FA concentrations may reduce the transformation of free cholesterol to esterified cholesterol, as well as the expression of LDL receptors (Woollett *et al.*, 1992; Dietschy, 1998), which would provoke a decrease in hepatic cholesterol concentrations. It may be thought that this reduction in the hepatic cholesterol uptake is correlated with an increase on plasma concentrations, but no difference was found in plasma cholesterol concentrations (Fig 13).

Therefore, one of the hypotheses for the lack of hypocholesterolemic effect of plant sterols may be the enhanced absorption of medium chain SFA as well as the reduction in cholesterol output.

3.3 EFFECT OF THE ADDITION OF PECTIN TO A HIGH-SATURATED FAT DIET IN GUINEA PIGS

Pectin is one of the soluble fibers with hypocholesterolemic properties, used in a great number of studies in animals (Fernandez, 1995; Garcia-Diez *et al*., 1996; Shen *et al*., 1998) and humans (Everson *et al*., 1992; Pereira *et al*., 2004). It is thought that this kind of fiber binds bile acids, reducing their re-absorption and also that of cholesterol. However, to achieve hypocholesterolemic effects, high-methylated pectin should be used (Terpstra *et al*., 1998; Dongowski & Lorenz, 2004).

3.3.1 BODY WEIGHT

As with phytosterols, no studies have been conducted to study if pectin also shows some hypocholesterolemic effects when added to a high-saturated fat diet.

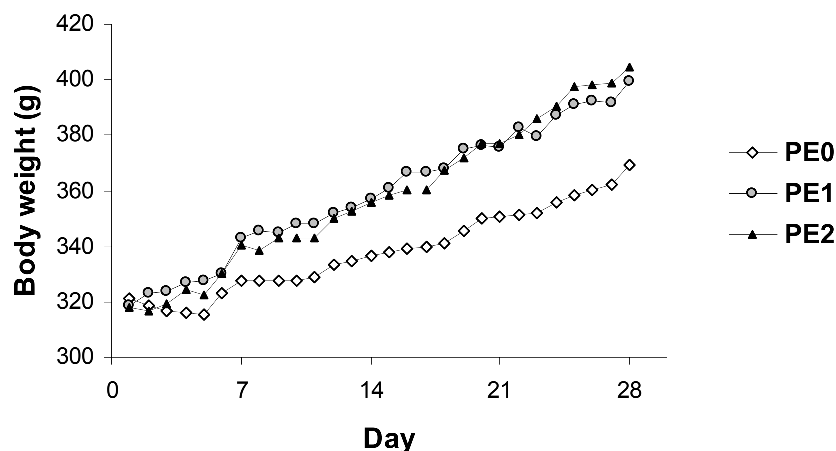

Fig. 14. Mean body weight curves for different pectin levels

We found a negative effect of pectin supplementation on food consumption. The addition of pectin led to higher body weight (Fig. 14), in agreement with Anderson *et al.* (1994), although no difference was found in other studies (Vergara-Jimenez *et al.*, 1998; Roy *et al.*, 2000).

We also found an increase in feed efficiency with high pectin concentrations, which is in disagreement with previous studies (Vergara-Jimenez *et al.*, 1998; Roy *et al.*, 2000). The unexpected increase in feed efficiency and body weight may be due to an early breakdown of pectin, although we used high-methylated pectin, less fermentable than low-methylated pectin (Judd & Truswell, 1985; Gallaher *et al.*, 1993).

3.3.2 SATURATED FATTY ACID CONCENTRATIONS

No treatment effect was found on glucose and triacylglycerol concentrations, or in SFA excretion (Fig. 15). Some authors (Swain *et al.*, 1990) have shown that the effects of dietary fiber intake may counteract the effect of a saturated fat intake and thus reduce CHD events, but others (Ascherio *et al.*, 1996; Hu *et al.*, 2001; Wu *et al.*, 2003) did not find any fiber effect on SFA content in biological samples. A recent study (Kritchevsky & Tepper, 2005) reports a liver weight gain in rats fed with a fiber mixture.

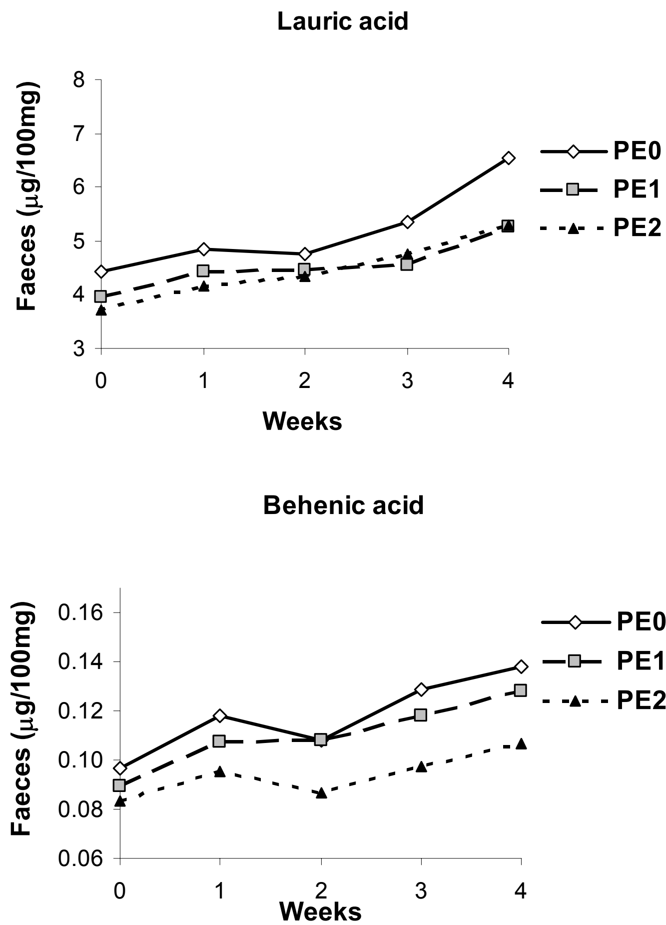

Fig. 15. Faecal concentrations of lauric and behenic acids along the study, for different doses of pectin treatment

We did not find a direct pectin effect on faecal FA concentrations (Fig. 15) or in liver (Fig. 16). However, in plasma the highest concentrations of medium chain SFA were found at intermediate doses of pectin (significant only for myristic) (Fig. 16).

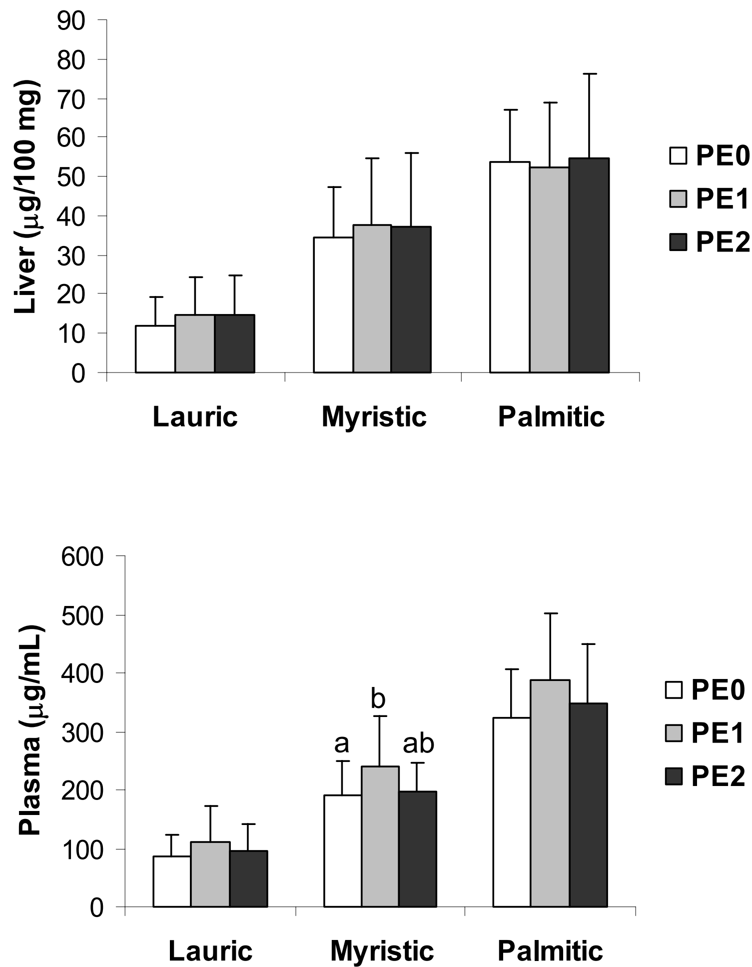

Fig. 16. Fatty acid contents in liver and plasma
Different letter in superscript means $p<0.05$

3.3.3 PHYTOSTEROL AND CHOLESTEROL CONCENTRATIONS

Fiber consumption reduced the excretion of cholesterol, as well as that of campesterol, β-sitosterol and stigmasterol ($p<0.05$). Moreover, bile acids were also decreased after pectin intake ($p<0.05$), but no differences were found in cholesterol metabolites.

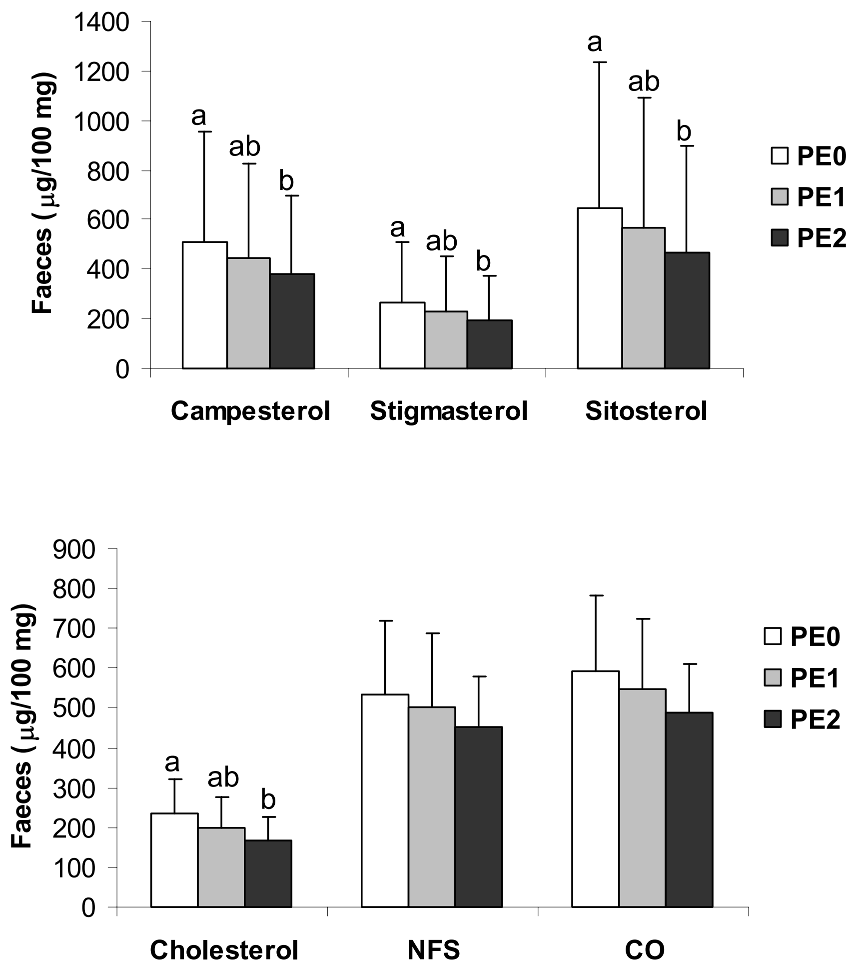

Fig. 17. Faecal concentrations (μg/100 mg) of the main plant sterols and cholesterol
NFS means neutral faecal steroids and *CO* cholesterol output.
Different letter in superscript means significantly different results ($p<0.05$).

Most of the studies reported (Pfeffer *et al*., 1981; Falk & Nagyvary, 1982; Everson *et al*., 1992; Shen *et al*., 1998) showed a decrease in cholesterol absorption and an interruption of enterohepatic cycle of bile acids after pectin supplementation, which provoke a decrease in plasma cholesterol concentration. Nevertheless, we found increases in campesterol and cholesterol in plasma, and in hepatic campesterol in animals fed pectin, the highest concentrations occurring at intermediate doses of pectin (Fig. 18).

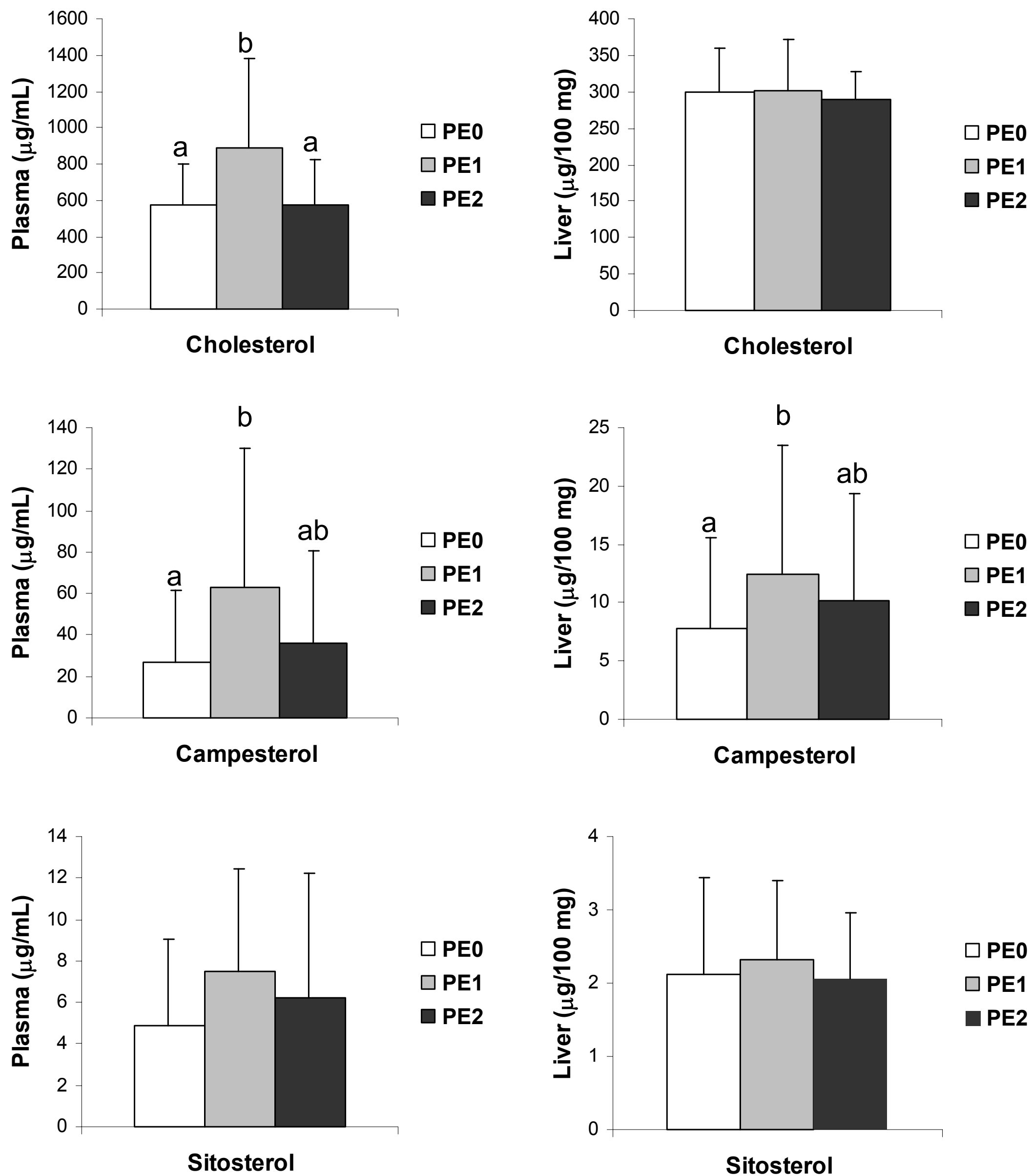

Fig. 18. Cholesterol, campesterol and β-sitosterol concentrations in plasma and liver
Different letter means different results ($p<0.05$).

Our results might be explained taking into account that, in most of the previous studies, pectin was added to a low-fat diet (mainly unsaturated), whereas the diet used in our study was rich in SFA and cholesterol. Some possible interactions between pectin and the source of fat added may have ocurred, since pectin may enhance mucosal proliferation in the lower part of the intestine (Fukunaga *et al.*, 2003), and this

effect may be modulated by the source of fat (Gorbach & Goldin, 1987; Chapkin *et al.*, 1993).

In addition, another explanation would be an early breakdown of pectin, although high-methylated pectin was used. The main support for this hypothesis is that animals fed pectin had higher body weight and feed efficiency than those from the control group. However, since the PE2 dose was almost double than the PE1 dose, it should be expected a higher body weight in the PE2 group, but no difference was found. Therefore, it may be hypothesised that the animals from the PE2 group had some remains of intact pectin in intestine that may show its physiological effects. This would also explain that plasma cholesterol concentrations in PE1 were higher than in PE2.

However, we cannot ascertain whether the mechanism underlying our findings was due to some abnormalities in the quality of pectin, or to some interactions with the fat of diets.

3.4 EFFECT OF THE COMBINATION OF PECTIN AND PHYTOSTEROL ON THE LIPID PROFILE

There are many studies in which the combined effect of several functional ingredients was studied. Phytosterol has been combined with compounds such as glucomannan (Yoshida *et al.*, 2006), soy protein (Lukaczer *et al.*, 2006), vegetable protein and high-viscosity fiber (Jones *et al.*, 2005), and in all these studies the concentrations of cholesterol-LDL were reduced. For instance, Yoshida *et al.* (2006) studied the effects of phytosterol and glucomannan separately and in combination and they reported an additive effect, but not synergic.

In our study, we did not find any additive nor synergic effect of phytosterols and pectin on cholesterol concentrations (data not shown). The only significant interactions were for campesterol and β-sitosterol concentrations in plasma ($p<0.05$). As shown in Fig. 19, in all groups, both campesterol and β-sitosterol concentrations increased with higher doses of phytosterols in diets.

In the control group, the increase of campesterol and β-sitosterol found in plasma was dose-dependent to their amount in diets. In PE2, animals from PH0 and PH1 showed an increase in plant sterol concentrations. However, between PH1 and PH2 no differences were found in campesterol and in β-sitosterol concentrations. In the PE1 group, campesterol concentrations in plasma had a similar profile to what was found in the control group, but the concentrations in this group were higher. In this group, β-sitosterol concentrations were higher in animals fed pectin and phytosterols than with animals fed only phytosterols.

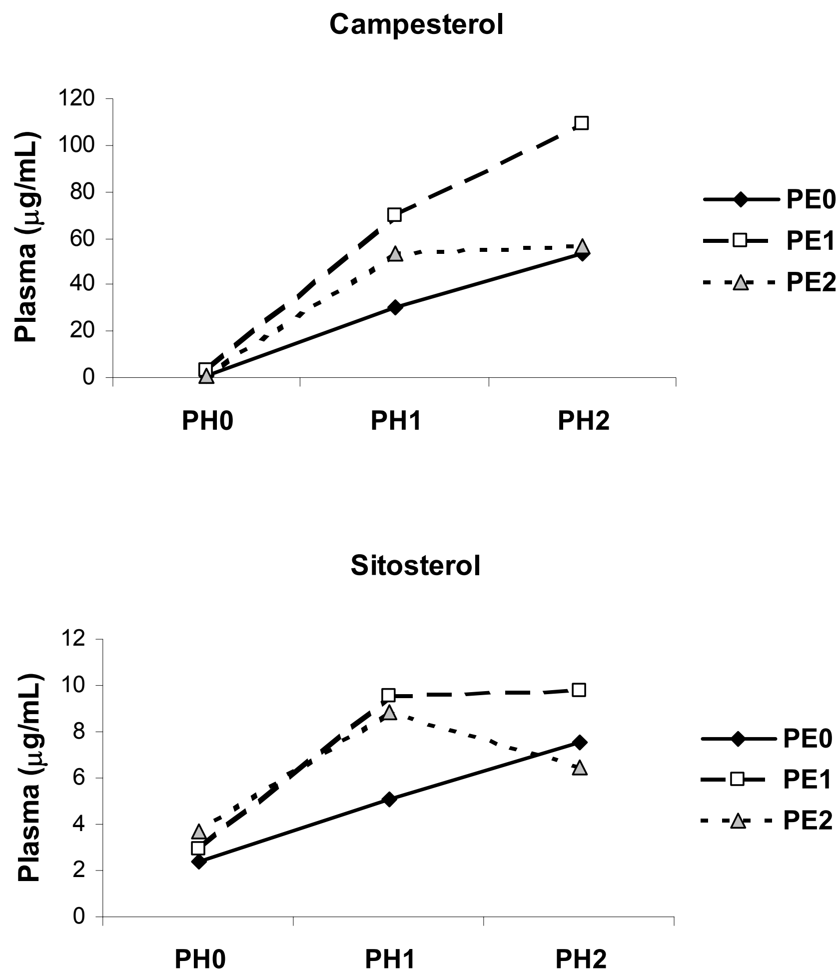

Fig. 19. Campesterol and β-sitosterol concentrations in plasma, for the combination of different doses of pectin and phytosterols

These results support what we already suggested for the pectin effect, that an explanation could be an early breakdown of pectin. Animals from PE2 showed enhanced phytosterol absorption since the concentrations were higher than those of control group. However, no differences were found in phytosterol concentrations between animals from PE2/PH1 and PE2/PH2 since this dose of pectin may show some effect by blocking plant sterol absorption.

PE1 enhanced the absorption of phytosterols, since the graphs for PE1 and PE0 are close related, although animals from PE1 showed the highest concentrations of campesterol and β-sitosterol in plasma. However, comparing the results between campesterol and β-sitosterol, some differences can be seen. While campesterol increased dose-dependent between PH0, PH1 and PH2, β-sitosterol showed similar concentrations in animals from PE1/PH1 and PE1/PH2, maybe due to that the campesterol is better incorporated in the animal body than β-sitosterol.

To conclude, in our study, the addition of pectin to saturated diets supplemented with phytosterols enhanced the absorption of phytosterols. Nevertheless, this effect was more important at intermediate doses of pectin, since in the highest dose of pectin, there might be some pectin left in the intestine that showed some activity by blocking the absorption of these compounds.

FINAL CONCLUSIONS

Several conclusions can be made from the work of this thesis:

- The addition of plant sterols esterified with UFA to a diet rich in high-saturated fat and cholesterol, modified the absorption of SFA, depending on the chain length. The excretion of medium-chain SFA (lauric and myristic) was reduced, whereas that of long-chain SFA (behenic and arachidic) was increased.

- A saturated diet with esterified plant sterols led to an increase in medium chain SFA concentration in liver and a decrease in plasma, suggesting an accumulation of lauric and myristic acids in liver.

- Animals fed with phytosterols showed an increase cholesterol excretion, although cholesterol output decreased, suggesting an increase in absorption of compounds from cholesterol metabolism.

- Animals fed phytosterols showed a decrease in hepatic cholesterol concentrations compared with animals from control group. This reduction was not due to a reduced synthesis since cholesterol precursors did not show any difference between treatments. Plasma cholesterol concentrations did not show differences between treatments.

- Pectin provoked an increase in body weight, suggesting an early breakdown of pectin. However, although in the PE2 group the concentration of pectin was double than in the PE1, no difference was found in body weight between the two groups, suggesting that part of the pectin in PE2 could reach the intestine and show its physiological effects.

- Animals from the pectin-groups presented lower cholesterol and plant sterol excretion, with the highest values at intermediate doses of pectin.

- Animals from intermediate dose of pectin (PE1) showed the highest cholesterol concentrations in plasma. No differences were found in hepatic cholesterol levels.

- Animals fed pectin showed the highest concentrations of campesterol in liver and plasma, finding the highest in animals fed intermediate doses.

- No pectin effect was found on FA excretion.
- No synergic effect of pectin and phytosterol was found in cholesterol levels.
- The only interactions found between phytosterols and pectin were in campesterol and β-sitosterol concentrations in plasma. Their concentrations were enhanced by intermediate doses of pectin, supporting the hypothesis that an early hydrolysis occurred.

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APPENDIX

OTHER PUBLICATIONS

Other papers are produced during the PhD process:

- Bakery products enriched with phytosterols, α-tocopherol and β-carotene. Sensory evaluation and chemical comparison with market products
  Joan Quílez, Joan Antoni Ruiz, **Gemma Brufau** & Magda Rafecas
  Food Chemistry (2006) 94; 300-405.
  Impact Factor (2005): 1,811

- Nuts: source of energy and macronutrients
  Gemma Brufau, Josep Boatella & Magda Rafecas
  British Journal of Nutrition (2006) *Accepted*
  Impact Factor (2005): 2,967

- Evaluation of lipid oxidation after ingestion of bakery products enriched with phytosterols, β-carotene and α-tocopherol
  Gemma Brufau, Joan Quílez, Miguel Angel Canela, *et al.*
  Clinical Nutrition (2004) 23; 1390-1397
  Impact Factor (2005): 2,296

- Bakery products enriched with phytosterol esters, α-tocopherol and β-carotene decrease plasma LDL-cholesterol and maintain β-carotene concentrations in normocholesterolemic men and women.
  Joan Quílez, Magda Rafecas, **Gemma Brufau**, *et al.*
  Journal of Nutrition (2003) 133; 3103-3109
  Impact Factor (2005): 3,689

- Effect of the consumption of pectin and phytosterols on sterol levels supplemented to a saturated diet in guinea pigs
  Gemma Brufau, Miguel Angel Canela, Joan Quílez & Magda Rafecas
  Atherosclerosis Supplements (2006) 7; S451.
  Impact Factor (2005): 8,963

- Fatty acid profile after plant sterol and pectin consumption enriched into a saturated diet in guinea pigs
  Magda Rafecas, **Gemma Brufau**, Miguel Angel Canela & Rafael Codony
  Atherosclerosis Supplements (2006) 7; S451.
  Impact Factor (2005): 8,963

- A stanol yoghurt drink alone or combined with a low-dose OTC statin lowers non-HDL, but also lowers TGS & elevates HDL in metabolic syndrome patients
  Jogchum Plat, **Gemma Brufau**, Margreet Dasselaar & Ronald P Mensink
  Atherosclerosis Supplements (2006) 7; S452.
  Impact Factor (2005): 8,963

CONGRESS COMMUNICATIONS

International congresses

- Phytosterols: recent insights
  M. Rafecas, **G. Brufau**, R. Codony
  4th. Euro Fed Lipid Congress – Fats, Oils and Lipids for a Healthier Future
  Madrid 01-04/10/06
  Oral Communication

- Effect of the consumption of pectin and phytosterol on sterol levels supplemented to a saturated diet in guinea pigs
  G. Brufau, M.A. Canela, J Quílez, M. Rafecas
  XIV International Symposium on Atherosclerosis
  Roma 18-22/06/06
  Poster

- Fatty acid profile after plant sterols and pectin consumption enriched into a saturated diet in guinea pigs
  M. Rafecas, **G. Brufau**¸ M.A. Canela, R. Codony
  XIV International Symposium on Atherosclerosis
  Roma 18-22/06/06
  Poster

- A stanol yogurt drink alone or combined with a low-dose OTC statin lowers non-HDLc, but also lowers TGs and elevates HDLc in metabolic syndromes patients
  J. Plat, **G. Brufau**, M. Dasselaar, R.P. Mensink
  XIV International Symposium on Atherosclerosis
  Roma 18-22/06/06
  Poster

- Determination of sterol content in liver and feces by capillary gas-chromatography
  G. Brufau, R. Codony, M. Rafecas
  26th. ISF World Congress “Modern Aspects of Fats and Oils – A Fascinating source of knowledge”
  Praga 25-28/09/05
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- A rapid method to determine plant sterols in human plasma
  G. Brufau, M.A. Canela, H Agell, R. Codony, M. Rafecas
  3rd. EuroFed Congress’ Oils, Fats and Lipids in a Changing World
  Edinburgh 5-8/09/04,
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<u>*National congresses*</u>

- Efecto de la bollería industrial enriquecida con fitosteroles, α-tocopherol y β-caroteno sobre el perfil lipídico plasmático en individuos sanos
  I. Megías, J. Quílez, P. García-Lorda, M. Bulló, J.A., Ruiz, **G. Brufau**, M. Rafecas, J.A. Salas-Salvadó
  XIX Congreso Nacional de la Sociedad Española de Nutrición Parenteral y Enteral
  Madrid 14-16/05/03
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- Productos de bollería enriquecidos con fitosteroles: un alimento funcional para rebajar el nivel de cholesterol plasmático
  M. Rafecas, **G. Brufau**, I. Megías, R. Balançà, J. Quílez, J. Ruiz
  V Congreso de la Sociedad Española de Nutrición Comunitaria
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EFECTE DE LA INGESTA DE FITOSTEROLS I PECTINES SOBRE EL PERFIL LIPÍDIC EN CONILLS D’ÍNDIES

Resum de la memòria presentada per **Gemma Brufau Donés**, per optar al títol de Doctor en Farmàcia

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Barcelona, 28 de Setembre del 2006

ABREVIACIONS

ACAT	Acil-coenzima A: colesterol aciltransferasa
AG	Àcids grassos
AGCC	Àcids grassos de cadena curta
AGI	Àcids grassos insaturats
AGMI	Àcids grassos monoinsaturats
AGPI	Àcids grassos poliinsaturats
AGS	Àcids grassos saturats
AGt	Àcids grassos trans
AHA	American Heart Association
Apo	Apolipoproteïna
CETP	Proteïna transferidora d'ésters de colesterol
GM	Grau de metilació
HDL	Lipoproteïna d'alta densitat (*high density lipoprotein*)
HMG-CoA	3-hidroxi-3-metilglutaril-coenzima A
INC	Instituto Nacional de Consumo
LCAT	Lecitin: colesterol acil transferasa
LDL	Lipoproteïna de baixa densitat (*low density lipoprotein*)
Lp	Lipoproteïna
LPL	Lipoprotein lipasa
NCEP	National Cholesterol Education Program
VLDL	Lipoproteïna de molt baixa densitat (*very-low density lipoprotein*)

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INTRODUCCIÓ

1 MODULACIÓ DE LES CONCENTRACIONS SANGUÍNIES DE COLESTEROL A PARTIR DE LA DIETA

Han estat descrits un gran nombre de factors nutricionals capaços d'influir sobre diversos factors de risc cardiovascular, com ara els lípids plasmàtics (Hu i col., 2001), la pressió sanguínia i els pes corporal (De Backer i col., 2003). Per exemple, un consum elevat de greix, ric en àcids grassos saturats i *trans*, així com també elevades quantitats de colesterol en la dieta s'associen amb un elevat risc de patir malalties cardiovasculars (Heinonen i Piironen, 1991; Johansson i col., 1996; Dietschy, 1998; Elmstahl i col., 1999; De Roos i col., 2001; Dixon i Ernst, 2001), mentre que el consum de greix ric en àcids grassos insaturats, així com el de fibra alimentaria s'associa amb una reducció en risc cardiovascular (Liu i col., 2002). A més a més, el consum de seleni i de vitamines antioxidants, com la vitamina A, C i E, també s'associa amb reduccions en el risc de patir malalties cardiovasculars (Gerber i col., 2000; Osler i col., 2001).

Les recomanacions nutricionals a España (Serra-Majem i col., 2001) (Taula 1) aconsellen reduir el consum total de greix, però especialment es recomana reduir el consum de greix ric en àcids grassos saturats i el de colesterol (Mann, 1987; Grundy i Denke, 1990; Grundy i col., 2002). En els següents apartats, es discutiran alguns components de la dieta que poden modular el perfil lipoproteic.

Taula 1. Situació actual del consum diari a España

	Recomanacions nutricionals (Serra-Majem i col., 2001)	Ingesta real (INC*, 2006)
Energia (Kcal)	---	2634
Contingut hidrats de carboni (% energia)	50-55	41.9
Contingut proteïna (% energia)	---	14.2
Contingut lípids (% energia)	30-35	41.5
- Contingut AGS (% energia)	7-8	11.9
- Contingut AGMI (% energia)	15-20	18.9
- Contingut AGPI (% energia)	5	6.8
Colesterol (mg/dia)	<300	440
Fibra total (g)	>25	21
- Fibra insoluble (g)	---	12.4
- Fibra soluble (g)	---	8.0

* Instituto Nacional de Consumo

Colesterol

El colesterol de la dieta té molt poc efecte en la modificació de les seves concentracions sanguínies. No obstant, un elevat consum pot provocar increments en les concentracions de colesterol total i colesterol-LDL (Mattson i col., 1972; Beynen i Katan, 1985; Howell i col., 1997). Aquest increment en la colesterolèmia és degut al fet que un increment en el colesterol hepàtic suprimeix els receptors per a les partícules LDL en el fetge.

La magnitud d'aquest efecte varia entre individus, donat que hi ha una gran variació interindividual en l'absorció de colesterol i en la seva homeostasi hepàtica (Beynen i Katan, 1985; Katan i col., 1986; Katan i Beynen, 1987). Les recomanacions nutricionals de les organitzacions americanes *"American Heart Association"* (AHA) i *"National Cholesterol Education Program"* (NCEP) recomanen reduir el consum de colesterol a menys de 300 mg/d en individus sans i menys de 200 mg/d en individus hipercolesterolemics i en aquells amb alguna malaltia coronària preexistent (Expert Panel on Detection, 1993; Krauss i col., 2000).

Àcids grassos saturats

Els àcids grassos saturats (AGS) incrementen les concentracions sanguínies de colesterol, de manera molt més marcada que el propi colesterol de la dieta (Elmstahl i col., 1999). No obstant, aquest efecte varia notablement en funció de la longitud de la cadena dels àcids grassos (Aro i col., 1997; Ginsberg i col., 1998). Per exemple, els àcids grassos de cadena curta (AGCC) com el butanoic (4:0), hexanoic (6:0), octanoic (8:0) i l'AG de cadena mitja decanoic (10:0), són oxidats ràpidament en el fetge a acetil CoA. Aquests AG no modifiquen ni la composició lipídica en el fetge, ni la concentració de colesterol lliure o esterificat en els hepatòcits. Per tant, aquests AG són biològicament neutres, respecte a la regulació de les concentracions de colesterol. A més, l'àcid esteàric (un AG de cadena llarga amb 18 àtoms de carboni) també pertany a aquest grup (Kris-Etherton i Yu, 1997).

Per altra banda, els àcids grassos làuric (12:0), mirístic (14:0) i palmític (16:0) incrementen la concentració de colesterol-LDL en el torrent sanguini (Temme i col., 1996; Kris-Etherton i Yu, 1997), donat que inhibeixen l'activitat del receptor de les LDL en el fetge (Woollett i col., 1992; Dietschy, 1998) i que estimulen la producció de colesterol-LDL.

El mecanisme per a explicar com aquests AG modifiquen les concentracions de colesterol és que una vegada aquests AG es troben en el fetge, redueixen la transformació de colesterol no-esterificat a colesterol esterificat. Tenint en compte que l'expressió dels receptors LDL està regulada per la concentració de colesterol no-esterificat, concentracions elevades d'aquests AGS disminueixen l'expressió dels receptors hepàtics de les LDL, incrementant per tant, les concentracions sanguínies de colesterol (Woollett i col., 1992; Dietschy, 1998).

Les recomanacions de l'AHA proposen limitar el consum de greix ric en àcids grassos saturats a menys d'un 10% del contingut energètic total en individus sans (Expert Panel on Detection, 1993; Krauss i col., 2000). No obstant, el límit es situa en un 7% en individus amb concentracions elevades de colesterol-LDL o en individus amb altres factors de risc cardiovascular (Expert Panel on Detection, 1993). Tot i que s'han portat a terme diverses campanyes per a mostrar a la població que el consum de dietes riques en greixos amb elevat contingut en àcids grassos saturats és un factor de risc molt important per a patir malalties cardiovasculars, el consum segueix sent molt

elevat (Instituto Nacional de Consumo, 2006). Per tant, sembla obvi que modificar els hàbits alimentaris en la població és una tasca difícil.

Àcids grassos insaturats

Les tres principals sèries homòlogues dels àcids grassos insaturats (AGI) són els àcids grassos monoinsaturats (AGMI) de la sèrie n-9, i els àcids grassos poliinsaturats (AGPI) de les sèries n-6 i n-3. S'ha vist en diversos estudis que quan es substitueixen els AGS per AGI, les concentracions de colesterol total i colesterol-LDL es redueixen (Hegsted i col., 1965; Mensink i Katan, 1992; Howard i col., 1995). Aquests efectes es podrien explicar pel simple fet que amb la substitució es redueix el contingut en AGS en la dieta. No obstant, en un gran nombre d'estudis s'ha demostrat que aquests AG tenen efectes per si mateixos (Howard i col., 1995).

S'ha suggerit que els AGMI, especialment l'àcid oleic ($C_{18:1n\text{-}9,\ cis}$), redueix les concentracions de colesterol a través de dos mecanismes. Per un banda, incrementa la captació hepàtica de partícules LDL donat que bloqueja la depressió provocada per concentracions elevades de colesterol. Per l'altra banda, incrementa l'activitat de l'enzim CETP, el qual incrementa la concentració intracel·lular de colesterol esterificat (Kurushima i col., 1995).

També s'han descrit dos mecanismes que podrien explicar els efectes hipocolesterolemiants dels AGPI n-6. Aquests AG incrementen l'expressió dels receptors hepàtics de les LDL, així com també l'activitat de l'enzim 7α-hidrolasa (enzim responsable de la conversió de colesterol a àcids biliars) (Fernandez i West, 2005).

Finalment, els olis rics en AGPI n-3, també poden reduir les concentracions sanguínies de colesterol i triacilglicerols, donat que aquests AG poden actuar en diferents rutes metabòliques. Aquests AG redueixen la lipogènesi i la secreció de partícules VLDL; incrementen l'activitat de la lipoprotein lipasa (LPL) i estimulen el transport revers del colesterol a través de les lipoproteines HDL (Nestel, 2000; Fernandez i West, 2005).

No obstant, el consum d'AGPI per sobre les recomanacions nutricionals, podria suposar un risc per a la salut donat que aquests AG són més oxidables que els menys

insaturats. De totes maneres, aquest efecte no ha estat descrit quan el consum d'aquests AG forma part d'una dieta equilibrada (Kratz i col., 2002).

Àcids grassos trans

Un gran nombre d'estudis epidemilògics han descrit associacions entre els àcids grassos *trans* (AGt) i la mortalitat deguda a malalties coronàries tant en els Estats Units com a Europa (Willett i col., 1993; MacDonnald i col., 1995; Ascherio i col., 1996; Hu i col., 1997; Pietinen i col., 1997; Oomen i col., 2001). Aquests AG poden incrementar les concentracions de colesterol-LDL (Lichtenstein i col., 1993; Judd i col., 1994) i reduir les de colesterol-HDL (Mensink i Katan, 1990; Judd i col., 1994), en comparació amb un consum isocalòric d'AGI *cis*. A més a més, els AGt incrementen la concentració de lipoproteina(a) [Lp(a)] (Nestel i col., 1992), un important factor de risc cardiovascular (Utermann, 1989). Els AGt també incrementen les concentracions de triacilglicerols (Stampfer i col., 1996). També alteren el metabolisme dels AG essencials (linoleic i linolènic) i l'equilibri de prostaglandines. Finalment, estudis recents suggereixen que el consum d'AGt està associat amb un increment en la incidència de la diabetis tipus 1 (Lovejoy, 1999).

Greix total i hidrats de carboni

Els estudis epidemiològics dels últims 25 anys han demostrat que el nivell de greix aportat per la dieta està relacionat de forma positiva amb un increment en la mortalitat deguda a malalties cardiovasculars (Dietschy, 1998). És per aquesta raó, que una de les principals recomanacions consisteix a reduir el consum de greix total, especialment el consum de greix ric en àcids grassos saturats.

A més a més, també es recomana disminuir el consum d'hidrats de carboni simples (com ara sucres) (De Lorgeril i col., 1999). Les dietes amb baix contingut amb greix i amb un alt contingut en hidrats de carboni poden provocar efectes potencialment adversos, especialment en pacients diabètics tipus 2. Per exemple, aquest tipus de dietes poden incrementar els triacilglicerols plasmàtics, i reduir les concentracions de colesterol-HDL. També poden modificar la mida i la densitat de les partícules LDL, que esdevenen més petites i denses, i per tant més aterogèniques (Krauss i Dreon, 1995; Krauss, 1998; Grundy, 1999). De totes maneres, aquests

efectes adversos poden ser contrarestats amb la substitució parcial dels hidrats de carboni per greixos rics en AGI (Mensink i Katan, 1987).

2 ALIMENTS FUNCIONALS

Tal i com s'ha comentat en la secció prèvia, una gran part de la població en els països desenvolupats (Grundy i col., 2002), Espanya inclosa (Capita i Alonso-Calleja, 2003), hauria de disminuir el consum de greix total, i especialment el consum d'AGS, AGt i colesterol, per a reduir el risc de patir malalties cardiovasculars. Encara que s'haurien de modificar els hàbits alimentaris, els aliments funcionals poden esdevenir una eina molt útil per a reduir la incidència de les malalties cardiovasculars. No obstant, cal tenir present que els aliments funcionals s'haurien de consumir com a part d'una dieta equilibrada, i no per a contrarestar els efectes indesitjables d'una dieta no saludable.

Una de les definicions d'aliment funcional és: "Un producte alimentari que pot ser considerat com a funcional si s'ha demostrat satisfactòriament que posseeix efectes beneficiosos en una o més funcions fisiològiques, a més dels efectes nutricionals convencionals, essent importants per a millorar l'estat de salut humà i/o reduir el risc de patir certes malalties" (Contor, 2001).

En les següents seccions es resumiran els coneixements actuals sobre els fitosterols i la pectina (fibra soluble), dos components utilitzats com a ingredients funcionals per a millorar el perfil lipoproteic.

2.1 ESTEROLS I ESTANOLS VEGETALS

Ja en els anys 50 (Peterson, 1958), es va descriure que el consum de fitosterols estava relacionat amb reduccions en les concentracions de colesterol plasmàtic, però no va ser fins als anys 90 quan es va pensar que seria interessant utilitzar-lo com a ingredient funcional (Weststrate i Meijer, 1998; Hendriks i col., 1999).

Aquests productes es troben en els vegetals de forma natural, però també es poden obtenir durant el procés de refinat dels olis vegetals, concretament en el procés de desodorització. Al parlar de fitosterols s'entén tant els esterols tradicionals (els més

representatius són el β-sitosterol, l'estigmasterol i el campesterol), com els seus equivalents saturats anomenats estanols. Aquests últims són obtinguts per hidrogenació de les formes insaturades o a partir de matèries primeres com l'oli de fusta ("*tall-oil*").

La quantitat de fitosterols aportada per la dieta depèn del major o menor consum de greixos o olis vegetals i es situa entre els 170 i els 360 mg/persona/dia.

Els fitosterols han estat descrits com a agents capaços de reduir les concentracions sanguínies de colesterol-LDL en un 8-10% quan es consumeixen amb dosis entre un 1.5 i 3 g/dia, tant en individus normocolesterolèmics (Mensink i col., 2002; Quílez i col., 2003), en hipercolesterolèmics (De Graaf i col., 2002; Gylling i Miettinen, 2002), en pacients diabètics (Lau i col., 2005) com en nens (Amundsen i col., 2002; 2004). La majoria d'estudis han utilitzat margarines com a suport i els esterols esterificats amb àcids grassos, ja que la seva solubilitat en greixos augmenta, permetent dissolucions relativament concentrades. No obstant, últimament els fitosterols també s'han afegit a productes amb baix contingut en greix com per exemple en iogurts amb baix contingut en greix (Mensink i col., 2002).

S'han proposat diferents mecanismes per a explicar els seus efectes hipocolesterolemiants, tot i que el més àmpliament acceptat es basa en la baixa absorció d'aquests compostos a nivell intestinal (colesterol: 50%, β-sitosterol: <5%; sitostanol: pràcticament no s'absorbeix) (Ostlund i col., 2002). Aquests compostos poden desplaçar el colesterol de les micel·les intestinals, fet que provoca una reducció en la seva absorció i un increment en la seva excreció fecal. Aquest fet provoca una disminució del colesterol hepàtic i una retirada de les partícules LDL en sang.

És per totes aquests raons que es considera que l'ús dels fitosterols pot ser una eina molt útil per a reduir les concentracions de colesterol plasmàtic i per tant, reduir el risc de patir malalties cardiovasculars.

2.2 PECTINA

El concepte de fibra alimentaria ha evolucionat molt al llarg del temps, a mesura que ha augmentat el coneixement de les seves característiques, propietats fisiològiques i s'han desenvolupat mètodes adients per a determinar-la. Una definició

de *fibra alimentaria* seria "la part d'hidrats de carboni no digeribles i lignina que són intrínsecs i intactes a les plantes". Una altra definició seria per la *fibra funcional* que consisteix en "els hidrats de carboni aïllats i no digeribles que tenen efectes fisiològics funcionals en els humans". Finalment, la *fibra total* seria la suma de la fibra alimentaria i la fibra funcional (IOM, 2001).

En general, s'accepta que els efectes de la fibra insoluble es limiten a nivell intestinal, actuant com a agents laxants i incrementant el volum de les femtes. En canvi, les fibres solubles d'alta viscositat (com la pectina) són agents hipocolesterolemiants i hipoglucèmics. A més a més, la fibra soluble posseeix altres efectes derivats del fet que es fermenta en el colon generant àcids grassos de cadena curta (AGCC). Concretament, els AGCC majoritaris que es generen són els àcids acètic, propiònic i el butíric.

Les fibres solubles, entre elles la pectina, han estat descrites com agents amb capacitat hipocolesterolemiant, essent eficaces en la reducció de les concentracions sanguínies de colesterol-LDL. El mecanisme d'acció primari acceptat es deu a la seva capacitat per a lligar les sals biliars, fet que provoca un increment en la seva excreció i la interrupció del cicle enterohepàtic. Això incrementa la síntesi d'àcids biliars a expenses del colesterol hepàtic, el qual disminueix de manera significativa. Paral·lelament i per aquesta raó, s'incrementa l'activitat de la HMG-CoA reductasa i el nombre de receptors en membrana per a l'apoB i l'apoE, fet que implica una retirada del colesterol lligat a les LDL en sang.

Encara que hi ha certa variabilitat en l'eficàcia hipocolesterolemiant d'aquest tipus de fibra soluble, la majoria d'estudis han demostrat que després d'ingestes de 10 g/dia, s'aconsegueixen reduccions en les concentracions de colesterol total entre 5-10% i les de colesterol-LDL entre un 8 i un 15% (Haskell i col., 1992; Brown i col., 1999). Un altre efecte addicional de la pectina és la capacitat de reduir la glucèmia en persones amb hiperglucèmia (Giacco i col., 2002).

Per a que la pectina sigui un eficaç agent hipocolesterolemiant i hipoclucèmic, ha de tenir unes determinades característiques fisico-químiques. El grau de metilació (GM) és defineix com el grau d'esterificació dels grups carboxil de la pectina amb metanol (Dongowski i col., 2004). Són necessàries pectines molt metilades (amb un GM superior al 50%) per a observar efectes fisiològics.

En la Taula 1 es pot observar que el consum de fibra a Espanya, especialment de fibra soluble, és insuficient; per tant la seva addició en aliments funcionals seria en principi favorable.

OBJECTIUS

Donat que tant els fitosterols com la pectina han estat descrits com agents capaços de reduir les concentracions de colesterol total i colesterol-LDL, es va pensar que seria interessant estudiar els efectes de l'addició dels dos components de manera conjunta, donat que al mercat hi ha un gran nombre de productes enriquits amb algun d'aquests compostos. Si els aliments funcionals enriquits amb fitosterols i pectines tinguessin efectes sinèrgics, es podrien reduir les dosis necessàries per aconseguir els efectes desitjats. Per tant, el principal objectiu d'aquesta tesi va ser **estudiar en el perfil lipídic, la possible sinèrgia d'una dieta amb alt contingut en àcids grassos saturats, en la qual s'hi han addicionat fitosterols i pectines, en conills d'índies. Els resultats es compararan amb els efectes dels fitosterols i la pectina de forma separada.**

La dieta utilitzada tenia un alt contingut en AGS i colesterol degut a diverses raons. Primer, amb l'elecció d'aquesta dieta es va voler simular la situació real als països occidentals, donat que la majoria de la població en aquests països no segueix les recomanacions nutricionals, on el consum de greix, especialment el de greix saturat i colesterol és elevat (Taula 1) (Kris-Etherton i col., 2002; Simopoulos, 2002). Segon, el consum dels aliments funcionals s'hauria de fer sempre per a compensar una dieta saludable, i no per a contrarestar els efectes d'una dieta no saludable. No obstant, els aliments funcionals estan amplament utilitzats en països on el consum de greix saturat i colesterol és alt. Finalment, fins al moment, no existeixen estudis que hagin estudiat els efectes d'aquests ingredients funcionals quan la dieta base és rica en greix saturat i colesterol.

El model animal escollit va ser el conill d'índies, donat que ha estat proposat com un dels millors models animals per estudiar el metabolisme del colesterol i de les lipoproteines en humans per diverses raons (Fernandez i Volek, 2006). (i) Tenen elevades proporcions LDL/HDL. (ii) En el fetge, tenen concentracions més elevades de colesterol lliure que de colesterol esterificat. (iii) Tenen diversos enzims comuns amb els humans, com són la proteïna transferidora d'ésters de colesterol (CETP), la lecitin colesterol acil transferasa (LCAT) i la lipoprotein lipasa (LPL). (iv) Tenen similars proporcions de síntesi i catabolisme del colesterol. (v) Tal i com ocorre en els humans,

el domini dels receptors de les LDL pel reconeixement de l'apolipoproteina B-100, diferencia entre receptor normal i receptor defectuós degut a causes congènites. (vi) L'edició de l'RNA missatger per la apoB en el fetge és negligible, comparat amb altres espècies. (vii) Necessiten vitamina C aportada amb la dieta. (viii) Les femellas de conills d'índies tenen concentracions de HDL més elevades que els mascles. (ix) Les femelles de conills d'índies en els quals se'ls ha extret el sistema reproductiu tenen un perfil lipídic similar a les dones post-menopàusiques. (x) Durant l'exercici físic, les concentracions plasmàtiques de triacilglicerols plasmàtics disminueixen i les de colesterol-HDL incrementen. (xi) Els conills d'índies responen a intervencions alimentaries i tractaments farmacològics, reduint les concentracions de colesterol-LDL.

A partir de l'objectiu principal proposat, van sorgir diversos objectius secundaris:

- Estudiar la influència d'una dieta altament saturada enriquida amb fitosterols, sobre el perfil lipídic en plasma, fetge i femtes.
- Estudiar els efectes d'una dieta altament saturada enriquida amb pectina, sobre el perfil lipídic en plasma, fetge i femtes.
- Estudiar la possible sinèrgia del consum de una dieta altament saturada enriquida amb fitosterols i pectina.

Finalment, per poder assolir aquests objectius específics, va sorgir un nou objectiu:

- Validar un mètode analític per a determinar el contingut d'esterols vegetals i colesterol en mostres de fetge.

SECCIÓ EXPERIMENTAL

1 DISSENY EXPERIMENTAL

El disseny experimental va ésser tal i com es descriu a continuació:

Animals

Els animals es van mantenir al sistema d'estabulació de la Facultat de Farmàcia (Universitat de Barcelona). Les pràctiques de cria i utilització dels animals d'experimentació es van realitzar d'acord amb l'article 5 de la "Llei de protecció dels animals utilitzats per l'experimentació i per altres finalitats científiques" de la Generalitat de Catalunya (Llei 5/1995, 21 de juny). Tots els procediments van ser aprovats pel Comitè Ètic d'Experimentació Animal de la Universitat de Barcelona.

Per a estudiar els efectes dels diferents tractaments en el perfil lipídic, es va utilitzar un disseny factorial 3x3. Es van utilitzar 72 femelles de conill d'índies Dunkin Hartley, les quals van ser assignades aleatòriament als diferents tractaments (8 animals per grup). Es van mantenir dos animals per gàbia en un cicle de llum de 12 hores (llum des de les 08:00 a les 20:00), sota condicions controlades d'humitat i temperatura.

La duració total de l'estudi va ser de 5 setmanes. Abans de començar, tots els animals van rebre el pinso control, sense ni pectina (PE0) ni fitosterols (PH0) afegits, durant una setmana, amb la finalitat que els animals es familiaritzessin amb la dieta base. Després d'aquesta setmana, els animals van ser assignats a un grup i es van mantenir amb aquesta dieta durant 4 setmanes, amb lliure accés a l'aigua i a l'aliment. Es va controlar el pes corporal, el consum d'aigua i de pinso durant tot l'estudi.

Dietes

Es van utilitzar tres nivells de fitosterols (0, 1.37 i 2.45%, p/p) i tres nivells de pectina (0, 3.67 i 6.93%, p/p) (Taula 2). Es van escollir aquestes dosis d'acord amb estudis ja publicats.

Els fitosterols utilitzats estaven esterificats amb AGI, donat que l'esterificació i d'aquests compostos incrementa la seva eficàcia i la facilitat per incorporar aquests ingredients en la matriu de l'aliment (Weststrate i Meijer, 1998; Hendriks i col., 1999). La pectina utilitzada va ser "*GENU-type Freeze pectin*", amb més d'un 50% de grups carboxils metilats. Es va escollir pectina amb un alt grau de metilació donat que és necessari per aconseguir efectes hipocolesterolemiants (Gallaher i col., 1999; Dongowski i col., 2002). Aquests ingredients funcionals es van afegir a una dieta base la qual tenia un alt contingut en AGS, a fi de simular la dieta consumida en la majoria de països desenvolupats. Amb aquest objectiu, es va afegir un 0.33% (p/p) de colesterol a totes les dietes.

Taula 2. Composició de les dietes

	Ingredients[a] (g/100g)								
Dietes	**PE0/PH0**	**PE0/PH1**	**PE0/PH2**	**PE1/PH0**	**PE1/PH1**	**PE1/PH2**	**PE2/PH0**	**PE2/PH1**	**PE2/PH2**
Pectina	0	0	0	3.67	3.67	3.67	6.93	6.93	6.93
Fitosterols[b]	0	1.27	2.45	0	1.27	2.45	0	1.27	2.45
Proteïna	18.3	18.5	17.7	18.6	18.9	18.0	18.7	18.9	18.1
Greix	15.9	17.6	19.6	15.9	17.4	19.7	15.6	17.4	19.5
AGS[c]	11.3	8.7	9.8	12.1	9.2	10.0	12.4	9.2	9.5
HC[d]	38.4	40.0	39.4	35.3	36.2	33.6	33.1	30.5	29.2
Fibra total	13.7	12.8	12.4	15.9	16.3	16.3	19.2	18.9	18.7
Minerals i vitamines[e]	6.5	6.7	6.5	6.7	6.7	6.7	6.7	6.8	6.7

[a] A totes les dietes se'ls va afegir un 0.33% de colesterol. [b] Els fitosterols es van afegir a la dieta basal. La barreja de fitosterols estava formada per un 6.4% de brassicasterol, un 24.9% de campesterol, un 1.0% de campestanol, un 18.5% de estigmasterol, un 45.6% de β-sitosterol, un 1.8% de sitostanol, un 0.9% de δ-5-avenasterol i un 1.0% d'altres esterols. [c] AGS significa àcids grassos saturats. [d] HC significa hidrats de carboni. [e] La barreja de minerals i vitamines es van ajustar d'acord amb els requeriments alimentaris dels conills d'índies.

Tots els percentatges estan expressats com p/p.

1.1 PREPARACIÓ DE LES MOSTRES

Els animals es van sacrificar després de 4 setmanes de tractament, per punció cardíaca després de ser anestesiats amb halotà. Les mostres sanguínies es van obtenir per extracció cardíaca. El fetge va ser extret després del sacrifici i es va homogeneïtzar immediatament. Les femtes es van recollir tres vegades per setmana i es van liofilitzar. Es van mantenir totes les mostres a –80ºC fins al moment de l'anàlisi.

1.2 MÈTODES ANALÍTICS

Es van utilitzar els següents mètodes analítics:

- Determinació quantitativa i qualitativa dels principals àcids grassos en el pinso i en les mostres biològiques (femta, plasma i fetge).
- Determinació quantitativa i qualitativa del colesterol i els principals fitosterols en fetge i plasma.
- Determinació qualitativa i quantitativa dels esterols vegetals, colesterol i dels seus metabòlits (coprostan-3-ol i coprostan-3-ona) i els àcids biliars primaris i secundaris en femtes.

2 RESULTATS

Para a poder determinar el contingut de colesterol i esterols vegetals en el fetge, va ser necessari posar a punt un mètode analític. Aquest va donar lloc a la següent publicació:

- Rapid and quantitative determination of total sterols of plant and animal origin in liver samples by gas-chromatography
  Gemma Brufau, Rafael Codony, Miguel Angel Canela i Magda Rafecas
  Chromatographia (En premsa)
  Factor d'impacte (2005): 0.959

Dels resultats obtinguts a partir de l'estudi, es van generar 4 articles més, recollits a continuació:

- A high-saturated fat diet enriched with phytosterol and pectin affects the fatty acid profile in guinea pigs
  Gemma Brufau, Miguel Angel Canela i Magda Rafecas
  Lipids (2006) 41; 159-168
  Factor d'impacte (2005): 1.905

- Phytosterols, but not pectin, added to a high-saturated fat diet, modify saturated fatty acid excretion related to chain length
  Gemma Brufau, Miguel Angel Canela i Magda Rafecas
  The Journal of Nutritional Biochemistry (*Acceptada*)
  Factor d'impacte (2005): 2.459

- Plant sterols and pectin, added to a high-saturated fat diet, do not show the hypocholesterolemic activity in guinea pigs
  Gemma Brufau, Miguel Angel Canela, Joan Quílez i Magda Rafecas
  Clinical Nutrition (*Enviada*)
  Factor d'impacte (2005): 2.296

- Reduction in cholesterol *output* after plant sterol and pectin supplementation in a high-saturated diet in guinea pigs
  Gemma Brufau, Miguel Angel Canela, Rafael Codony i Magda Rafecas
  Lipids (*Enviada*)
  Factor d'impacte (2005): 1.905

2.1 A HIGH-SATURATED DIET ENRICHED WITH PHYTOSTEROL AND PECTIN AFFECTS THE FATTY ACID PROFILE IN GUINEA PIGS

Títol: Una dieta molt saturada enriquida amb fitosterols i pectina afecta al perfil d'àcids grassos en conills d'índies

Autors: Gemma Brufau, Miguel Ángel Canela i Magda Rafecas

Revista: Lipids (2006); **41**: 159-168

Resum: Aquest article presenta els resultats d'un estudi, l'objectiu del qual va ser el d'estudiar els efectes de varies dosis de pectina i fitosterols en el guany de pes corporal i en el contingut d'àcids grassos (AG) en femelles de conill d'índies. Els tractaments es van obtenir a partir de l'addició de pectina i fitosterols a una dieta de conills d'índies (rica en àcids grassos saturats), seguint d'un disseny factorial 3x3, amb tres nivells de pectina (0, 3.67 i 6.93%) i tres nivells de fitosterols (0, 1.37 i 2,45%). Es van utilitzar 72 femelles de conill d'índies Durkin Hartley i es van assignar aleatòriament (8 animals/grup) a cada grup, amb una duració de l'estudi de 4 setmanes.

El consum diari de pectina va conduir a un increment del pes corporal ($p<0.001$) i en l'eficiència del pinso ($p<0.025$), però no es va observar cap diferència ni en el guany de pes ni en el consum d'aliment després de l'addició de fitosterols.

Es va trobar una relació inversa entre l'addició de fitosterols i el contingut de làuric, mirístic i palmític en femta, i un efecte positiu en la seva concentració en plasma i fetge; encara que no es va observar cap diferència en el contingut d'esteàric. L'absorció aparent dels AG es va estudiar a partir del *ratio* femta/dieta, per tant es va poder comparar l'absorció aparent dels diferents AG, i es va trobar una relació inversa entre l'addició de fitosterols i aquestes ratios, especialment pel làuric i mirístic.

2.2 PHYTOSTEROLS, BUT NOT PECTIN, ADDED TO A HIGH-SATURATED DIET, MODIFY SATURATED FATTY ACID EXCRETION IN RELATION TO CHAIN LENGTH

Títol: Els fitosterols, però no la pectina, afegits a una dieta molt saturada, modifiquen l'excreció d'àcids grassos saturats en funció de la longitud de la cadena

Autors: Gemma Brufau, Miguel Ángel Canela i Magda Rafecas

Revista: The Journal of Nutritional Biochemistry (2006). *Acceptada*

Resum: El principal objectiu d'aquest article va ser el d'estudiar com l'excreció d'àcids grassos saturats (AGS) es modificada durant el consum d'una dieta altament saturada en la quan s'han afegit fitosterols i pectina. Presentem els resultats d'un estudi longitudinal durant 4 setmanes, en conills d'índies. A les dietes se'ls va addicionar un 0.33% de colesterol, i es van diferenciar en el contingut de pectina (tres concentracions) i en el contingut de fitosterols (tres concentracions) afegits. Es van utilitzar 72 femelles de conill d'índies Dunkin Hartley que van ser assignats aleatòriament en els grups de tractament (8 animals/grup).

Es va trobar una relació inversa entre el consum de fitosterols i l'excreció de làuric (12:0) i mirístic (14:0), però un efecte positiu amb l'excreció de l'àcid araquídic (20:0) i behènic (22:0). L'addició de fitosteroles no va tenir un efecte clar en l'excreció dels àcids palmític (16:0) i esteàric (18:0). També hi va haver una relació inversa entre l'addició de pectina i l'excreció dels AGS, però aquestes diferències no van ser significatives. Aquests resultats suggereixen que l'addició de fitosterols a una dieta molt saturada estimula l'absorció dels àcids grassos més aterogènics (làuric i mirístic) després d'una setmana de tractament, comparat amb només la dieta saturada.

2.3 PLANT STEROLS AND PECTIN, ADDED TO A HIGH-SATURATED FAT DIET, DO NOT SHOW THE HYPOCHOLESTEROLEMIC ACTIVITY IN GUINEA PIGS

Títol: Els fitosterols i la pectina, afegits a una dieta rica en greix saturat, no mostren cap activitat hipocolesterolèmica en conills d'índies

Autors: Gemma Brufau, Miguel Ángel Canela, Joan Quílez i Magda Rafecas

Revista: Clinical Nutrition (2006). *Enviada*

Resum: **Introducció i objectius.** Aquest article presenta els resultats d'un estudi, l'objectiu del qual va ser el d'estudiar diferents dosis de pectina i fitosterols en el contingut d'esterols en plasma i fetge en conills d'índies quan es van afegir a una dieta occidental. **Mètodes.** Els tractaments van seguir un disseny factorial 3 x 3, amb tres nivells de pectina (0, 3.67 i 6.93%) i tres nivells de fitosterols (0, 1.37 i 2.45%). 72 femelles de conill d'índies Dunkin Hartley es van assignar aleatòriament als grups problema (8 animals/grup). La duració de l'estudi va ser de 4 setmanes. **Resultats.** No es van trobar diferències en les concentracions de colesterol plasmàtic, mentre que es van trobar reduccions en les concentracions de colesterol hepàtic després de l'addició de fitosterols. A més, no es van trobar diferències després de la ingesta de pectina. Les concentracions plasmàtiques de fitosterols van augmentar en el plasma i el fetge després del consum de fitosterols, i les concentracions més elevades es van trobar una altra vegada en els animals alimentats a dosis intermèdies de pectina. **Conclusions.** Els nostres resultats suggereixen que una dieta saturada pot alterar les propietats hipocolesterolèmiques dels esterols vegetals i la pectina.

2.4 *REDUCTION IN CHOLESTEROL* OUTPUT *AFTER PLANT STEROL AND PECTIN SUPPLEMENTATION IN A HIGH-SATURATED DIET IN GUINEA PIGS*

Títol: Reducció del colesterol *output* després de l'addició d'esterols vegetals i pectina en una dieta molt saturada en conills d'índies

Autors: Gemma Brufau, Miguel Ángel Canela, Rafael Codony i Magda Rafecas

Revista: Lipids (2006). *Enviada*

Resum: El principal objectiu va ser el d'estudiar si l'absorció aparent dels fitosterols i colesterol es modifica després d'afegir esterols vegetals i pectina a una dieta saturada. Els animals utilitzats van ser72 femelles de conill d'índies Dunkin Harley. Es va addicionar tres nivells de pectina (0, 3.67 i 6.93%) i tres de fitosterols (0, 1.37 i 2.45%) a una dieta molt saturada de conills d'índies durant un període de 4 setmanes.

La pectina no va modificar l'absorció aparent dels fitosterols però va augmentar la del colesterol ($p<0.05$). L'addició de fitosterols va disminuir l'absorció aparent (definit com el ratio femta/dieta) del β-sitosterol i stigmasterol, però va augmentar l'absorció del campesterol. Els fitosterols també van disminuir l'eliminació total de colesterol.

Els fitosterols afegits a una dieta molt saturada poden estimular l'absorció de campesterol, encara que l'absorció de colesterol va disminuir, l'eliminació total de colesterol va disminuir. Aquests resultats suggereixen que una dieta Occidental pot modificar els efectes hipocolesterolèmics de fitosterols i pectina.

2.5 RAPID AND QUANTITATIVE DETERMINATION OF TOTAL STEROL OF PLANT AND ANIMAL ORIGIN IN LIVER SAMPLES BY GAS-CHROMATOGRAPHY

Títol: Ràpida determinació dels esterols totals d'origen vegetal i animal en mostres de fetge per cromatografia de gasos

Autors: Gemma Brufau, Rafael Codony, Miguel Ángel Canela i Magda Rafecas

Revista: Chromatographia (2006). *En premsa*

Resum: Es descriu un mètode ràpid i simple per a quantificar esterols per cromatografia de gasos (CG). Els mètodes més habituals per a determinar esterols inclouen una extracció lipídica, saponificació, extracció de la fracció insaponificable i el seu fraccionament. Aquest article proposa una saponificació directa de les mostres biològiques per a estalviar temps i dissolvents.

Les mostres es van homogeneïtzar amb etanol, el qual contenia una barreja d'antioxidants, seguit d'una etapa de saponificació amb KOH 1N, extracció dels insaponificacles, i finalment, purificació utilitzant un cartutx d'extracció en fase sòlida (SPE). L'anàlisi cromatogràfic es va realitzar utilitzant un columna capil·lar ZB-1 (100% metilpolisiloxà) i un programa de temperatura del forn que inclou dues rampes des de 245 a 290ºC. Els analits es van identificar utilitzant el 5α-colestà com a patró intern.

Es va determinar la repetitibilitat, la recuperació i la linearitat (n=8). Els valors de repetitibilitat van ser [mitjana μg/100 mg (CV)]: per l'esqualè [0.68 (12.99%)], pel colesterol [190.82 (2.34%)], pel latosterol [0.48 (3.05%)], pel campesterol [3.90 (2.55%)] i pel β-sitosterol [0.27 (19.69%)]. En resum, aquest mètode ofereix perspectives interessants per l'anàlisi quantitatiu d'aquests esterols no només en mostres de fetge, sinó que també en altres matrius biològiques.

3 DISCUSSIÓ GENERAL

Tal i com s'ha anat recollint en la revisió bibliogràfica d'aquesta tesi, les dietes occidentals (amb continguts en àcids grassos saturats i *trans* superiors a les recomanacions nutricionals i inferiors en àcids grassos poliinsaturats n-3 i n-6) (Kris-Etherton i col., 2002; Simopoulos, 2002) incrementen el risc associat a les malalties cardiovasculars, donat a que augmenten les concentracions de colesterol total i colesterol-LDL (Spady i col., 1993, Mustad i col., 1997; Stamler i col., 2000; Hu i col., 2001). Per tant, les recomanacions actuals proposen limitar el consum d'àcids grassos saturats a menys del 10% del consum energètic total (Krauss i col., 2000; Serra-Majem i col., 2001).

Encara que sempre s'hauria de seguir una dieta d'acord amb les recomanacions nutricionals, els aliments funcionals poder ser molt útils per millorar el perfil lipoproteic. Durant els últims anys, s'ha demostrat l'eficàcia hipocolesterolèmica dels fitosterols (Mensink i col., 2002; Katan i col., 2003; Miettinen i Gylling, 2004) i de la fibra soluble (Galibois i col., 1994; Brown i col., 1999; Dongowski i Lorenz, 2004). No obstant, fins al moment, no hi ha resultats sobre els efectes de l'addició d'aquests ingredients funcionals a una dieta amb un elevat contingut amb àcids grassos saturats i colesterol.

Per tant, en la primera part d'aquesta secció es comentarà un nou mètode analític per a determinar esterols vegetals i animals en mostres de fetge. A continuació, es discutiran els efectes sobre el perfil lipídic de conills d'índies de l'addició de fitosterols (secció 2) i de pectina (secció 3) a una dieta saturada. En l'última secció es discutirà la influència del consum simultani de pectina i fitosterols.

3.1 VALIDACIÓ D'UN MÈTODE ANALÍTIC PER A DETERMINAR ESTEROLS D'ORIGEN VEGETAL I ANIMAL EN MOSTRES DE FETGE

Per a poder complir els nostres objectius, un dels principals problemes que ens vam trobar va ser la falta d'un mètode analític adequat per a determinar esterols en mostres de fetge. La majoria de mètodes descrits en bibliografia, presenten diverses

dificultats quan s'apliquen per a determinar fitosterols i colesterol, donat que es troben en un marge molt ample de concentracions en matrius complexes, com el fetge, fet que pot interferir amb la separació.

Les concentracions de fitosterols/fitostanols, colesterol i els seus precursors (com ara el latosterol) proporcionen informació rellevant en la regulació de la síntesi de colesterol i dels àcids biliars sota diverses condicions experimentals (Miettinen i Kesaniemi, 1989). Donat que els mètodes disponibles (Ntanios i Jones, 1998; 1999; Tatematsu i col., 2004) no inclouen els resultats de validació, hem desenvolupat un mètode que posseeix una gran selectivitat i sensitivitat.

Bàsicament, les mostres es van homogeneïtzar amb etanol, seguit d'una fase de saponificació utilitzant hidròxid sodi 1N, extracció dels compostos no saponificables i purificació utilitzant un cartutx d'extracció en fase sòlida. Finalment les mostres van ser injectades en un cromatògraf de gasos, utilitzant detector de ionització de flama.

Es va decidir evitar l'extracció lipídica abans de saponificar donat que Toivo i col. (2000) van descriure que una inicial extracció lipídica total no afecta significativament a la concentració d'esterols. Es va determinar la repetitibilitat, recuperació i linealitat i els valors es van trobar dins dels límits de variació acceptable, proposats per Hubert (1993).

El nostre mètode permet l'anàlisi d'esterols en fetge sense una extracció lipídica abans de la saponificació. A més, dóna la possibilitat de determinar la quantitat de diversos compostos que es troben en una ample rang de concentracions. Per tant, aquest mètode ofereix perspectives interessants per l'anàlisi quantitatiu d'aquests compostos, no només en mostres de fetge, sinó que també en altres mostres biològiques.

3.2 EFECTE DE L'ADDICIÓ DE FITOSTEROLS A UNA DIETA SATURADA SOBRE EL PERFIL LIPÍDIC DE CONILLS D'ÍNDIES

3.2.1 PES CORPORAL

Els primers resultats que es van obtenir van ser els referents al pes corporal dels animals. Els nostres resultats van mostrar que l'addició de fitosterols a una dieta molt saturada, no modifica el creixement dels animals ni el consum de pinso (Fig. 1), d'acord amb altres estudis ja publicats (Ewart i col., 2002; Hayes i col., 2002), tot i que en aquests estudis no es va utilitzar una dieta saturada. A més, els animals alimentats amb la dieta amb fitosterols no van mostrar cap diferència en les concentracions de glucosa ni de triacilglicerols.

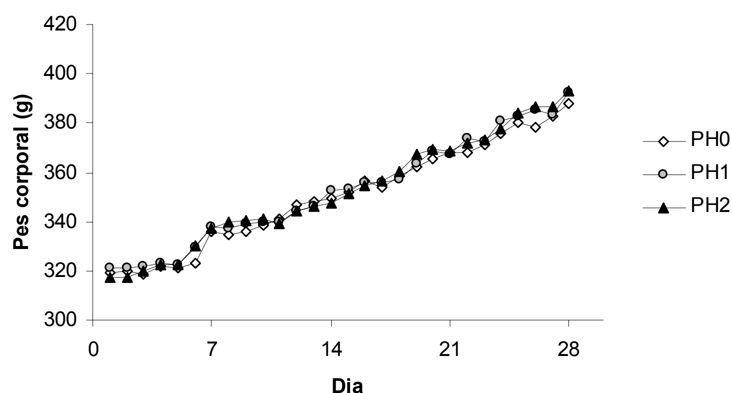

Fig. 1. Corbes de pes corporal per les diferents concentracions de fitosterols.

3.2.2 EXCRECIÓ FECAL DE COLESTEROL I FITOSTEROLS

Després del consum de fitosterols, les concentracions de β-sitosterol, estigmasterol i campesterol en femtes van incrementar (Fig. 2).

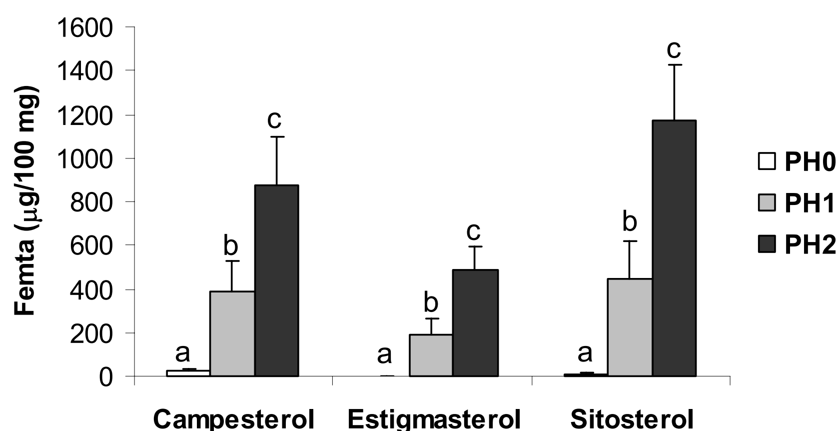

Fig. 2. Concentracions en femtes (mg/100g) dels principals fitosterols. *Camp* significa campesterol; *estig* significa estigmasterol i *sitost* significa β-sitosterol. Lletres diferents en el superíndex significa $p<0.05$

De totes maneres, els fitosterols en femtes, van estar altament correlacionats amb la quantitat en dietes, donat que la seva absorció va ser molt pobra. Per tant, més important que estudiar la concentració total en femtes, és estudiar l'absorció d'aquests compostos. Per a determinar-la, es va utilitzar el ratio del contingut fecal entre el contingut en la dieta, que ens dóna una idea de l'absorció aparent (Bonanome i Grundy, 1988; Dougherty i col., 1995; Baer i col., 2003). Aquest ratio ja ha estat utilitzat en molt estudis, on es van trobar resultats similars quan es va comparar l'ús del ratio amb el d'isòtops estables (Emken, 1994; Jones i col., 1999).

Donat que les concentracions fecals de fitosterols en el grup control va ser molt baix, no es va poder utilitzar en aquest grup, per tant la comparació es va restringir als grups PH1 i PH2. Tal i com es pot veure a la Fig. 3, tots els fitosterols analitzats (campesterol, estigmasterol i β-sitosterol) van tenir valors similars d'absorció aparent, encara que va ser més alta pel campesterol donat que s'absorbeix millor que la resta (Sanders i col., 2000). No obstant, és interessant notar que, mentre que els ratios de l'estigmasterol i del β-sitosterol tendeixen a ser més alts amb concentracions més elevades en la dieta, el ratio del campesterol disminueix amb les concentracions de fitosterols més elevades en la dieta (resultats no-significatius).

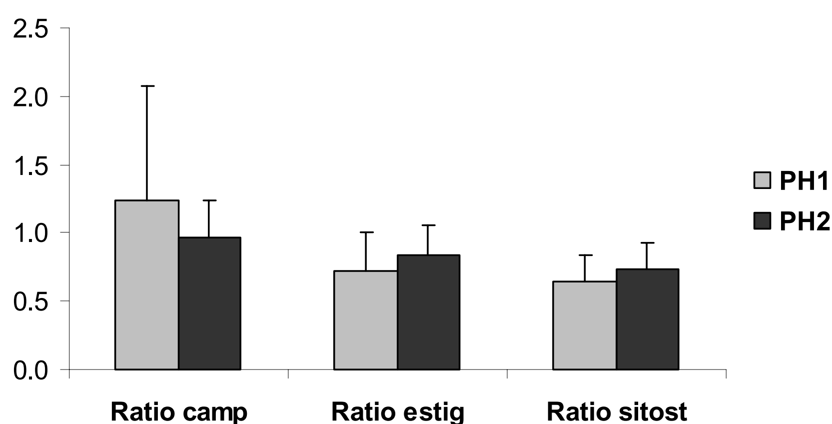

Fig. 3. Resultats de l'absorció aparent de fitosterols.
Camp significa campesterol; *stig* significa estigmasterol i *sitost* significa β-sitosterol

També es va trobar que l'excreció de colesterol, així com el ratio femta/dieta era superior després de la ingesta de fitosterols. Aquests resultats són similars als descrits per diversos autors (Ntanios i Jones, 1999; Ramjiganesh i col., 2001; Normen i col., 2006), els quals van suggerir que la disminució en la absorció de colesterol és un dels principals mecanismes pels que els fitosterols actuen com a agents hipocolesterolemiants. De totes maneres, creiem que millor que estudiar les concentracions totals de colesterol fecal, és estudiar el contingut d'esterols neutres fecals (definit com la suma de colesterol i dels seus metabòlits, coprostan-3-ol i coprostan-3-ona), donat que una vegada el colesterol es troba en el tracte intestinal, es metabolitzat per la flora colònica. També és interessant estudiar el valor del colesterol *output*, definit com la suma de àcids biliars fecals i esterols neutres, donat que aquest valor dóna una idea sobre el metabolisme del colesterol.

Es va trobar una disminució en el contingut d'esterols neutres fecals i en el de colesterol *output* després del consum de fitosterols (Fig. 4). Aquests resultats estan d'acord amb l'increment trobat en l'absorció aparent del campesterol, donat que l'absorció de fitosterols és un marcador de l'absorció del colesterol (Tilvis i Miettinen, 1986; Assmann i col., 2006).

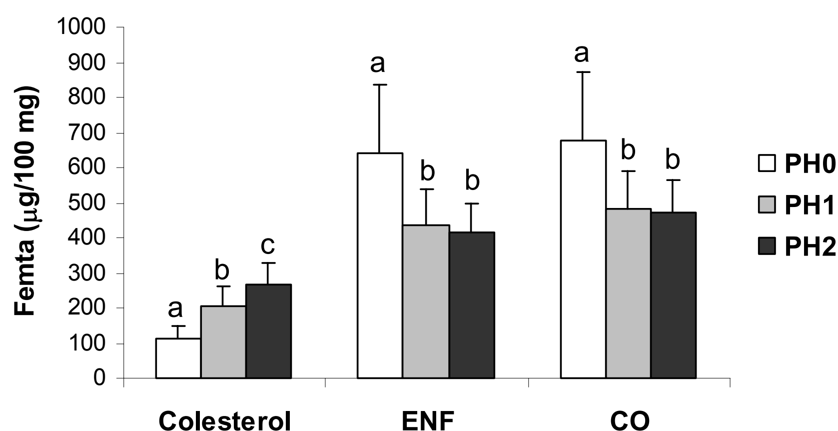

Fig. 4. Metabolisme del colesterol (μg/100mg).

ENF significa esterols fecals neutres i *CO* significa colesterol *output*.

Diferent lletra al superíndex significa $p<0.05$.

3.2.3 CONCENTRACIÓ D'ÀCIDS GRASSOS SATURATS

L'addició de fitosterols al pinso va provocar canvis en l'excreció dels AGS en funció de la longitud de la cadena. Els fitosterols van estimular l'excreció dels AGS de cadena llarga (behènic i araquídic), però van disminuir la dels AGS de cadena mitja (làuric i mirístic) (Fig. 5). A més a més, el patró d'excreció dels AG també va ser diferent en funció de la cadena. Es va trobar un patró d'excreció similar per tots els AGS en el grup control (Fig. 5). L'excreció va incrementar després de la primera setmana, després es va estabilitzar durant una setmana, i va tornar a incrementar les dues últimes setmanes. De totes maneres, l'efecte dels fitosterols va variar en funció dels AGS. L'excreció de l'àcid làuric en el grup control va incrementar al voltant d'un 77% al llarg de l'estudi, en el grup PH1 va ser d'un 29% i en el grup PH2 va ser només d'un 18%. En canvi, l'excreció més gran de behènic es va trobar en el grup PH2 (al voltant d'un 52%), mentre que en el grup control i en el grup PH1 van mostrar valors similars d'excreció (al voltant d'un 30%).

Aquestes diferències es poden explicar segons la diferent distribució dels compostos a la micel·la. Els fitosterols poden desplaçar els compostos de l'interior de la micel·la (com ara carotenoids hidrocarbonats), sense tenir cap efecte amb els compostos de la superfície (com ara tocoferols) (Borel i col., 1996; Plat i Mensink,

2001). L'àcid làuric, amb 12 àtoms de carboni, és menys liposoluble que l'àcid behènic (amb 22 àtoms de carboni) i és possible que estigui situat més a la superfície de la micel·la. Per tant, tal i com passava amb els carotens, els fitosterols poden ser més eficients desplaçant de la micel·la l'àcid behènic que l'àcid làuric. A més, els àcids palmític i esteàric no van mostrar un clar efecte de l'addició de fitosterols, els quals amb cadenes de 16 a 18 àtoms de carboni tenen una solubilitat intermèdia. Per tant, possiblement estan situats entre l'interior i la superfície de la micel·la.

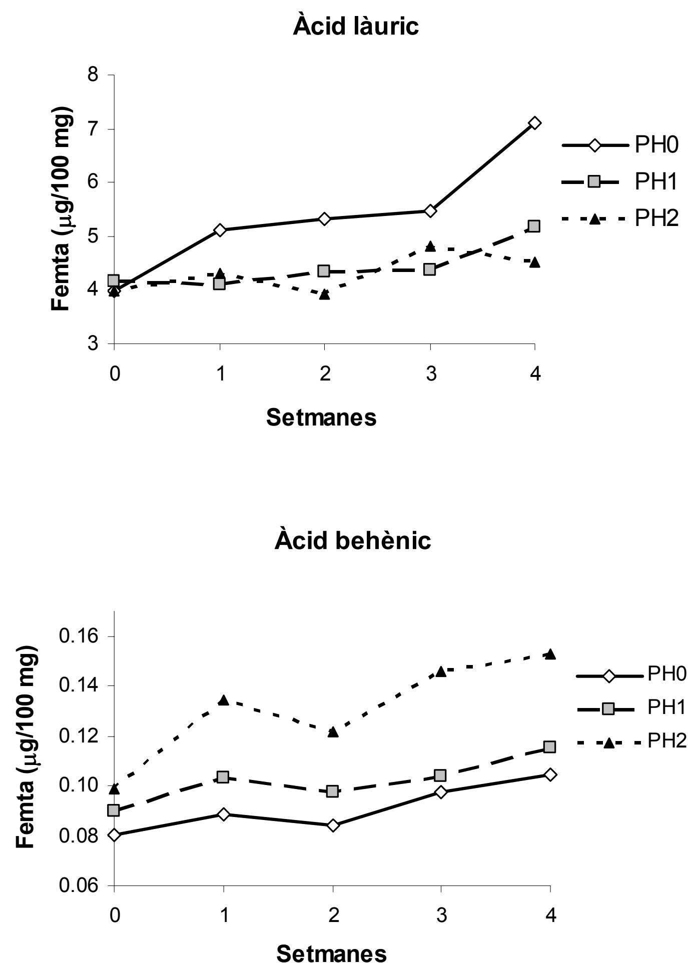

Fig. 5. Concentracions fecals dels àcids làuric i behènic durant l'estudi, en funció de la dosi de fitosterols afegida.

Els nostres resultats estan d'acord amb els publicats per Normen i col. (2006), que van trobar que una dieta rica saturada amb fitosterols disminueix més l'excreció de làuric i mirístic que de palmític i esteàric. De totes maneres, si enlloc de fitosterols,

s'enriqueix la dieta amb fitostanols, aquest efecte no apareix. A més a més, en el nostre estudi vam trobar un increment en les concentracions de làuric i mirístic en el fetge amb l'addició més gran de fitosterols, suggerint una acumulació d'aquests àcids grassos en aquest teixit. En canvi, no es van torbar diferències en les concentracions d'àcids grassos plasmàtiques (Fig. 6).

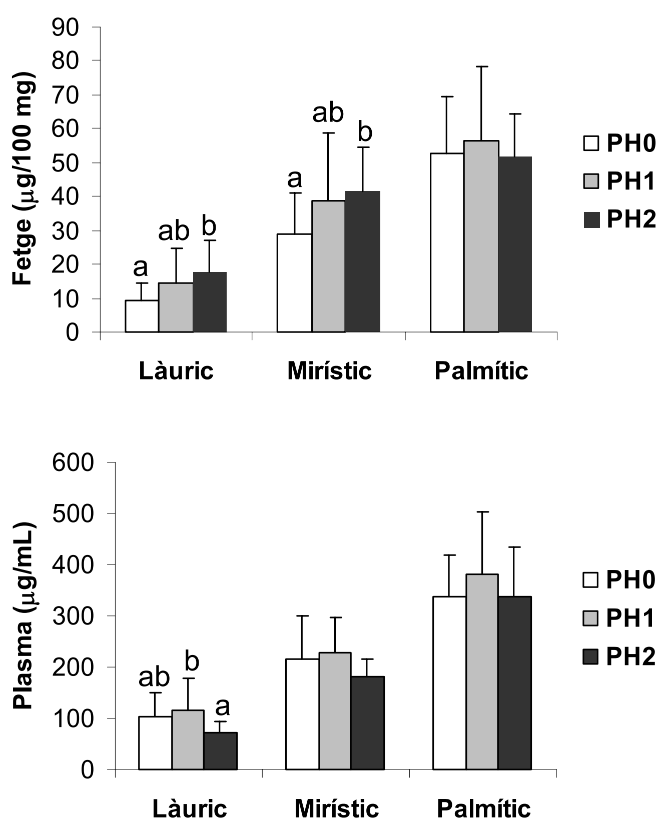

Fig. 6. Concentracions d'àcids grassos en el fetge i en el plasma
Lletres diferents en el superíndex signifiquen $p<0.05$

3.2.4 CONCENTRACIONS DE FITOSTEROLS I COLESTEROL EN PLASMA I FETGE

La concentració plasmàtica de fitosterols en el grup control (sense addició de pectina ni de fitosterols) va ser molt baixa (inferior a un 1%), donat que els fitosterols s'absorbeixen de manera molt pobra a l'intestí (entre 0.4 i 3.5%) (Ostlund, 2002), i no poden ser sintetitzats pels animals (Salen i col., 1970). També es van trobar

concentracions de fitosterols més elevades en el fetge d'animals alimentats amb fitosterols (Fig. 7). Especialment, les concentracions plasmàtiques de campesterol van ser molt superiors a les de β-sitosterol, d'acord amb els diferents patrons d'absorció trobats en femtes. La raó per aquests resultats pot ser el fet que la ACAT hepàtica té menys afinitat pel β-sitosterol que pel campesterol, fet que provoca que sigui excretat per la bilis més ràpidament (Tavani i col., 1982), i a més a més, el campesterol s'absorbeix millor a l'intestí. Per exemple, les concentracions de campesterol van ser 70 i 50 vegades més grans en els grups PH2 i PH1, respectivament, que en el control grup, mentre que la del β-sitosterol va ser només dos vegades (Fig. 7).

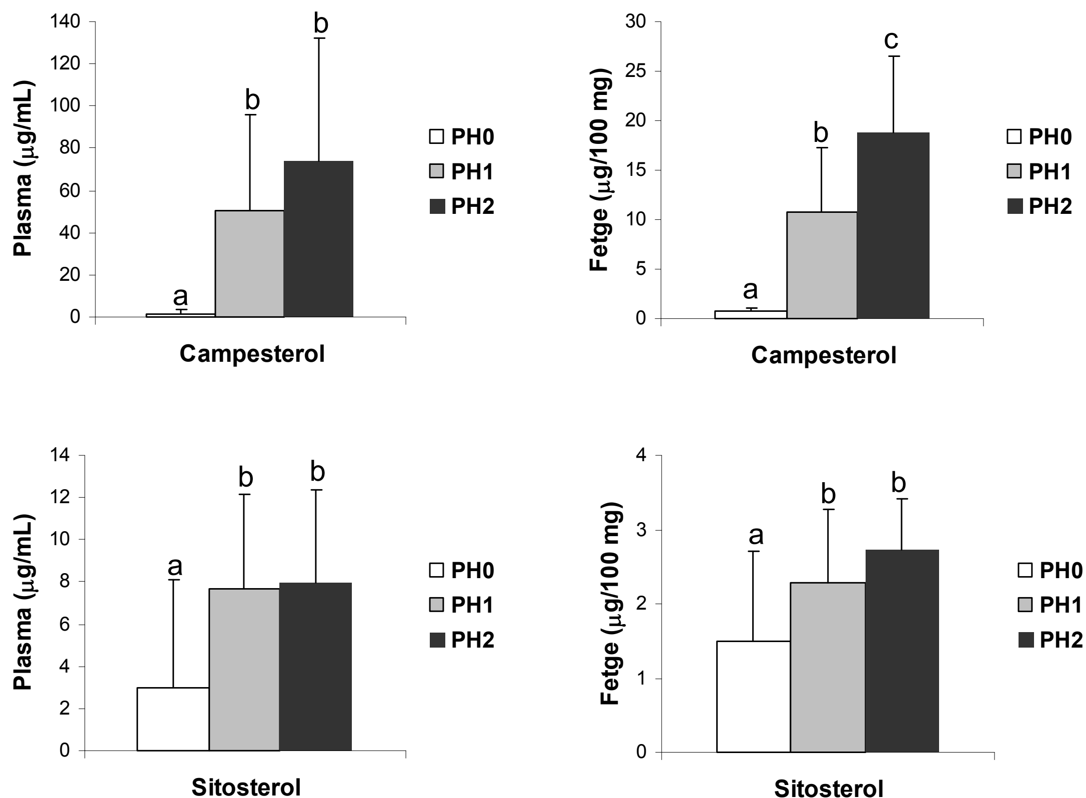

Fig. 7. Concentracions de fitosterols en el fetge i en el plasma.
Lletres diferents al superíndex signifiquen $p<0.05$.

Les elevades concentracions sanguínies de fitosterols suggereixen que l'absorció del colesterol es va incrementar, donat que les concentracions plasmàtiques de fitosterols són un indicador de l'absorció del colesterol (Tilvis i Miettinen, 1986;

Miettinen i Kesaniemi, 1989; Miettinen i col., 1990; Assmann i col., 2006). No es van trobar diferències en les concentracions plasmàtiques de colesterol, encara que els animals alimentats amb fitosterols van tenir concentracions lleugerament més elevades (diferències no significatives) (Fig. 8). Per contra, les concentracions hepàtiques de colesterol van ser inferiors ($p<0.05$), encara que la reducció observada no pot ser atribuïda a una disminució en la síntesi de colesterol, donat que no es van trobar diferències en els precursors del colesterol (latosterol i desmosterol).

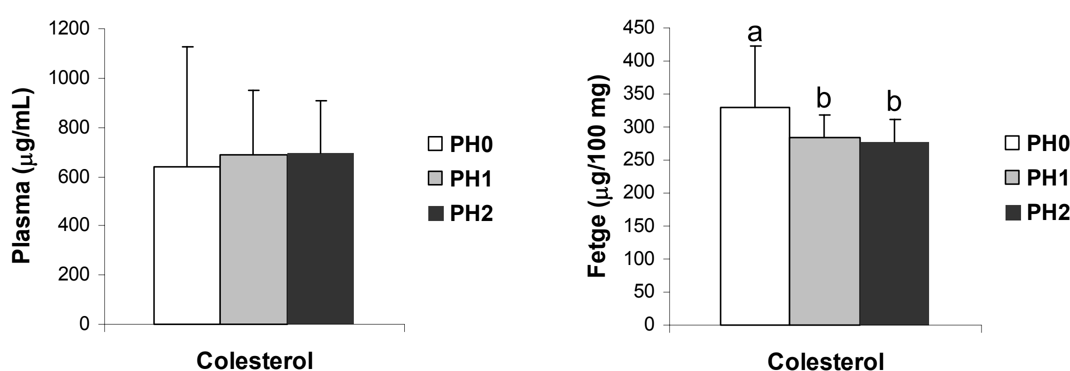

Fig. 8. Concentracions de colesterol en el plasma i en fetge.
Diferent lletra al superíndex significa resultats diferents ($p<0.05$).

Els nostres resultats semblen estar en desacord amb la majoria d'estudis publicats (Katan i col., 2003) els quals van trobar reduccions en les concentracions de colesterol plasmàtiques després del consum de fitosterols. De totes maneres, aquests estudis van utilitzar aliments amb baix contingut en greix i tal i com s'ha mencionat, en el nostre estudi es va escollir una dieta enriquida amb colesterol i greix saturat donat que són els principals components de les dietes occidentals (Kris-Etherton i col., 2002; Simopoulos, 2002).

Les concentracions de colesterol van estar molt correlacionades amb l'increment trobat en l'absorció dels àcids grassos de cadena mitja (especialment làuric i mirístic). Per tant, una possible explicació seria que els fitosterols, quan s'afegeixen a aquest tipus de dieta, estimulen l'absorció dels AGS de cadena mitja. En realitat, es va trobar un increment en l'absorció aparent d'aquests AG, correlacionats positivament amb el seu contingut en el fetge. Aquest increment en les concentracions d'AG pot haver

reduït la transformació de colesterol lliure a colesterol esterificat, així com també l'expressió de receptors per a les LDL (Woollett i col., 1992; Dietschy, 1998), el qual provocaria una disminució en les concentracions de colesterol hepàtiques. No obstant, no es van trobar diferències amb les concentracions plasmàtiques de colesterol (Fig. 8).

Per tant una de les nostres hipòtesis per la falta d'efecte hipocolesterolemiant dels fitosterols podria ser un increment en l'absorció dels AGS de cadena mitja, així com també com la reducció de colesterol *output*.

3.3 EFECTE DE L'ADICIÓ DE PECTINA EN UNA DIETA SATURADA SOBRE EL PERFIL LIPÍDIC DE CONILLS D'ÍNDIES

La pectina és una de les fibres solubles amb propietats hipocolesterolèmiques, utilitzada en un gran nombre d'estudis en animals (Fernandez, 1995; Garcia-Diez i col., 1996; Shen i col., 1998) i en humans (Everson i col., 1992; Pereira i col., 2004). Es creu que aquest tipus de fibra és capaç de lligar els àcids biliars, reduint llavors la seva reabsorció, així com també la del colesterol. De totes maneres, per a aconseguir els efectes hipocolesterolemiants, s'hauria d'utilitzar pectina d'elevat GM (Terpstra i col., 1998; Dongowski i Lorenz, 2004).

3.3.1 PES CORPORAL

Tal i com passava amb els fitosterols, no hi ha estudis que hagin evaluat els possibles efectes hipocolesterolemiants de la pectina quan forma part d'una dieta amb un alt contingut en AGS i colesterol. En el nostre estudi es va trobar una associació negativa entre l'addició de pectina a les dietes i el consum d'aliment. L'addició de pectina va conduir a un increment en el pes corporal dels animals (Fig. 9), d'acord que els resultats publicats per Anderson i col. (1994), encara que aquestes diferències en les corbes de pes corporal no es van trobar en altres estudis (Vergara-Jimenez i col., 1998; Roy i col., 2000).

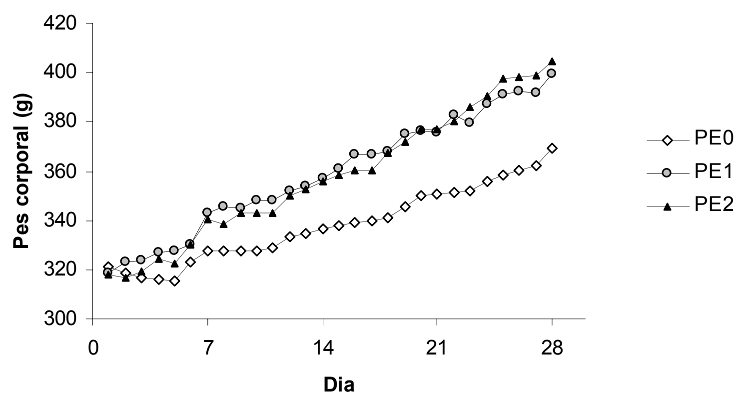

Fig. 9. Corbes de pes corporal per diferent concentracions de pectina.

En el nostre estudi també es va trobar un increment en l'eficàcia de l'aliment amb concentracions elevades de pectina, que està en desacord amb estudis anteriors (Vergara-Jimenez i col., 1998; Roy i col., 2000). Aquest inesperat increment en l'eficàcia de l'aliment i en el pes corporal pot ser degut a un trencament de la pectina abans d'assolir el seu lloc d'acció, tot i que la pectina que vam utilitzar era altament metilada.

3.3.2 CONCENTRACIÓ D'ÀCIDS GRASSOS SATURATS

L'addició de pectina no va tenir cap efecte en la concentració de glucosa, ni en la de triacilglicerols ni en l'excreció dels AGS, amb un percentatge d'excreció que es situa entre el 18 i el 25% en tots els grups. Alguns autors (Swain i col., 1990) han descrit que el consum de fibra pot contrarestar els efectes del consum d'una dieta rica en AGS i per tant reduir l'aparició de malalties cardiovasculars. En canvi, altres autors (Ascherio i col., 1996; Hu i col., 2001; Wu i col., 2003) no van trobar cap efecte del consum de la fibra en la concentració d'AGS en mostres biològiques. Un estudi recent (Kritchevsky i Tepper, 2005) va descriure que rates alimentades amb una mescla de diferents fibres experimentaven un increment de pes del fetge.

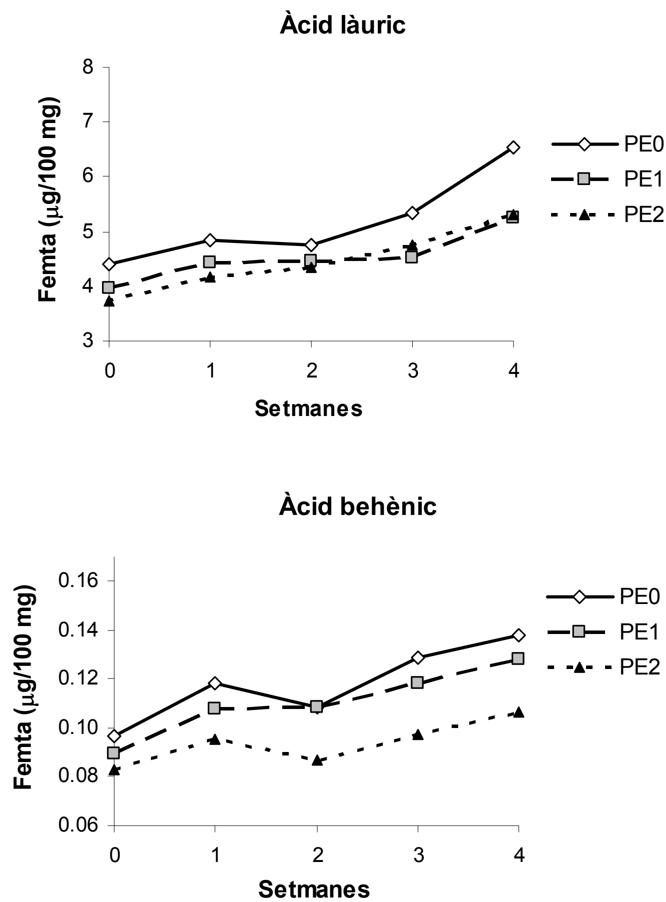

Fig. 10. Concentracions fecals dels àcids làuric i behènic al llarg de l'estudi, en funció de la concentració de pectina utilitzada.

De totes maneres, en el nostre estudi no vam trobar un efecte directe de la pectina en l'excreció d'AGS (Fig. 10) o en les concentracions en el fetge. En canvi en el plasma, les concentracions més elevades d'AGS es van trobar a les dosis intermèdies de pectina (efecte significant només per l'àcid mirístic) (Fig. 11).

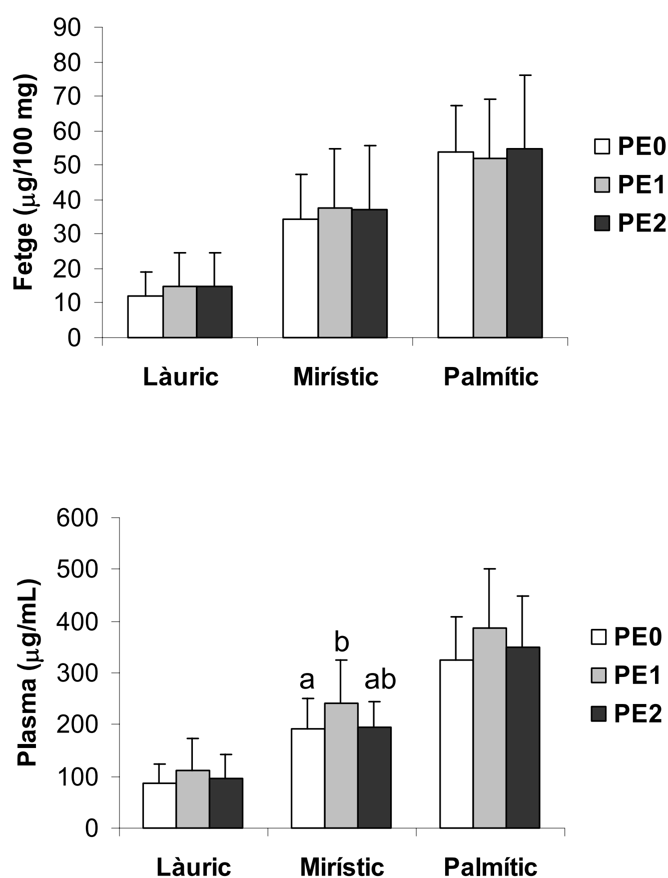

Fig. 11. Concentracions d'àcids grassos en el fetge i en el plasma
Lletres diferents al superíndex signifiquen $p<0.05$

3.3.3 CONCENTRACIONS DE COLESTEROL I FITOSTEROLS

El consum de fibra va incrementar l'absorció aparent de colesterol, així com també la de β-sitosterol i d'estigmasterol ($p<0.05$), sense tenir cap efecte en l'absorció aparent de campesterol. A més a més, la concentració fecal d'àcids biliars també es va veure reduïda després del consum de fibra ($p<0.05$), sense trobar diferències entre tractaments amb els metabòlits del colesterol (Fig. 12).

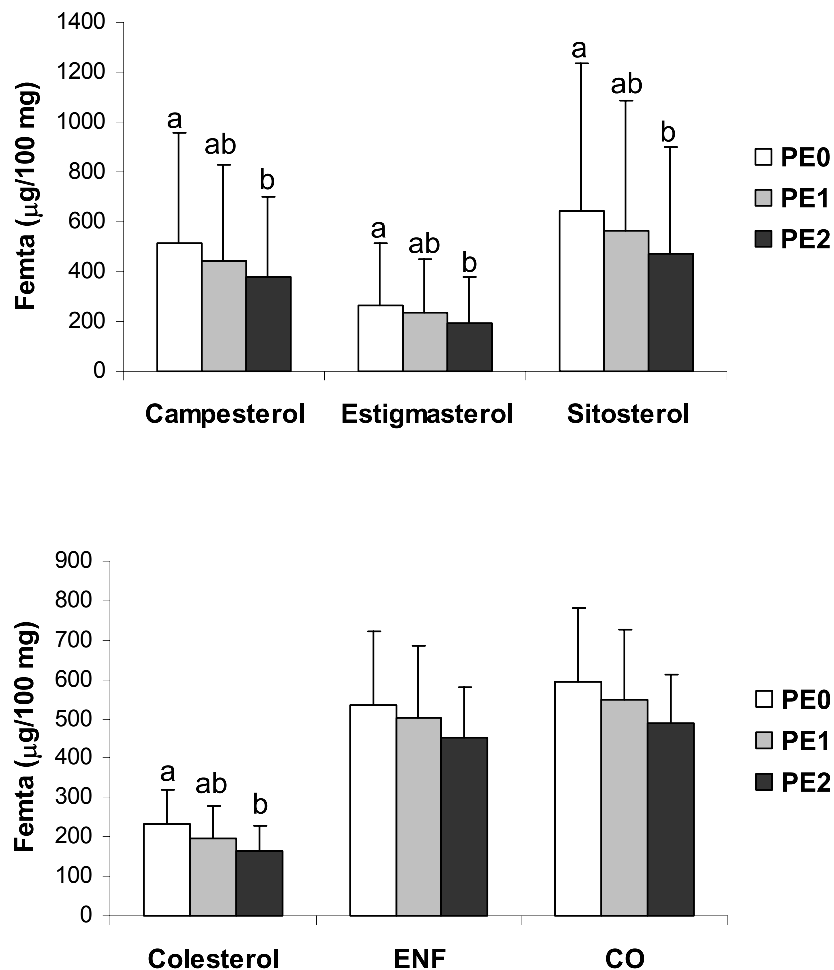

Fig. 12. Concentracions fecals (μg/100 mg) dels principals fitosterols i colesterol.
EFN significa esterols fecals neutres i *CO* significa colesterol *output*.
Lletres diferents signifiquen resultats diferents ($p<0.05$).

La majoria d'estudis publicats fins al moment (Pfeffer i col., 1981; Falk i Nagyvary, 1982; Everson i col., 1992; Shen i col., 1998) han mostrat que el consum de pectina provoca una reducció en l'absorció de colesterol i la interrupció de la circulació enterohepàtica, fet que condueix a reduccions en les concentracions sanguínies de colesterol. No obstant, en el nostre estudi, es van trobar increments en la concentració plasmàtica de campesterol i colesterol, i increments en la concentració hepàtica de campesterol després d'alimentar els animals amb dietes enriquides amb pectina, trobant les concentracions més elevades amb concentracions intermèdies de pectina (Fig. 13).

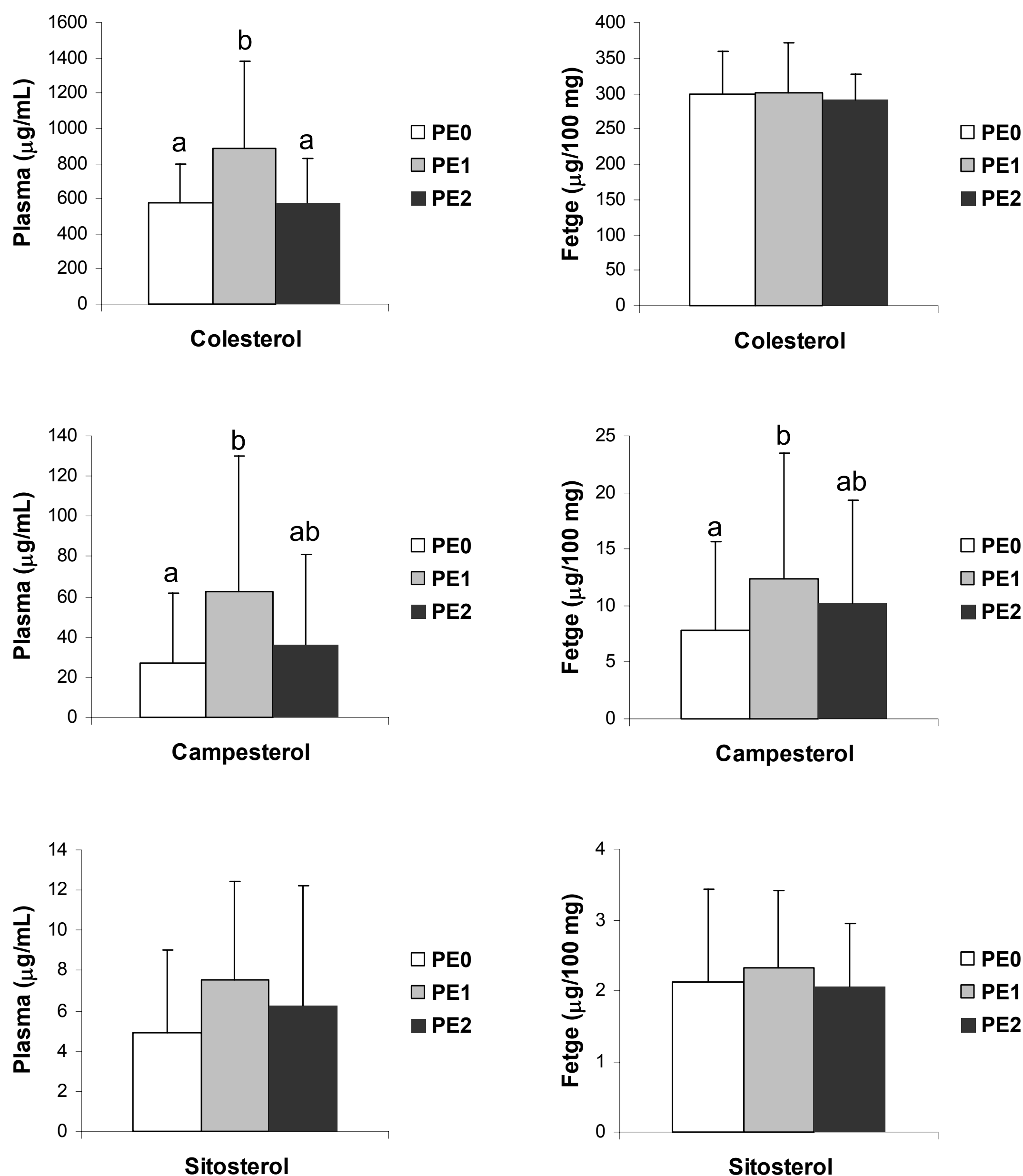

Fig. 13. Concentracions de colesterol, campesterol i β-sitosterol en plasma i fetge.
Lletres diferents signifiquen resultats diferents ($p<0.05$).

Els nostres resultats es podrien explicar tenint en compte que en la majoria d'estudis publicats fins al moment, la pectina es va addicionar a una dieta amb un baix contingut en greix (especialment insaturat), mentre que la dieta utilitzada en el nostre estudi va ser rica en AGS i colesterol. A més a més, és possible que es produïssin

algunes interaccions entre la pectina i el tipus de greix, donat que la pectina pot estimular la proliferació de la mucosa en les parts més inferiors del tracte intestinal (Fukunaga i col., 2003), i el tipus de greix podria modular aquest efecte (Gorbach i Goldin, 1987; Chapkin i col., 1993).

Una altra possible explicació pels nostres resultats seria que la pectina hagués sofert una hidròlisi abans d'arribar al lloc d'acció, tot i que la pectina utilitzada tenia una GM superior al 50%. Un dels fets que donen peu a aquesta hipòtesi és el fet que els animals alimentats amb fibra tenien pesos corporals superiors als del grup control. No obstant, tenint en compte que la dosi de pectina del grup PE2 era gairebé el doble que el grup PE1, es podria esperar que els animals del grup PE2 haguessin pesat més, però en canvi, no es van trobar diferències en els pesos corporals dels animals d'aquests grups. Per tant, es podria pensar que en grup PE2 tenia una part de la pectina intacta a l'intestí, la qual podria haver exercit els seus efectes hipocolesterolemiants. Aquesta hipòtesi també explicaria el fet que les concentracions de campesterol i colesterol fossin més elevades en el grup PE1 que en el grup PE2.

De totes maneres, no podem assegurar si el mecanisme que explicaria els nostres resultats són possibles alteracions en la qualitat de la pectina o interaccions entre la pectina i el greix de les dietes.

3.4 EFECTE DE LA COMBINACIÓ DE PECTINA I FITOSTEROLS SOBRE EL PERFIL LIPÍDIC

Hi ha una gran nombre de treballs publicats que han estudiat l'efecte combinat de diversos ingredients funcionals. Els fitosterols han estat combinats amb diversos components com ara el glucomanà (Yoshida i col., 2006), la proteïna de soja (Lukaczer i col., 2006), la proteïna vegetal i la fibra d'alta viscositat (Jones i col., 2005), i tots aquests estudis han descrit reduccions en les concentracions de colesterol-LDL. Per exemple, Yoshida i col. (2006) van estudiar els efectes del consum de fitosterols i glucomanà separadament i en combinació, i van trobar només efectes additius d'aquests compostos, però no sinèrgics.

En el nostre estudi, no vam trobar ni un efecte sinèrgic ni un efecte additiu de la combinació de pectina i fitosterols (no es mostren aquests resultats). Les úniques

interaccions significatives que vam trobar van ser per les concentracions plasmàtiques de campesterol i β-sitosterol ($p<0.05$). Tal i com es mostra a Fig. 14, en tots els grups, tant el campesterol com el β-sitosterol van incrementar quan l'addició d'aquests compostos en la dieta era superior.

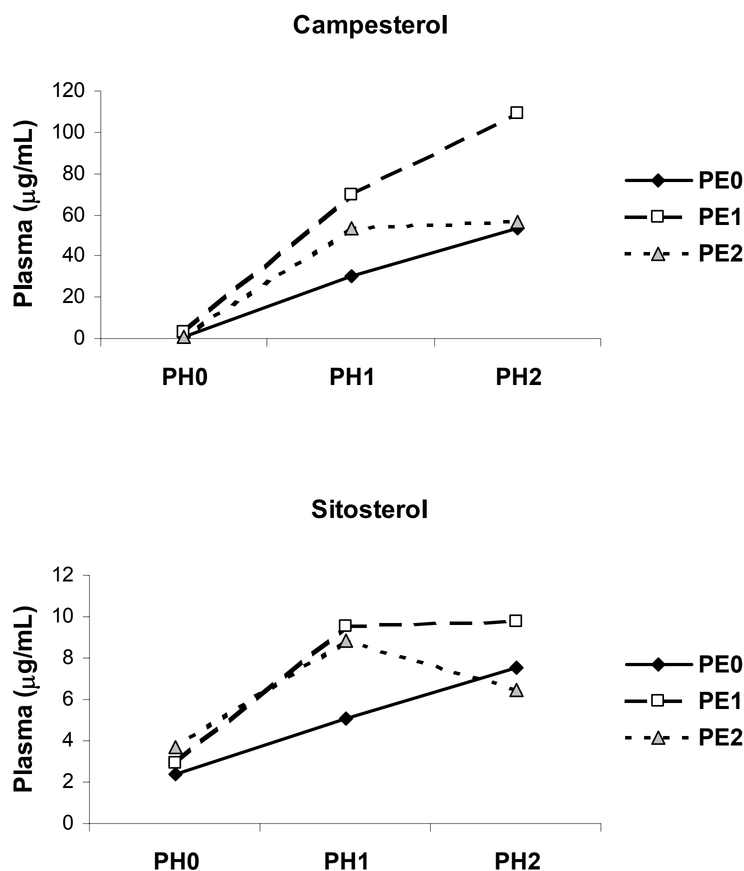

Fig. 14. Concentracions de campesterol i β-sitosterol en plasma, amb diferents combinacions de pectina i fitosterols.

En el grup sense pectina afegida, l'increment del campesterol i del β-sitosterol en el plasma va ser dosi-dependent a la seva quantitat en les dietes. En el grup PE2, comparant els animals del grup PH0 i PH1 es veu un increment en la concentració de fitosterols en plasma. En canvi, si es comparen els grups PH1 i PH2 no hi ha diferències en les concentracions de campesterol i β-sitosterol. En el grup PE1, les concentracions de campesterol en plasma tenen un perfil similar al que s'ha descrit pel grup sense pectina afegida, encara que les concentracions en aquest grup van ser superiors. En el grup PE1, les concentracions de β-sitosterol van ser superiors en els

animals alimentats amb pectina i fitosterols que en els animals alimentats només amb fitosterols.

Aquests resultats recolzen el que ja s'ha suggerit pels efectes de la pectina; possiblement la pectina es va hidrolitzar abans d'arribar al seu lloc d'acció. No obstant en el grup PE2, és possible que una part de la pectina romangués intacta i bloquegés en cert grau l'absorció d'aquests compostos, donat que no es van trobar diferències en les concentracions plasmàtiques de fitosterols al comparar els animals alimentats amb les dosis PH1 i PH2.

Els animals del grup PE1 també van absorbir els fitosterols millor que el grup control donat que les gràfiques dels animals del grup PE1 i PE0 són molt similars, però els del grup PE1 tenen concentracions més elevades pel campesterol i pel β-sitosterol en plasma. De totes maneres, comparant els resultats entre el campesterol i el β-sitosterol es pot veure que hi ha algunes diferències entre ells. Mentre que el campesterol va incrementar linealment amb la dosis de fitosterols de les dietes, les concentracions de β-sitosterol en els grups PE1/PH1 i PE1/PH2 van ser similars, probablement degut a que els campesterol s'incorpora millor a l'organisme animal que el β-sitosterol.

Per tant, en el nostre estudi, l'addició de pectina a dietes saturades enriquides amb fitosterols va provocar un augment en les concentracions plasmàtiques de fitosterols. No obstant, aquest efecte va ser més important a dosis intermèdies, ja que a concentracions més elevades de pectina, una part possiblement encara podia exercir la seva acció bloquejant l'absorció d'aquests compostos.

CONCLUSIONS FINALS

Després de la realització d'aquesta tesi doctoral es poden extreure les següents conclusions:

- L'addició de fitosterols esterificats amb AGI en una dieta amb elevat contingut en AGS i colesterol, modifica l'absorció aparent d'AGS en funció de la longitud de la cadena. Els àcids grassos de cadena intermèdia (làuric i mirístic) van incrementar la seva absorció, mentre que la dels de cadena llarga (behènic i araquídic) la van disminuir.

- L'addició de fitosterols esterificats amb AGI en una dieta amb elevat contingut en AGS i colesterol, condueix a un increment en la concentració d'AGS de cadena mitja (làuric i mirístic) en el fetge.

- Els animals alimentats amb fitosterols esterificats amb AGI, van veure reduïda l'absorció aparent de colesterol, tot i que el valor de colesterol *output* va disminuir, fet que suggereix un increment en l'absorció dels compostos derivats del metabolisme del colesterol. Aquest fet està estretament relacionat amb els valors d'absorció aparent trobats pels fitosterols (indicadors de l'absorció de colesterol), els qual també es van absorbir millor en els animals alimentats amb fitosterols.

- Els animals alimentats amb fitosterols van tenir concentracions hepàtiques de colesterol inferiors als animals del grup control. Aquesta reducció no va ser deguda a una reducció en la síntesi hepàtica de colesterol donat que no es van observar diferències en els seus precursors entre els diferents tractament. Les concentracions plasmàtiques de colesterol no van mostrar cap diferència entre els animals alimentats amb fitosterols dels animals del grup control.

- L'addició de pectina va provocar un increment en el pes corporal dels animals, suggerint un trencament prematur de la pectina. De totes maneres, encara que en el grup PE2 la concentració de pectina va ser el doble que

en el grup PE1, no es van trobar diferències en els pesos corporals dels animals d'aquests dos grups, fet que suggereix que una part de la pectina del grup PE2 va arribar intacta a l'intestí, on va exercir els seus efectes.

- Els animals dels grups alimentats amb pectina van tenir valors d'absorció aparent del colesterol i dels fitosterols superiors que els del grup control. No obstant, els animals dels grups alimentats amb dosis intermèdies (PE1) van ésser els que van mostrar els valors més elevats.

- Els animals del grup intermedi de pectina (PE1) van ser els que van tenir valors més elevats de colesterol en plasma. No obstant, no es van trobar diferències en les concentracions hepàtiques de colesterol.

- Els animals alimentats amb pectina van tenir concentracions més elevades de campesterol en el fetge i en el plasma, trobant les concentracions més elevades en els animals del grup intermedi (PE1).

- L'addició de pectina no va provocar cap modificació en l'absorció dels àcids grassos, ni tampoc en les concentracions dels àcids grassos en fetge o plasma.

- No es va trobar cap efecte sinèrgic de la pectina i fitosterols en les concentracions de colesterol, possiblement degut al possible trencament prematur de la pectina.

- Les úniques interaccions trobades entre els fitosterols i la pectina van ser per les concentracions plasmàtiques de campesterol i β-sitosterol. Els animals del grup intermedi de pectina van ser els que van tenir concentracions més elevades, fet que corrobora la hipòtesi d'una hidròlisi prematura de la pectina.

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