Review

Plant sterols: biosynthesis, biological function and their importance to human nutrition

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Abstract: Plant sterols are an essential component of the membranes of all eukaryotic organisms. They are either synthesised *de novo* or taken up from the environment. Their function appears to be to control membrane fluidity and permeability, although some plant sterols have a specific function in signal transduction. The phytosterols are products of the isoprenoid pathway. The dedicated pathway to sterol synthesis in photosynthetic plants occurs at the squalene stage through the activity of squalene synthetase. Although the activity of 3-hydroxymethyl-3-glutaryl coenzyme A (HGMR) is rate-limiting in the synthesis of cholesterol, this does not appear to be the case with the plant sterols. Up-regulation of HGMR appears to increase the biosynthesis of cycloartenol but not the Δ^5-sterols. A decline in sterol synthesis is associated with a suppression of squalene synthetase activity, which is probably a critical point in controlling carbon flow and end-product formation. The major post-squalene biosynthetic pathway is regulated by critical rate-limiting steps such as the methylation of cycloartenol into cycloeucalenol. Little is known about the factors controlling the biosynthesis of the end-point sterol esters or stanols. The commonly consumed plant sterols are sitosterol, stigmasterol and campesterol which are predominantly supplied by vegetable oils. The oils are a rich source of the steryl esters. Less important sources of sterols are cereals, nuts and vegetables. The nutritional interest derives from the fact that the sterols have a similar structure to cholesterol, and have the capacity to lower plasma cholesterol and LDL cholesterol. Since the morbidity and mortality from cardiovascular disease have been dramatically reduced using cholesterol-lowering drugs (statins), the interest in plant sterols lies in their potential to act as a natural preventive dietary product. Stanols (saturated at C-5) occur in low amounts in the diet and are equally effective in lowering plasma cholesterol and do not cause an increase in plasma levels, unlike the sterols which can be detected in plasma.
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INTRODUCTION

Plant sterols, also called phytosterols, have been reported to include over 250 different sterols and related compounds in various plant and marine materials.[1] The most common representatives are sitosterol, stigmasterol and campesterol (4-desmethyl sterols). The 4-methyl sterols and 4,4-dimethyl sterols are usually only minor components in most plant sources. Sitosterol is the principal sterol in plant materials, but in addition to its 22-dehydro analogue stigmasterol and campesterol, brassica- and avenasterols occur in many plant materials.

Chemical structures of these sterols are similar to cholesterol, differing in the side chain. For instance, sitosterol and stigmasterol have an ethyl group at C-24, and campesterol a methyl group at the same position. Small amounts of 5-saturated plant stanols, sitostanol and campestanol, occur in some plant materials, eg wheat and rye.[2] Cholestanol is formed analogously from cholesterol.

Plant materials and especially vegetable oils contain free and esterified steryls, and steryl glycosides which can be esterified to acylated steryl glycosides.[3] Free sterols, and to some extent also steryl glycosides and acylated steryl glycosides, are incorporated into cell membranes. Like cholesterol in mammalian cells, they perform an important role in the structure and function of cell membranes. Plant steryl esters are

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located intracellularly and they represent mostly a storage form of sterols, analogously to cholesteryl esters in mammalian organism. Thus the vegetable oils are rich in plant steryl esters. In addition to their important role in maintaining adequate function of plant cell membranes, plant sterols are precursors of a group of plant growth factors.

The usual human diet contains currently around 200–300 mg day^{-1} of plant sterols.[4] The higher the dietary intake of plant sterols from the diet, the lower is cholesterol absorption and the lower is the serum cholesterol level. In fact, isolated dietary plant sterols have been used in poorly soluble, mainly crystalline form since the early 1950s to lower serum cholesterol level in hypercholesterolemic patients.[5]

In the 1970s the interest in using plant sterols in large doses for the treatment of hypercholesterolemia decreased partly because of the variable effects seen, the consumption of some plant sterol products was difficult, and they increased serum concentrations of plant sterols to the levels seen in sitosterolemia. At that time, this clinical condition was described for the first time. It was subsequently shown to be an inheritable disorder characterised by enhanced absorption of cholesterol and plant sterols, resulting in the early development of atheromatosis and coronary heart disease as a result of an increase in total serum plant sterols.

In the 1970s, plant stanols, a mixture of sitostanol and campestanol, were shown to be virtually unabsorbable and were capable of lowering serum cholesterol much more effectively than the respective sterol mixture in experimental animals. Ten years later the stanol ester mixture was shown to be hypolipidemic in hypercholesterolemic patients, even though no consistent effect was observed in all studies with this poorly soluble stanol preparation. However, the preparation of fat-soluble stanyl esters showed a consistent 10–15% reduction in serum total cholesterol when consumed as stanyl ester mayonnaise or margarine.[6]

These recent observations have renewed the research interest in plant sterols, as well as attempts to develop new functional foods, especially those with an increased plant sterol content for the lowering of serum cholesterol. Since effective serum cholesterol lowering using hypolipidemic drugs has led to a dramatic reduction in the mortality and morbidity for a cardiovascular diseases in primary and secondary prevention studies, and has been shown to be economically beneficial, the importance of the dietary treatment of hypercholesterolemia has been given particular emphasis. Diets rich in plant materials have become recommended in the majority of public health education programmes, not only because of the presence of endogenous and exogenous plant sterols and stanols, but also because of the presence of antioxidants. Diets lacking in these components have been strongly associated with the development of atheromatous arterial disease.[7]

CHEMICAL AND PHYSICAL PROPERTIES OF PLANT STEROLS

Plant sterols are steroid alcohols. They resemble cholesterol, the predominant sterol found in animals, both in their chemical structure and their biological function. Plant sterols and cholesterol are biosynthetically derived from squalene and form a group of triterpenes.[8] They are made up of a tetracyclic cyclopenta[*a*]phenanthrene ring and a long flexible side chain at the C-17 carbon atom. The four rings (A, B, C, D) have *trans* ring junctures, forming a flat α system. The side chain and the two methyl groups (C-18, C-19) are angular to the ring structure and above the plane, thus having β stereochemistry.[9] Moreover, since 20*R* conformation is preferred in the side chain, the sterols create planar surfaces at both the top and the bottom of the molecules, which allow for multiple hydrophobic interactions between the rigid sterol nucleus and the membrane matrix.[10,11] Generally, the hydroxyl group at C-3 also has β stereochemistry. The chemical name for cholesterol is 5α-cholesten-3β-ol, which contains one double bond compared to the basic sterol compound 5α-cholestan-3β-ol (Fig 1).

Plant sterols can be divided on structural and biosynthetical grounds into 4-desmethyl sterols, 4α-monomethyl sterols and 4,4-dimethyl sterols.[1] The 4,4-dimethyl sterols and 4α-methyl sterols are plant sterol precursors and exist at lower levels than the terminal products 4-desmethyl sterols.[12] The 4-desmethyl sterols may be categorized into Δ^5-sterols, Δ^7-sterols and $\Delta^{5,7}$-sterols according to the position and number of double bonds in the B ring.[10]

Most plant sterols, eg campesterol and sitosterol, have a Δ^5 bond and an additional one-carbon or two-carbon substituent in the side chain at C-24 (Fig 2).

These substituents are introduced by *trans*-methylation reactions. Methyl and ethyl substituents may have α or β chirality. Most 24-ethyl sterols are 24α-epimers, whereas 24-methyl sterols occur as mixtures of 24α-epimers and 24β-epimers.[1,12] Alkylation of C-24 is a reaction specific to plants.[8] Another characteristic of plant sterols is the frequent presence of *trans*-Δ^{22} double bonds in the side chain, as eg in stigmasterol (Fig 2). Double bonds exist in *cis* configuration to a lesser extent.[13] As representatives of minor plant sterols, the structures of Δ^7-avenasterol as a Δ^7-sterol, ergosterol as a $\Delta^{5,7}$-sterol, sitostanol as a saturated

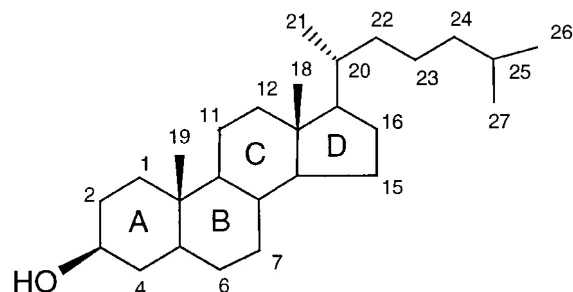

Figure 1. Chemical structure of 5α-cholestan-3β-ol.

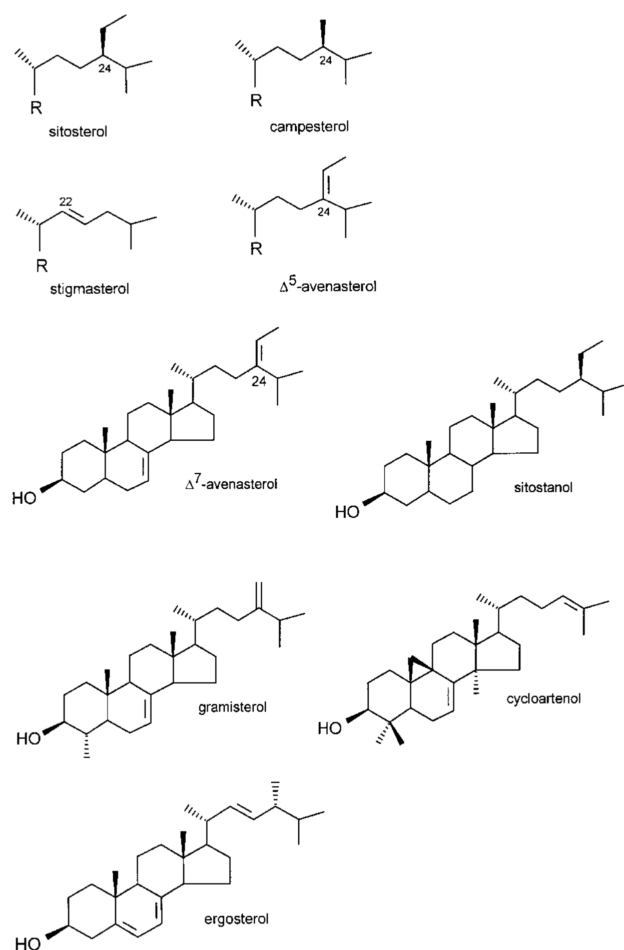

Figure 2. Examples of structures of major plant Δ^5-sterols with a similar sterol nucleus (R): sitosterol (24α-ethylcholest-5-en-3β-ol), campesterol (24α-methylcholest-5-en-3β-ol), stigmasterol (24α-ethylcholest-5,22-en-3β-ol) and Δ^5-avenasterol (*Z*-24-ethylidenecholest-5-en-3β-ol); and of minor sterols: Δ^7-avenasterol (*Z*-24-ethylidenecholest-7-en-3β-ol), sitostanol (24α-ethylcholestan-3β-ol), ergosterol (24β-methylcholest-5,7-22-en-3β-ol), gramisterol (4α-methyl-24-methylenecholest-7-ene-3β-ol) and cycloartenol (9,19-cyclo-4,4,14α-trimethylcholest-24-en-3β-ol).

sterol, gramisterol as a 4α-monomethyl sterol and cycloartenol as a 4,4-dimethyl sterol are shown in Fig 2.

The 3-hydroxyl group of free sterols may be esterified by a fatty acid or a phenolic acid to give steryl esters, or it may be β-linked to the 1′-position of a carbohydrate to form either steryl glycosides or acylated steryl glycosides (Fig 3). In acylated steryl glycosides the 6′-position of the carbohydrate is esterified with a long-chain fatty acid.

Plant sterols are amphiphilic and occur as membrane constituents. They are primarily encountered in the plasma membrane, the outer membrane of mitochondria and the endoplasmic reticulum, and to a large extent determine the properties of these membranes. Free sterols fit in membranes, because the overall length of free sterols is virtually the same as that of a phospholipid monolayer, ie 2.1 nm.[10]

In bulk the plant sterols are solid, eg sitosterol, campesterol and stigmasterol have melting points of 140, 157–158 and 170 °C respectively.[1] The bigger its

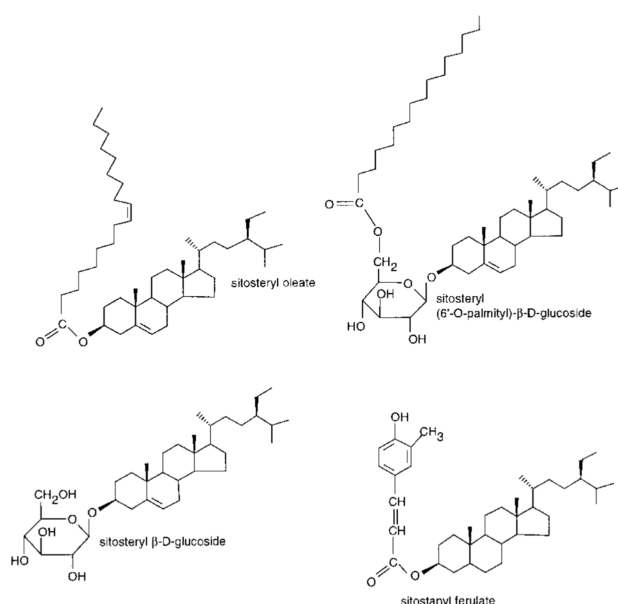

Figure 3. Examples of steryl conjugate structures.

side chain, the more hydrophobic a sterol becomes. Thus plant sterols with 28 or 29 carbon atoms are more hydrophobic and have lower micellar solubilities than cholesterol with 27 carbon atoms.[10,16] Side chain double bonds make sterols more hydrophilic.[16] However, free sterols and steryl esters are soluble in non-polar solvents such as hexane, while polar modifiers are needed to solubilise steryl glycosides.[12]

The oxidation of plant sterols is the most important chemical reaction they undergo controlling process and storage stability. The oxidation mechanism of cholesterol has been extensively studied and it is suggested that the oxidation mechanism of other Δ^5-sterols follows the same scheme. Although the products of cholesterol oxidation are well established, much less is known about the plant sterol products.[17] In the presence of heat, light, metal contaminants and oxygen, preformed radicals easily attack the double bond in the ring structure, beginning an autocatalytic free radical chain reaction following the same chemistry as the oxidation of other unsaturated lipids. Reactive oxygen species and oxidative enzymes may also begin plant sterol oxidation. The primary product, the 7-hydroperoxide, is formed after an allylic hydrogen atom has been abstracted at C-7. The allylic hydroperoxide undergoes epimerisation to form both 7α- and 7β-hydroperoxides from which the major oxidation products 7β- and 7α-hydroxy compounds, as well as 7-keto compounds, are derived. Other important products include 5,6α- and 5,6β-epoxy compounds and 3β,5,6β-triols (Fig 4).[17–19]

Oxygen attack may also occur at the side chain at tertiary carbons; however, products such as 20- and 25-hydroxy compounds are formed in minor amounts. It has been postulated that a sterol with an ethylidene double bond, eg Δ^5-avenasterol, may be protective against oxidation at frying temperatures.[20,21] Moreover, sterols esterified with ferulic or other phenolic

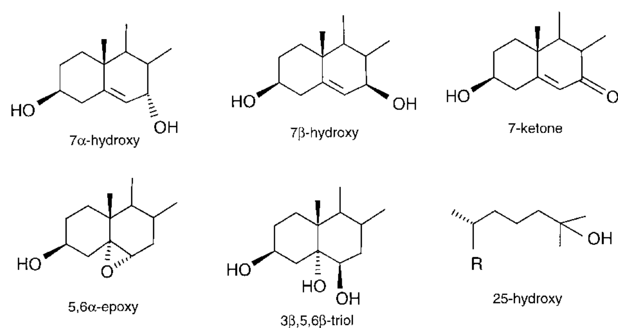

Figure 4. Functional groups of major oxidation products of sterols (R, sterol nucleus).

acids should act as antioxidants because of the properties of the acid moiety.[22,23]

BIOLOGICAL FUNCTIONS IN PLANTS

Sterols regulate the fluidity of membranes and probably play a role in the adaptation of membranes to temperature. A free hydroxyl group in free sterols is an important factor enabling its specific interactions with phospholipids and proteins in membranes.[3] Steryl glycosides and acylated steryl glycosides co-occur with free sterols in various membranes with the carbohydrate group oriented in the aqueous region.[14,24] In addition, sterols participate in the control of membrane-associated metabolic processes, which involves the action of a few specific sterols. Sterols also play an important role in cellular and developmental processes in plants as precursors to the brassinosteroids. They also act as substrates for a wide variety of secondary metabolites such as the glycoalkaloids, cardenolides and saponins.

Sterol molecules are incorporated into membranes. From isolated membrane studies it has been shown that the side chain extends into the hydophobic core and interacts with fatty acyl chains of phospholipids and proteins with the free 3-hydroxyl facing the aqueous phase. The typical plant sterols (sitosterol, stigmasterol and campesterol) act in membranes to restrict the motion of fatty acyl chains. All plant sterols are able to regulate membrane fluidity, but with different efficiencies. Sitosterol and campesterol are the most efficient. In comparison, stigmasterol with its *trans*-oriented double bond at C-22 has a reduced ordering effect.

The stereochemistry of C-24 also interferes with the sterol–membrane interaction.[25] Sitosterol and campesterol reduce membrane permeability effectively in soybean phospholipid bilayers.[26] Stigmasterol is much less efficient. Experiments in these artificial membranes have shown a good correlation between acyl chain ordering and water permeability.

Sterols also play a role in cellular differentiation and proliferation. Their accumulation in seeds and oils is likely to provide a reservoir for the growth of new cells and shoots. Active sterol synthesis occurs following the germination of the seed and then gradually decreases as the seed matures.[27] The roles of steryl esters also include storage and transport; this is supported by their presence in soluble forms, such as in lipoprotein complexes.[3,15]

Plant sterols modulate the activity of ATPase in the membrane of maize roots.[28] Cholesterol and stigmasterol stimulate the export of H^+ at low concentrations, whereas all other sterols act as inhibitors. In this regard they may function in the same way as cholesterol can in activating the Na^+/K^+-ATPase of animal cells. Specific sterol molecules may also participate in some signal transduction events as is the case for cholesterol in mammalian cells.

BIOSYNTHESIS OF STEROLS

The phytosterols are products of the isoprenoid biosynthetic pathway (Fig 5). Isopentenyl pyrophosphate (IPP) serves as the fundamental building block for the biosynthesis of all the terpenoids, including sterols which are C_{30} triterpenoids. In the case of the synthesis of sterols it has been shown that the IPP derives exclusively from the mevalonic acid pathway, as opposed to the pyruvate/glyceraldehyde-3-phosphate pathway which appears to be responsible for the synthesis of terpenes of plasticidal origin.[29] However, it has been reported that in germinating corn seedlings, mevalonic acid may not be an intermediate in the synthesis of the phytosterols,[27] with other pathways able to direct carbon flux from sugars and amino acids into IPP. This observation throws doubt on the pivotal role of 3-hydroxymethyl-3-glutaryl coenzyme A reductase (HMGR) in the plant sterol pathway.

The conversion of IPP into dimethylalkyl diphosphate (DMAPP) serves as the primer for elongation through a prenyl transferase, FPP synthase, that converts the metabolite to farnesyl pyrophosphate (FPP), which is the branch point for the synthesis of sesquiterpenes and triterpenes (sterols). Direction into

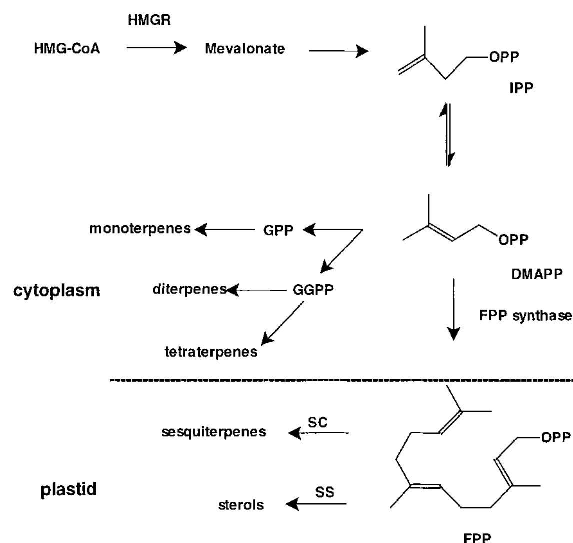

Figure 5. Biosynthesis of isoprenoids in plants from IPP/DMAPP.

the sterol pathway is controlled by the activity of squalene synthetase. Sterols are exclusively synthesised in the cytoplasm

In plants the sterol pathway (Fig 6) consists of a sequence of more than 30 enzyme-catalysed reactions, all of which are found in the plant membranes.[24,30] The enzymic steps taken by plants with a photosynthetic apparatus convert squalene oxide into cycloartenol, whereas non-photosynthetic fungi convert squalene oxide into lanosterol and finally ergosterol.[31] Both lanosterol and cycloartenol can be converted into Δ^5-24-alkyl sterol end-products.

Plants are thought to transform sterols by a series of interacting pathways. The major post-squalene biosynthetic pathway is regulated by critical rate-limiting steps such as the methylation of cycloartenol[32] into cycloeucalenol. The 9β, 19-cyclopropane ring of cycloeucalenol is opened by cycloeucalenol obtusifoliol isomerase (COI) to form obtusifol. Two classes of fungicides widely used in agriculture have been found to inhibit COI and obtusifoliol 14-demethylase (OBT14DM). Inhibition of both enzymes leads to the formation of unusual sterols at the expense of the Δ^5-sterols (Fig 7). A further alkylation occurs at the C-24 alkyl chain to give the end-product sterols which are always produced as a mixture. However, the end-products are always stereochemically pure 24α-alkyl derivatives. Various pathways have been proposed leading from cycloartenol to 24-methyl and 24-ethyl sterol end-products. All the sterolic enzymes may lack a high degree of sterol specificity, and multiple pathways have been considered as converging into the pathway that leads to sitosterol formation.[24] Experiments on corn, sunflower and bean show differences in K_m values with a range of substrates, but enzyme studies and the similarity in the distribution of sterols in several plants indicate a fairly uniform route for the synthesis of stigmasterol from cycloartenol.[33]

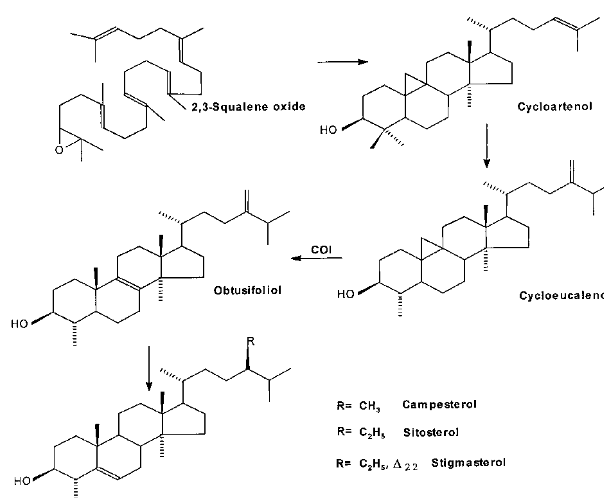

Figure 7. Synthesis of 9β, 19-cyclopropyl steroids following treatment with inhibitors of cycloencalenol obtusi foliol isomerase (COI) and obtusi foliol 14-demethylase (OBT14DM).

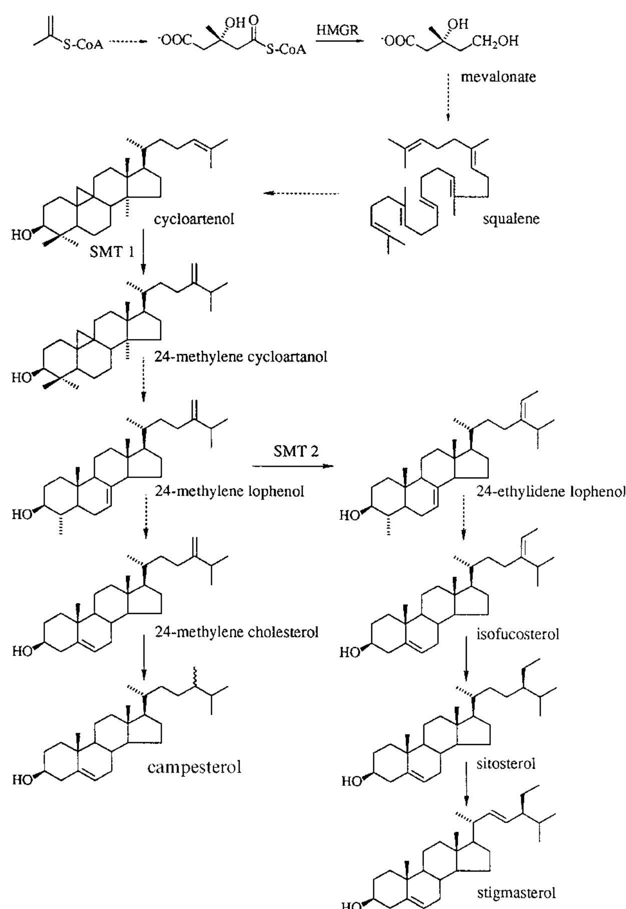

Figure 6. Synthesis of sterols by photosynthetic plants.

Although in most higher plants the major end-products of the synthesis have a free 3β-hydroxyl group, sterols are also found conjugated by esterification (by a long-chain fatty acid) or are β-linked to the 1-position of a monosaccharide (usually glucose) to form steryl glucosides. Acyl steryl glucosides can also be formed by esterification of the 6-position of the sugar moeity. Very little is known about the biosynthetic and regulatory steps leading to their production.

Pre-squalene pathway: 3-hydroxymethyl-3-glutaryl coenzyme A reductase (HMGR)

In the case of the synthesis of the mammalian sterols the rate-limiting step in their biosynthesis is at an early stage in the isoprenoid pathway as a result of the activity of HMGR.[34] Much of the effort in understanding the regulation of phytosterol biosynthesis has been focused on the role played by HMGR. Some studies have suggested that HMGR activity is a key step in the synthesis of sterols, but not all of the evidence supports the view that it is a rate-limiting or regulatory step.

There is frequently a good correlation between the level of HMGR activity and the rate of sterol production.[35,36] If HMGR activity is amplified, the levels of Δ^5-sterols are not increased beyond the levels

of the free sterols that can be incorporated into membranes, but the levels of cycloartenol and related intermediates are increased.

It would seem that there are arrays of isozymes in the isoprenoid pathway that are dedicated to the production of specific classes of isoprenoids, with each array independently regulated. Tobacco was transformed by part of the hamster HMGR gene that encodes a soluble HGMR activity. The levels of sterols and elicitor-inducible sesquiterpenes (synthesised in the cytosol) and carotenoid and phytol (synthesised in the chloroplast) were measured and compared with non-transgenic plants. It was only in the transgenic plants that the levels of sterols were significantly increased and the predominant sterol found was cycloartenol. This suggests that HMGR activity is not limiting for the synthesis of the common Δ^5-sterols but is limiting for cycloartenol synthesis.[37] As carotenoid and phytol synthesis was unaltered in the transgenic plants, it suggests that their synthesis is likely to occur independently of sterol biosynthesis. It has been suggested that plastids could independently regulate their own isoprenoid biosynthetic needs, or alternatively there may be co-ordination between a cytosolic and plasticidic pathway.[38]

In tobacco cell suspension cultures there is a steady accumulation of sterols corresponding to the rapid phase of plant cell growth. Elicitation of the sesquiterpene phytoalexins causes a suppression of sterol synthesis and the accumulation of sesquiterpenes.[39,40] Although a correlation between the induction of sesquiterpene biosynthesis and HMGR activity has been observed, the induction of HMGR activity was rapid and transient whilst the accumulation time course for sesquiterpenes was much more protracted.[36,41,42] A seven-amino-acid conserve sequence has been identified from a sequence of HGMR isozymes associated with sesquiterpene biosynthesis in the potato, tomato and in tobacco, and sesquiterpene cyclase enzymes from tobacco and *Hyoscyamus muticus*. This sequence is absent in HGMR proteins associated with sterol synthesis. A model has been proposed of how specific targeting of HGMR isoforms to phytoalexin production or sterol production might arise.[43,44]

This work suggests that the regulation of sterol and sesquiterpene synthesis is downstream of mevalonate and HGMR activity. The decline in sterol synthesis is associated with a suppression of squalene synthase activity and an induction of a sesquiterpene cyclase enzyme activity. Since these two enzymes are positioned at a putative branch point in the pathway, the induction of one enzyme and the suppression of another are regarded as an important control mechanism for controlling carbon flow and end-product formation.

There are multiple genes coding HMGR in plants whose number varies according to the plant, from two in *Arabidopsis* to over 12 in the potato.[45,46] It is currently thought that there are unique roles for each of the genes. The multigene families exhibit different tissue and developmental patterns of expression and respond differently to environmental stimulii.[47,48] The HMG1 gene in *Arabidopsis* utilises two different transcription start sites, resulting in a short (HMGR1S) and a long (HMGR1L) form.[44] The short form is expressed at relatively high levels throughout the plant, perhaps being required for normal cell growth (ie sterol synthesis). The long form is only expressed in specific tissues and at low levels.

An observation that may be important in the regulation of specific isoforms of HGMR is that there is an N-linked glycosylation site present in all the isoforms of HGMR that are associated with the production of elicited sesquiterpenoids, whereas it is absent from isoforms of HGMR that are associated with rapidly growing tissues or the constitutive synthesis of sterols.[44] The available evidence suggests that HMGR is targeted exclusively to the endoplasmic reticulum in plants. It is thought that differences in the N-terminal sequences of the isoforms of HMGR and the extent of glycosylation may target the enzyme to different domains of the endoplasmic reticulum or to different organelles, allowing selectivity in the synthesis of specific families of terpenoids.[29] However, this hypothesis remains to be confirmed.

Wound-induced steroid accumulation in potato is correlated with the expression of the HMG1 gene, whereas elicitor-induced sesquiterpene phytoalexin production is correlated with HMG2 and HMG3 gene expression. HMG1 was not expressed under these circumstances.[46] Studies in tobacco (Fig 8) and *Tabernaemontana divaricata* have shown a co-ordinated regulation of multiple enzymes in response to fungal induction of phytoalexins, including a transient induction of HGMR, IPP isomerase, FPP synthase and specific terpenoid synthases, and a co-ordinated suppression of sterol synthase.[49,50]

Similar results have been obtained in pathogen- or elicitor-challenged potato tuber discs. Wounding of the tissue stimulates carbon flow into the sterol biosynthetic pathway. When the studies were combined with pulse-labelling experiments and enzyme activity measurements, it suggested that squalene synthetase was a major control point for the regulation of sterol biosynthesis in plants.[51]

An understanding of the factors that regulate squalene synthetase activity and gene expression is much more limited compared with other terpene cyclases. The plant enzyme is membrane-bound and is present in low amounts. However, work on the mammalian squalene synthetase gene has indicated that the genes are regulated by their transcription rate in response to exogeneous sterols and inhibitors of sterol biosynthesis.[52,53] In contrast, in tobacco it has been shown that squalene synthetase mRNA levels are not altered in cells that have been treated with elicitors.[38] This suggests that there is some sort of post-translational control of the enzyme activity. This

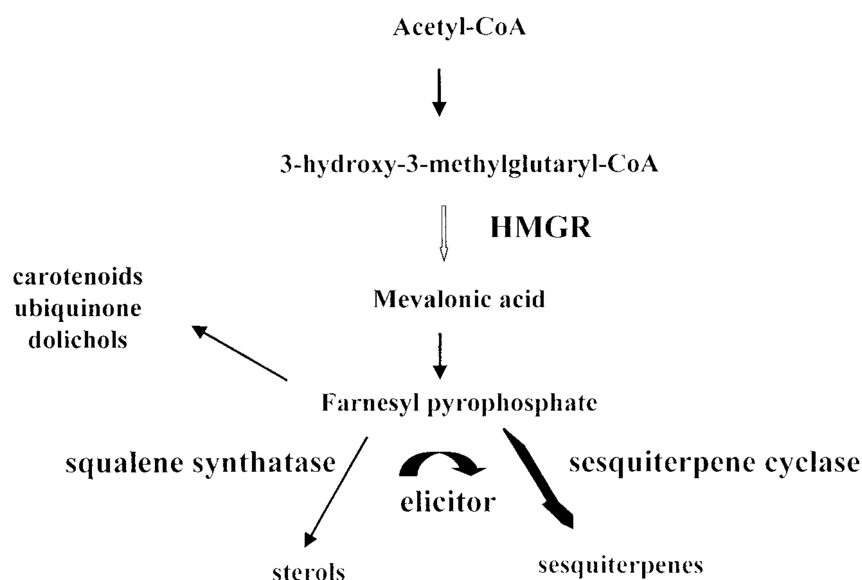

Figure 8. Biochemical changes occurring in elicitor-treated tobacco cell suspensions.

is known in mammalian systems but still has to be elucidated in plants.

FPP synthase is also expressed by multiple genes. Two different isoforms are obtained from the FPS1 gene of *Arabidopsis*.[54] The major isoform encodes a cytoplasmic form of the enzyme, whilst a minor isoform is targeted to the mitochondria.

Post-squalene pathway

The major pathway of relevance to the food chain leads to a plant sterol profile characterised by a mixture of stigmasterol, sitosterol and campesterol. Conversion of cycloartenol to sitosterol and stigmasterol involves two methylation steps, and the resulting products are stereochemically pure 24α-ethyl sterols.

The post-squalene biosynthetic pathway is characterised by critical rate-limiting steps such as the methylation of cycloartenol.[32] Two different families of methyl transferases have been identified in the biosynthesis of the 24-methyl and 24-ethyl sterols. Experiments in tobacco have resulted in the characterisation of a gene which encodes a cycloartenol C-24 methyl transferase (SMT1). Another gene codes for the enzyme SMT2 (SAM-24-methylene lophenol-C-24^1-methyl transferase) which is responsible for the ability of plants to synthesise 24-ethyl sterols (Fig 6). Similar results have been obtained in *Oriza sativa*.[55] The identification of these two distinct methyl transferases, each catalysing a separate step in the pathway of sterol biosynthesis, has opened up new avenues to study the role these enzymes might play as rate-determining steps in plant sterol biosynthesis.

Interestingly, studies on the action of specific inhibitors have shown that inhibitors of cycloeucalenol obtusifoliol isomerase (COI) have little impact on the growth of maize seedlings, even though 95% of the typical Δ^5-sterols are replaced by 9β,19-cyclopropyl steroids (Fig 7), and the position of accumulation of the free sterols can shift from the plasma membrane to the endoplasmic reticulum.[56]

Measurements of HGMR and SMT activities in corn seedlings showed that HGMR activity was not correlated with development or Δ^5-sterol production, whereas SMT was associated with Δ^5-sterol production.[33] Blades produce mainly Δ^5-24-ethyl sterols, whereas sheaths, which develop from the blades, produce mainly $\Delta^{5,23(24)}$-24-methyl sterols. The difference in the sterol profiles between the sheath and blade is related to a shift in C-24 biomethylation as the sheath develops. This suggests that the SMT may be a key regulatory enzyme in phytosterol synthesis. In sunflower and corn it has been found that of those enzymes that act on cycloartenol the SMT has the highest degree of sterol specificity and is a relatively slow-acting enzyme, which supports its key role. Expression of the *Arabidopsis* gene for SMT2 in tobacco produces a sixfold increase in microsomal SMT2 activity and modification of the 24-alkyl-Δ^5-sterol composition. Campesterol/sitosterol ratios, which are close to unity in the wild type, fall to 0.01–0.3. In addition, transgenic plants showing the lowest ratios have a radically decreased level of campesterol and a corresponding increased level of sitosterol and are characterised by reduced growth.[55]

The chirality of 24α-ethyl sterols results from the action of a 24-ethyl reductase. The ability of some plants to produce a mixture of the α- and β-epimers suggests that different sterol biomethylation pathways have evolved, or alternatively that there are isozymes of SMTs which are expressed differently during the plant's development.

Sitosterol down-regulates the activity of C-24 methyl transferase activity in the sunflower, which is consistent with the feedback control of the first step in the pathway.[57]

Currently it is thought that the enzymes that control phytosterol production respond to changes in substrate concentrations. Metabolite production in excess of the requirements of the enzymic steps is incorporated into membranes and into oil storage bodies as

esters. When the levels rise to a point where there is no effective sink, then the enzymes controlling their biosynthesis are down-regulated

Mutants of the sterol biosynthetic pathway

In contrast to the extensive studies that have been undertaken on the characterisation of mutants that are defective in ergosterol biosynthesis, as well as the cloning of all the relevant genes in the ergosterol pathway,[58] most of the genes characterising the sterol biosynthetic pathway are unknown.[59] This situation will change dramatically with the commercial and scientific interest that exists in characterising the pathway of sterol biosynthesis.

Experiments with UV-mutagenised protoplast-derived tobacco mutants which were resistant to sterol inhibitors of the later stages of the biosynthetic pathway show a dramatic alteration in sterol composition and very significant increases in the proportion of cyclopropyl sterols (5–15-fold). However, 24-alkyl-Δ^5-sterols also accumulate.

In these mutants, extra sterol accumulates predominantly as sterol esters associated with cytoplasmic lipid storage bodies.[62] The mutant cells regulate their free sterol composition in order to maintain membrane integrity. The phenotype of the mutation does not require continuous selection with inhibitors, but the altered sterol composition is attenuated in regenerated plants and is dampened in successive generations.[60] Genetic studies have also shown that the alteration in sterol composition is segregated as a single semidominant mutation.

Biochemical analysis indicates that HMGR activity was increased up to fourfold. It has yet to be shown whether these mutants affect HMGR activity directly or indirectly or some other downstream enzymic activity.

Identification of specific genes in *Arabidopsis* is well advanced and a plant sterol mutant (STE1) has recently been isolated.[61] This mutant is defective in the Δ^7-sterol C-5 desaturase and accumulates Δ^7-sterols,[63] but has no specific phenotype.

Turnover

In mammalian systems, regulation of cholesterol synthesis has been shown to involve the co-ordinated synthesis of HMG-CoA synthetase, HMGR and FPP synthetase, although the degradative turnover of these proteins is regulated independently of each other.[64,65] This type of co-ordinated regulation undoubtedly exists in plants. Nothing is presently known about the degradative turnover of the sterol biosynthetic enzymes.

Potential strategies for enhancement

The nutritional interest in manipulating levels of individual sterols in food plants is focused entirely on the efficacy of the sterol to reduce plasma cholesterol levels effectively. The manipulation of enzymes in the pre-squalene synthase pathway is likely to lead to the production of cycloartenol and related sterols. It is not known how effective these sterols are in reducing plasma cholesterol. In addition, they are not commonly consumed in the amounts that would be necessary to produce effects, and detailed issues in relation to the potential safety of the product could be raised.

The post-squalene pathway offers most prospects for manipulation and should be studied in further detail. In particular, more information is required on:

- the rate-determing role of the methyl transferase enzymes in the pathway;
- the genes responsible for the synthesis of stanols, and the factors regulating the activity of the enzymes responsible for their production in plants;
- understanding the steps leading to the esterification of sterols and their storage in the plant cell, and the enzymes involved;
- detailed biochemical characterisation of the mutants that lead to the overproduction of sterols.

PLANT FOOD SOURCES

Important food sources

In general, vegetable oils and products derived from oils are regarded as the richest natural sources of sterols, followed by cereal grains, cereal-based products and nuts.

At the level of total diet, oils, fats and cereal products have been shown to be the most important sources. In the UK, oils and fats were significant contributors to the estimated daily intakes of brassicasterol (81%), campesterol (59%), Δ^7-stigmasterol (54%), sitosterol (44%), Δ^7-avenasterol (44%) and Δ^5-avenasterol (39%). Bread and other cereals were also important sources of several sterols, accounting for a further 36% of the intakes of sitosterol and Δ^5-avenasterol and 50% of the intake of Δ^7-avenasterol.[66] Recent studies have found that the total estimated daily intake of plant sterols ranges from 146 to 405 mg.[4,66–69] Intake values depend on type of food intake, such that some vegetarians can have almost an intake of 1 g day^{-1} of plant sterols, whereas others may consume even less than the non-vegetarian population. A diet rich in shellfish, eg clams, oysters and scallops, can contain brassicasterol, Δ^5-avenasterol, 22-dehydrocholesterol and campesterol, reaching the daily intake of certain vegetarians.[68,70] Plant stanol intake seems to be roughly 10% of the usual dietary plant sterol intake.[71]

Factors causing variation and inconsistency in the results should be borne in mind when sterol contents and compositions of different natural sources are evaluated and compared. Analytical methods developed recently enable more specific determination of the various components (See next section). On the other hand, sample preparation techniques, eg which bound sterol structures are liberated and included, have significant effects on the results.[72–74] Further-

Table 1. Plant sterols in edible oils (g kg^{-1})

Food item	*Total sterols*	*Sitosterol*	*Campesterol*	*Stigmasterol*	*Δ^5-Avenasterol*	*Brassicasterol*	*Reference*
Corn oil							
Crude	8.09–15.57	9.89	2.59	0.98	0.36	tr	75–79
Refined	7.15–9.52	6.90	1.58	0.76	0.22	tr	75, 79
Cottonseed							
Crude	4.31–5.39	4.00	0.26	tr	0.05	tr	75, 77
Refined	3.27–3.97	3.03–3.43	0.20–0.31	tr–0.04	0.04–0.09	tr	77, 156
Olive							
Extra virgin	1.44–1.50	1.18–1.21	0.05	0.01	0.17–0.18	—	84, 156
Pomace	2.61–2.82	2.21–2.36	0.09–0.10	0.05–0.06	0.08	0.01	84, 156
Palm							
Crude	0.71–1.17	0.72	0.23	0.04	0.02	tr	75, 76
Refined	0.49–0.61	0.30–0.35	0.10–0.18	0.06–0.07	tr	tr	2, 75
Rapeseed							
Crude	5.13–9.79	2.84–3.58	1.56–2.48	0.02–0.04	0.13–0.19	0.55–0.73	75–77, 79, 84, 120
Refined	2.50–7.31	1.38–3.73	0.76–2.70	tr	0.06–0.23	0.27–0.54	75, 79, 83
Rice bran							
Crude	32.25	17.45	6.58	2.52	3.55	—	75
Refined	10.55	5.71	2.15	0.82	1.16	—	75
Soybean							
Crude	2.29–4.59	1.22–2.31	0.62–0.76	0.45–0.76	—	—	75–77, 79, 91, 121
Refined	2.21–3.28	1.23–1.73	0.47–0.82	0.47–0.52	0.01–0.02	—	2, 75, 79
Sunflower							
Crude	3.74–7.25	4.65	0.69	0.75	0.28	—	75, 77

more, inaccurate sample description may hinder exact comparisons.

Vegetable oils

Total sterol contents. Based on earlier studies, the majority of crude vegetable oils contain 1–5 g kg^{-1} of plant sterols. The most significant exceptions are corn, rapeseed, rice bran and wheat germ oils which contain higher amounts of sterols.[75,76] The range for 13 commercial crude oils was from 1.29 to 15.57 g kg^{-1} when desmethyl, monomethyl and dimethyl sterols were included.[77] Later reports support the above generalisation. The total sterol contents reported for corn oil range from 8.09 to 15.57 g kg^{-1} and for rapeseed oil 5.13 to 9.79 g kg^{-1}.[75–79] In rice bran oil the total sterol content was 32.25 g kg^{-1} and in wheat germ oil 19.70 g kg^{-1}.[75] In Table 1, sterol contents of some crude and refined oils are given. The effects of refining processes are discussed later (see industrial processing).

Proportions of different sterols. The predominant sterol class in vegetable oils is 4-desmethyl sterols. Their proportion was above 85% in most of the 13 crude oils analysed.[77] Sitosterol usually contributes more than 50% of desmethyl sterols. The other most significant desmethyl sterols include campesterol, stigmasterol, Δ^5-avenasterol, Δ^7-avenasterol and Δ^7-stigmastenol,[2,80,81,83,84] although their proportions vary in the literature depending on which sterols are included in the total contents and on the specificity of the analytical method used. Brassicasterol is a typical sterol for rapeseed and other *Cruciferaceae*. Stanols occur in significant amounts in corn bran and fibre oil (see cereal products).

The 4,4-dimethyl sterols of rice bran oil have recently been the focus of research (see cereal products). Ferulic acid esters of cycloartenol, 24-methylene cycloartanol and cyclobranol, together with some desmethyl steryl ferulic acid esters, are components of a commercial product γ-oryzanol which is produced from rice bran. Among commonly used oils, olive and linseed oils contain substantial amounts of dimethyl sterols, with cycloartenol and 24-methylene cycloartanol being the main components.[77,85] The contents of monomethyl sterols are usually low. They varied from 70 to 780 mg kg^{-1} in 13 crude oils.[77] The main monomethyl sterols are usually obtusifoliol, gramisterol, cycloeucalenol and citrostadienol.[77,86,87]

Bound sterol structures. Sterols of vegetable oils occur mainly as free sterols and esters of fatty acids, mainly as esters of linoleic and oleic acids. Esters with phenolic acids, mentioned previously, are discussed in more detail later (see cereal products). Glycosides, possibly present in crude oils, are removed in refining.[88] The proportions of free sterols and steryl fatty acid esters vary widely among different oils. Free sterols dominated in soybean, olive and sunflower oils (57–82%), but in canola, avocado and corn oils, free sterols represented only 33–38% of total plant sterols.[89] These figures are in line with previous data.[77,78]

Some differences in the sterol compositions of the free sterol and steryl fatty acid ester fractions have been reported. More stigmasterol and less campesterol were

found in the free sterol fraction of peanut oil, whereas the free sterol fraction of corn oil contained less Δ^5-avenasterol and more campesterol than the ester fraction.[78] The steryl ester fractions of sesame oils had a higher percentage of the 'early' sterols in the biosynthetic pathway (the methyl sterols, Δ^7-sterols and campesterol), compared to free sterol fractions that were characterised by a higher percentage of the late sterols.[87]

Variation. Genetic factors as well as growing and storage conditions may affect the sterol contents of oil seeds, and further variation may be caused by different processing conditions (see industrial processing). When 17 soybean genotypes were compared, a high variation was obvious in the sterol contents. Sitosterol was always the main sterol, but the total sterol contents ranged from 6 to 51% of unsaponifiables, and sitosterol accounted for 35–59%, stigmasterol 12–20% and campesterol 6–22% of the total sterols.[90] In a later study, experimental soybean lines, produced by hybridisation breeding, and one standard cultivar were compared.[91] The increase or decrease in the total content generally did not alter the relative percentage composition of the individual sterols, even though the absolute amounts varied. However, the percentage of sitosterol, the primary plant sterol in soybean oil, was lower in two oils; the total variation was from 0.93 to 1.71 g kg^{-1}, whereas in the standard cultivar the corresponding concentration was 1.22 g kg^{-1}.

Abidi *et al*[92] compared different lines of genetically modified canola varieties differing in their fatty acid compositions. Plant sterol composition was markedly influenced by genetic modification. Brassicasterol contents of modified oils ranged from 0.85 to 1.89 g kg^{-1} as compared to 2.00 g kg^{-1} for the control; campesterol ranged from 2.05 to 2.64 g kg^{-1} (control 4.21 g kg^{-1}) and sitosterol from 4.57 to 5.09 g kg^{-1} of oil (7.82 g kg^{-1} for the control). Variation in the total sterol contents of three olive varieties produced on the same farm was pronounced; the total sterol contents ranged from 1.19 to 1.88 g kg^{-1} of oil.[93]

Cereal products

Sterol contents and composition. Plant sterols occur in cereals as free sterols, steryl esters of fatty and phenolic acids and glycosides. Glycosides are, however, usually not included in the reported contents because they are not liberated in the sample preparation methods used (see sample preparation). Their proportion is, however, substantial.[2,73,94] When different cereals are compared, considerable differences may be seen in both their sterol contents and sterol compositions in terms of individual sterols and bound sterol classes. Furthermore, sterols are unevenly distributed within the kernel, leading to varying sterol contents in milling products. Differences caused by genetic factors have also been shown. When seven oat cultivars grown at three different locations in Sweden were compared, a statistically significant difference in the total sterol content between cultivars was observed, but no effect was found for cultivation location.[95]

When methods including acid hydrolysis were applied for cereals cultivated in the Nordic countries, rye was shown to be the best source of sterols and oats the poorest[2,96] (Table 2).

The total sterol contents in rye, wheat, barley and oats grains were 1100, 760, 830 and 520 mg kg^{-1} respectively.[96] Corn is reported to contain 1780 mg kg^{-1} of sterols.[75] Sitosterol is the main component (Fig 9). Other major desmethyl sterols include campesterol, stigmasterol, avenasterols and Δ^7-stigmastenol, and in the case of rye, wheat and corn, campestanol and sitostanol are also found.[2,96,97] Monomethyl and dimethyl sterols occur in lower amounts.[98] The significance of dimethyl sterols in rice will be discussed in more detail later in this section. Germ and bran fractions are the best sources of plant sterols. However, there are only a few recent sets of data for their total sterol contents, because the focus of recent studies has been on compositions and contents of specific sterol classes. The total sterol content of wheat bran was reported to be 0.89–1.54 g kg^{-1} and that of rice bran 13.25 g kg^{-1} of bran.[75] The total sterol contents determined for wheat germ, wheat bran and oat bran were 42.38, 44.92 and 9.43 mg g^{-1} of lipids respectively.[2]

Considerable variation is seen also in sterol contents of other milling products. In the case of rye and wheat it has been clearly shown that the sterol contents in milling products correlate with their ash contents[96] (Fig 10). It is also important to notice that not only sterol contents but also their compositions may be highly different in different parts of the kernel. This is clearly seen in the case of steryl phenolic acid esters. Furthermore, stanols were found especially in the bran fractions of rye, wheat and corn.[2,96,97]

Bound sterol structures. In addition to the total sterol contents or distribution of individual sterols, differences in the binding of sterols in different cereals and grain fractions are of interest. In rye, sterol esters accounted for 47% and steryl glycosides for 22% of sterols.[94] The corresponding figures for barley were 45% and 14%. On the other hand, free sterols accounted for 58% of the sterols in wheat, whereas oats were especially rich in glycosides, their proportion being 41%. Palmer and Bowden[99] found that in sorghum, 4-desmethyl sterols occurred as free sterols, esters and glycosides, but monomethyl sterols were entirely free. The relative proportions of the various 4-desmethyl sterols in the free, esterified and glycosylated forms were similar. However, a higher proportion of stigmasterol appeared to be free rather than as esters or glycosides.

Corn has been widely studied for its steryl phenolic acid esters. They have been shown to be highly localised within cereal seeds.[97] Analyses of dissected tissues from corn and wheat indicated that ferulates were associated mostly with inner pericarp tissues.

Table 2. Plant sterols in cereals grains, vegetables and fruits (mg kg^{-1} of fresh weight)

Food item	*Total sterols*	*Sitosterol*	*Campesterol*	*Stigmasterol*	*Δ^5-Avenasterol*	*Δ^7-Avenasterol*	*Reference*
Rye	910–1100	421	159	17	46	24	82, 96
Wheat	603–690	309	88	15	23	14	82, 96
Oats	329–520	167–282	24–40	12–15	32–91	24	82, 95, 96
Barley	586–830	289	111	150	145	17	82, 96
Corn	1780	1200	320	210	—	—	75
Brussels sprouts	430	340	80	3.8	—	—	110
Broccoli	390	310	69	11	—	—	110
Cauliflower	400	260	95	37	—	—	110
Carrot	160	110	22	28	—	—	110
Potato	38	27	2.3	3.8	—	—	110
Wax beans	170	89	13	51	—	—	72
Apple	130	130	3.6	1.0	—	—	110
Orange	240	200	30	10	—	—	110

Later, Seitz[100] confirmed that sitostanyl ferulate of corn was associated mostly with this tissue. Therefore corn fibre, which is a pericarp-enriched fraction obtained by wet-milling of corn, and corn bran, a pericarp-enriched fraction obtained by dry-milling, have been of special interest.

Seitz[97] showed that the main steryl phenolic acid esters in corn were sitostanyl and campestanyl ferulates, and lesser amounts of sitosteryl and campesteryl ferulates were found. Corn also contains minor amounts of sitostanyl and campestanyl *p*-coumarates. Later, Norton[101] identified more steryl cinnamic acid derivatives from corn bran. A total of 16 compounds appeared to be steryl cinnamic acid derivatives; sitosteryl, sitostanyl, stigmasteryl, campesteryl, campestanyl, Δ^7-sitosteryl and Δ^7-campesteryl ferulates were identified. Esters of sitostanol and campestanol accounted for 80.8% of the sterols in these ester structures, confirming previous studies. Only 10.7% are made up of the three sterols that are dominant in most corn tissues, sitosterol, campesterol and stigmasterol. A small percentage of Δ^7-steryls (2.3%) was found and at least two coumarates, Δ^7-campestenyl and campesteryl *p*-coumarate. Detectable amounts of cycloartenol and 24-methylene cycloartenol, main steryl components in phenolic acid esters of rice, were not found. In a later study, sitostanyl ferulate was shown to account for 64–85% of the ester fraction in oil from corn bran and related fractions.[102]

The concentrations of these esters in corn grain fractions have recently been determined, but the results of the published reports are not consistent. In a chloroform extract of the inner pericarp of yellow corn the sitostanyl ferulate content was 270 mg kg^{-1}.[100] In different types of corn the total content of ferulate plant sterol esters ranged from 25 to 225 mg kg^{-1} of seed or from 0.89 to 4.11 g kg^{-1} of oil.[103] Considerable differences have been noticed in corn bran and fibre. Corn fibre, a product of the wet-milling process and consisting of hull and other cell wall material, seems to be the best starting material for obtaining an oil rich in phenolic acid esters from corn.[104]

In corn bran the total amount of phenolic acid esters was 93.3 mg kg^{-1} of fresh weight.[101] Quantitation of steryl ferulate and *p*-coumarate esters from corn gave a yield of esters from bran and related products which ranged from 70 to 540 mg kg^{-1} of bran.[102] Steryl phenolic acid esters accounted for 1100 mg kg^{-1} of fibre (67 g kg^{-1} of oil), whereas the proportion of fatty acid esters was 1900 mg kg^{-1} of fibre (90.5 g kg^{-1} of oil) and that of free sterols 390 mg kg^{-1} (19.2 g kg^{-1}).[105] When oil was extracted from corn fibre and corn bran prepared under controlled laboratory conditions, higher ester contents were found in corn fibre oil compared to corn bran oil.[104] During both industrial and laboratory-scale wet-milling, almost all

Figure 9. Plant sterols in cereals (mg kg^{-1}).[96]

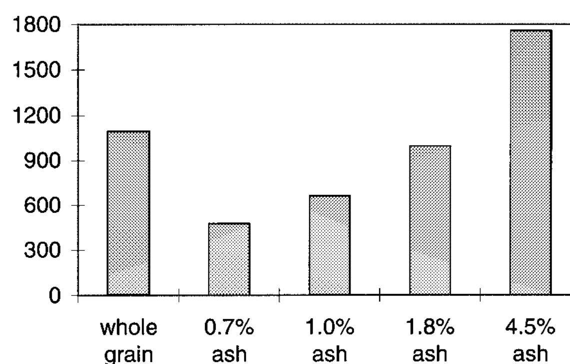

Figure 10. Plant sterols in milling products of rye (mg kg^{-1}).[96]

the phenolic acid esters were recovered in the fibre fraction. During laboratory-scale dry-milling, below 20% of the esters were recoved in the bran (pericarp) and the rest in the grits (9.7–15.6%). In this study the total steryl phenolic acid ester contents for corn kernel, bran and fibre were in the ranges 3.51–4.11, 8.2–14.0 and 16.8–57.1 g kg^{-1} of oil respectively.

When oils are produced from corn bran, processing conditions have shown to be of great importance. In different unrefined oils the range was from 0.18 to 8.6 g kg^{-1} of oil.[103] The method of extraction, the particle size of the bran and the heat treatment are of special significance.[102,104–106]

In rice bran the major sterols occurring as esters of phenolic acids are cycloartenol, 24-methylene cycloartenol, campesterol, sitosterol and cycloartanol.[101,102,107] Xu and Godber[108] purified and identified 10 components of γ-oryzanol in rice bran oil. Three major components were cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesteryl ferulate as in earlier studies, but five components not reported before were also identified.

The total concentration of steryl ferulate and *p*-coumarate esters in rice bran was reported to be 3.4 g kg^{-1} of bran or 15.7 g kg^{-1} oil.[102] When various cereals were compared, brown rice contained the highest amount of ferulate esters (15.3 g kg^{-1} of oil, 456 mg kg^{-1} of seed).[103] On the other hand, the yield of ferulate esters in oil extracted from rice bran is highly dependent on the extraction and processing conditions.[102,107,109] Refined oils from different manufacturers contained total γ-oryzanol levels of 115–787 mg kg^{-1}.[107] The proportion of cycloartenyl ferulate was 134 and 274 mg kg^{-1} and that of 24-methylene cycloartanyl ferulate 52 and 48 mg kg^{-1} in the steryl ester fractions of two oils of different origin.[102] For cyclobranyl ferulate and campesteryl ferulate the corresponding figures were 215 and 201 mg kg^{-1} and 352 and 242 mg kg^{-1} respectively. Saska and Rossiter[109] reviewed γ-oryzanol contents reported for rice bran oil and showed also a wide range in the contents. The highest reported values are above 25 g kg^{-1} of oil, whereas as little as 1 g kg^{-1} is reported for commercial oil marketed in the United States.

Sitostanyl and campestanyl ferulates, and lesser amounts of sitosteryl and campesteryl ferulates, were also found in wheat, rye and triticale grains.[97] Later, some ferulate esters were found in dehulled wheat and barley, but none were found in seeds with the hulls attached, which may be attributed to cultivar differences.[103] Their content in wheat without a hull was 53 mg kg^{-1} of seed (5.23 g kg^{-1} of oil) and in barley 3.9 mg kg^{-1} (0.44 g kg^{-1}).

Vegetables, fruits and berries

Because of their high moisture contents, vegetables, fruits and berries are generally not regarded to be as good sources of sterols as cereals. However, considerable variation within this group has been found.[96,110] In vegetables analysed in Finland, the total sterol contents ranged from 50 to 370 mg kg^{-1} of fresh weight and the range was from 250 to 4100 mg kg^{-1} of dry matter.[96] The highest content was found in cauliflower. In fruits and berries the corresponding ranges were 60–750 and 370–2930 mg kg^{-1} respectively, the concentration on a dry weight basis being above 1000 mg kg^{-1} in most products. Peels and seeds, usually excluded from the edible portion, were shown to contain more sterols than edible parts (Piironen *et al*, unpublished). In Sweden the median total amount of plant sterols in 22 vegetables was 160 mg kg^{-1} of the edible portion (range 38–500 mg kg^{-1}).[110] Concentrations in the upper range, ie more than 300 mg kg^{-1}, were found for broccoli, Brussels sprouts, cauliflower, green olives and black olives. Sitosterol occurred at the highest concentrations, the median concentration being 82 mg kg^{-1} and the highest concentration 480 mg kg^{-1}. For fruits the median concentration of total sterols for 14 items was 160 (13–440) mg kg^{-1}; only passion fruit contained more than 300 mg kg^{-1}. The sterol contents of some selected items are given in Table 2.

As in the other food groups, free sterols, steryl esters and glycosides are found in vegetables and fruits. The relative proportion of steryl esters in different species ranges from a low precentage to being the dominant steryl lipid present.[15] Their amount in the tissue of a particular species may vary depending on a number of factors, such as maturity, light, tissue type or physiological status (eg germination and senescence). It has been observed in a number of species that the proportion of stigmasterol in the esters was lower than in the free sterol fraction.

Other foods

The plant sterol contents of nuts are not well known, although nuts are generally regarded as good sources. The total sterol contents determined in different nuts range from 290 to 2200 mg kg^{-1}.[72,75,111] In the nutrient database of the US Department of Agriculture the sterol contents for peanuts, cashew nuts and almonds are 2.20, 1.58 and 1.43 g kg^{-1} respectively.

Ripening and post-harvest changes

Few data are available on quantitative changes in plant sterol contents during ripening or post-harvest processes. Some data on tomato, pepper fruit and apples are available. The effects of chilling have also been studied.

During tomato fruit ripening, free sterols, steryl esters, acylated steryl glycosides and steryl glycosides in outer pericarp tissue from Rutgers tomato fruit were determined, ranging from mature green to red ripe, and from three isogenic non-ripening mutants.[112] A large increase in the plant sterol content occurred with ripening. The content of free sterols, steryl glycosides and particularly that of steryl esters increased in Rutgers tomatoes. On a fresh weight basis the increase was 2.6-, 1.9- and 12-fold in the content of free sterols, steryl glycosides and steryl esters respectively. Only a

small change was seen in acylated steryl glycosides (1.1-fold decrease). The increases in steryl esters, free sterols and steryl glycosides appeared substantially greater when calculated on a dry weight basis, and the level of acylated steryl glycosides showed a small increase. These changes were shown to be 'ripening-specific' by comparison with the non-ripening mutant, ie they were much more pronounced with ripening than merely with aging of the fruit. A dramatic change was also seen in the sterol composition. In Rutgers tomatoes the stigmasterol/sitosterol ratio increased in the four sterol classes with ripening, but only a small increase was seen in the mutants. In the free sterol fraction the ratio increased from about 0.5 to 2.5.

In mature green tomato fruits stored at chilling (2 °C) and non-chilling (15 °C) temperatures for up to 12 days, the total sterol content increased during storage at both 15 and 2 °C. The increase in free sterols was greater in chilled compared to non-chilled tomatoes, whereas the reverse was true for steryl esters.[113] After 12 days at 15 °C the content of free sterols increased almost twofold and those of steryl esters more than fourfold. Chilling had no effect on free or acylated glycosides. The stigmasterol/sitosterol ratio increased in all steryl classes dramatically with storage at 15 °C but only slightly with storage at 2 °C. In free sterols the ratio changed from 0.46 to 3.34 at 15 °C.

The results obtained in the above studies cannot be generalised for all vegetables and fruits. When changes of sterols in pericarp tissue of three cultivars of bell pepper fruit at three stages of ripening were determined, the results differed markedly from those obtained for tomatoes.[114] Only minor changes in sterol contents and compositions were seen with ripening. Chilling and rewarming altered the relative proportions of different sterol classes in both tomato[115] and bell pepper fruit,[116] but the patterns of changes were different.

Only small changes in sterol contents were observed when apples were stored at 12 and 3.5 °C in air or at 3.5 °C in 2 kPa O_2.[117] In air at 12 °C, sitosterol declined about 4% during fruit ripening (about 50 days). At 3.5 °C in air (64 days) and 2 kPa O_2 (62 days), sitosterol increased by 16%.

Processing

The effects of processing, especially that of refining, on plant sterols in oils, has been extensively studied. On the other hand, only a few studies concerning other processes, either industrial processes or different food preparation methods at home, have been published.

Plant sterol contents may change in different processes either because of the removal of sterols or as a result of chemical reactions. A significant quantity of sterols in the original plant material may be lost by removing sterol-rich parts such as peels or seeds. In oil refining, some sterols are also removed. Reactions leading to decreased sterol contents, changes in sterol compositions or reaction products of sterols include oxidation reactions, hydrolysis, isomerisation and other intramolecular transformations, and dehydration.

Oxidation of plant sterols is the reaction of major concern during processing and storage of plant sterol-containing materials. Oxidation of cholesterol has been extensively studied because of possible harmful effects of its oxidation products. They may have cytotoxic, atherogenic, mutagenic and carcinogenic properties and may inhibit cholesterol biosynthesis and membrane function.[118] Much is not known about the oxidation of plant sterols, but because of structural similarities with cholesterol, their stability towards oxidation is also of interest.

Industrial

Oil refining. The magnitude of losses of minor components in oil refining depends to a large extent on the refining conditions used. Therefore evaluating the significance of different refining steps based on earlier reports should probably be done with caution. In the refining processes, plant sterols are partly removed together with other components. Furthermore, they may react by oxidation, isomerisation and other intermolecular transformation reactions, dehydroxylation, hydrolysis and dehydrogenation.

In earlier studies, sterol losses range from 10 to 70% depending on the oil and processing conditions employed. Changes in composition regarding the three main sterols are considered negligible.[75,88] Total sterols decreased by 36% in fully refined corn oil compared with the crude oil. Refining of soybean oil led to an 18% reduction and of rapeseed oil to a 24% reduction.[119] The relative proportions of the individual sterols were essentially not altered during the refining process. Prior *et al*[120] reported that refining had no effect on the sterol isomer ratio of canola oil. In this study the total content of sterols in canola oil was reduced by 15% and by a further 1% on degumming and bleaching respectively. On the other hand, bleaching was shown to cause changes in the composition of olive oil also.[85] Among desmethyl sterols, Δ^5-avenasterol suffered the most noticeable diminution. In the case of the methyl sterols, citrostadienol was decreased, whereas amongst the dimethyl sterols, 24-methylene cycloartanol was decreased.

As temperature and processing time increased in physical refining, sterols of soybean oil were progressively lost and the surviving sterols were partially isomerised. Periods of 1.5–3 h at 240–300 °C brought about major changes in the content and composition of unsaponifiable matter.[121] In the crude oil the total sterol content was 3900 mg kg^{-1}, whereas acid-degummed and bleached oil contained 3200 mg kg^{-1} of sterols. In phosphoric acid-degummed, bleached and physically refined oil the sterol content was 2600 mg kg^{-1} after physical refining at 240 °C for 2 h, but only 1600 mg kg^{-1} if the temperature was 260 °C. Campesterol and stigmasterol were less stable than

sitosterol. The absolute level of Δ^7-stigmastenol increased from 28 to 118 mg kg^{-1}. This was concluded to be due to isomerisation of sitosterol. There are some indications that severe physical refining also brings about isomerisation of Δ^5-avenasterol to Δ^7-avenasterol.

It is of special interest that free sterols and different bound sterol classes are removed to varying extents. Glycosides[88] and esters with phenolic acids[22,102] are largely removed in the refining processes. Degumming, alkali refining, bleaching and deodorisation removed 51% of oryzanols.[22] The results of another study indicated that compared to corn bran and unrefined oils, refined oils contained only traces of the two major phenolic acid stanyl esters.[102] On the other hand, steryl fatty acid esters were lost to a much smaller extent than free sterols.[77,119]

Autoxidation does not seem to be of special significance in refining of oils. Autoxidation products were reported to be formed in neutralisation and storage.[88] Later, analysis of crude and freshly refined soybean oil showed no detectable levels of oxidation products of sitosterol at the detection limit of 0.2 mg kg^{-1}.[122] Saturated sterols may be formed on hydrogenation; quantifiable amounts of sitostanol (60 and 70 mg kg^{-1}) were found in hydrogenated soybean and coconut oils.[82] Modification of 4,4′-dimethyl sterols during the hydrogenation of edible vegetable oils led to isomerisation of the double bonds in the side chain.[123]

The sterol losses and transformations occurring during raffination are also used for identification of refined oils. Formation of 5,23- and 5,24-stigmastadienol was studied.[124] Recently, many papers on the dehydroxylation reactions of sterols have been published.[85,119,125–127]

Bleaching and deodorisation can be determined by analysis of dehydroxylation products of sterols.[125] All Δ^5-unsaturated sterols have an OH group in position 3, consequently most sterols occurring in oils and fats form 3,5-steradienes.[127] The dehydroxylation product of sitosterol is $\Delta^{3,5}$-stigmastadiene. Stigmasterol has a further double bond in position 22 in the side chain, so that dehydration yields a triene. The term steradiene is commonly used to include all these compounds. They are mainly produced during bleaching earth treatment.[126] A variety of factors, such as the amount of earth and its activity, and bleaching temperature, affect this reaction.[125,126] With charcoal, no dehydroxylation products were formed.[125] In deodorisation, passage of steam produces olefinic products and strips such material at the same time.[125,126] When stigmastadiene contents of oils, fats and margarines were determined, they varied in different oils from 1.6 to 134.8 mg kg^{-1}. Low amounts were found in oils labelled as non-refined or cold-pressed olive oil.[127] Neither the usual oil production procedures of virgin olive oil or crude vegetable oils nor long-term storage produced measurable amounts of stigmastadiene.[126] In commercial olive oils in Spain the range was between 2 and 10 mg kg^{-1} for chemically refined oils and between 15 and 45 mg kg^{-1} for physically refined ones; in refined pomace olive oils the contents were 7–200 mg kg^{-1} when 20 samples were examined.

Industrial frying operations. Significant sterol losses may occur at deep-frying temperatures. The susceptibility of different sterols was studied in model systems.[128] The most important factor determining the stability of sterols was the ring structure of the steryl moiety, when free sterols and steryl esters were added to rapeseed oil and paraffin oil at a level of 0.1% and heated at 180 °C. Saturated sitostanol was the most stable and ergosterol, having two double bonds in the ring structure, the most labile sterol among those studied. After heating for 24 h 2–73% of the sterols added to rapeseed oil remained.

Considerable sterol losses were found when soybean oil was heated at 180 °C for 8 h per day with cooling to room temperature overnight and taking samples after 24, 48, 72 and 96 h of the total heating time.[129] The losses were higher in a hydrogenated oil than in a deodorised oil which was concluded to be caused by destruction of protective compounds during hydrogenation. The sitosterol content of hydrogenated soybean oil decreased from 1520 to 1200 mg kg^{-1} of oil after 96 h of heating.

The significance of oxidation reactions during deep-frying has been studied by Dutta and Appleqvist.[130] When sterol compositions of original oils were compared with those of oils used for the frying of potato products at 250 °C for 2 days and with the oil in products fried in them, no marked differences were found. The oils compared were rapeseed oil/palm oil blend, regular sunflower oil and high-oleic sunflower oil. However, sterol oxides were found in both the oils and French fries. The sterol oxide content was 39–191 mg kg^{-1} in lipids extracted from French fries. Frying in a rapeseed oil/palm oil blend resulted in the highest amount of oxidation products.[131] A later paper by the same authors reports results obtained when the frying temperature was 200 °C for 15 min. Rapeseed oil/palm oil blend contained 41 mg kg^{-1} of total sterol oxides before frying operations. This increased after 2 days of frying to 60 mg kg^{-1}. The other oils contained 40 and 46 mg kg^{-1} before frying and 57 and 56 mg kg^{-1} after 2 days. In French fries the sterol oxide contents were 32, 37 and 54 mg kg^{-1} in lipids, corresponding to 2.4–4.0 mg kg^{-1} in the product.[131] Potato chips fried in palm oil, sunflower oil and high-oleic sunflower oil contained 5, 46 and 35 mg kg^{-1} of sterol oxides in lipids respectively.[132]

Home

Very few sets of data are available on the stability of plant sterols in different food preparation procedures used at home. Based on the few publications there are, significant sterol losses only occur at temperatures which are in the range of those used for deep-frying.

Normén *et al*[110] investigated sterol losses in the

cooking of 13 vegetables and fruits. Concentrations were 1.30 (0.55–6.95) g kg^{-1} dry weight for raw items and 1.28 (0.64–8.39) g kg^{-1} for cooked samples. They concluded that there was no significant difference between raw and cooked samples at a group level.

The effects of microwave and conventional heating on five oils have been investigated.[133] The oils were heated in a microwave oven at half power for 120 min so that the temperature remained at 170 °C, or in an electric oven at 180 °C for 120 min, or they were exposed to microwave energy for 60 min at intervals of 50 s below 40 °C. There were no significant differences in the sterol contents of untreated and treated oils.

Stability during storage

The stability of sterols during storage of foods has been little studied. Based on the results reviewed in the previous subsection and those few studies on storage stability, no significant changes in total sterol contents are likely to take place in most practical situations. However, after prolonged storage, some oxidation products may be found.

The stability of plant sterols in three wheat flours was investigated by Farrington *et al.* [134] After storage for 5 years at 12 °C the total extractable sterols remained unchanged. However, a decrease in free sterols and a corresponding increase in steryl esters were observed. The authors suggested that the acyl transferase activity of some lipases in the flour probably caused this esterification. As an example, the free sterols of weak flour decreased from 218 to 90 mg kg^{-1} in 60 months and its steryl esters increased from 86 to 214 mg kg^{-1}, whereas the total sterol content changed only from 367 to 359 mg kg^{-1}. The oxidation products of plant sterols were found in wheat flour samples after three storage periods (2, 8 and 36 months). The samples were, however, not from controlled storage experiments, but they represented different batches.[122] Analysis revealed that the samples contained variable levels of 5,6α-epoxysitosterol (5.4–55 mg kg^{-1} in lipids), 5,6β-epoxysitosterol (0.2–29 mg kg^{-1}), 7α-hydroxysitosterol (9.3–118 mg kg^{-1}) and 7β-hydroxysitosterol (9.7–126 mg kg^{-1}). A 2-month-old wheat flour sample was found to contain 35 mg kg^{-1} and the two older samples 24 and 328 mg kg^{-1} of total sitosterol oxides.

The above study also suggested that free sitosterol is stable during prolonged storage of refined soybean oil. Storage for 1 year at 4 °C caused no significant increase in the level of free sitosterol oxides.

Potato chips fried in cottonseed oil and stored in unopened foil bags for 150 days at 23 °C contained no detectable sitosterol oxidation products, but those kept at 40 °C for 95 days contained 7α-hydroxysitosterol, 7β-hydroxysitosterol and sitosterol β-epoxide.[136] French fries purchased from five fast-food restaurants contained α- and β-epoxides and 7α- and 7β-hydroxysterols.

During storage for 25 weeks in the dark, no considerable increase in sterol oxides was observed in chips fried in palm or sunflower oil.[132] Potato chips fried in palm oil had the lowest level of total sterol oxides, ranging from 5 to *ca* 9 mg kg^{-1} in lipids from time 0 to 25 weeks of storage. The corresponding ranges for chips fried in sunflower oil and in high-oleic sunflower oil were from 46 to 47 mg kg^{-1} and from 35 to 59 mg kg^{-1} respectively.

ANALYTICAL METHODS

General

The qualitative and quantitative analysis of plant sterols in a given food sample is generally carried out in order to evaluate its plant sterol contribution to overall dietary plant sterol intake. For this purpose, analytical data on plant sterols in foods are mainly obtained using methodology where sterols are analysed as free sterols after their hydrolysis from their conjugates.[137,138] In contrast, plant physiology studies have used methods to separate free sterols and their different conjugates.[1,15] Also, plant sterol analysis can be used for certain control purposes, eg to detect adulteration of vegetable oils.[139,140] When sterol analysis is carried out to detect adulteration, focus is on characteristic sterols of the given material, eg spinasterol in pumpkin seed oil.[140]

Currently, the most common methods for the determination of plant sterols involve extraction of the lipid fraction from homogenised sample material, followed by alkaline hydrolysis (saponification), extraction of the non-saponifiables, clean-up of the extract, derivatisation of the sterols, and separation and quantification of the sterol derivatives by gas chromatography (GC) using a capillary column. At the present time, many methods used in plant sterol analysis are derived from cholesterol analysis.

Many of the earlier data on the sterol contents of food were obtained using enzymatic and spectrophotometric methods, which suffer from problems with interferences and lack of specificity, ie only the total sterol amount can be determined.[137] Also, many contemporary chromatographic methods used may lead to serious errors in plant sterol values because of their inadequate sample preparation, eg steryl glycosides are overlooked in the hydrolysis step although they are widely present in foods of plant origin.[1] It must be pointed out that there are no official methods to determine plant sterols generally in foods. In order to minimise the methodology-based variation in plant sterol values, a well-validated methodology must exist and must be used. An important aid in evaluating different methods would be appropriate reference materials certified for their plant sterol values. Existence of reference samples would make it possible to use the same sample material in different laboratories to compare their analytical performance. To our knowledge, the only reference materials for plant sterols currently available are vegetable oil blend and animal fat matrices from the Community Bureau of

Reference (BCR) of the Commission of the European Communities.

Sample preparation

In foods, plant sterols occur as free sterols, steryl esters, steryl glycosides and acylated steryl glycosides (Fig 3). A sample preparation procedure should be capable of hydrolysing and isolating sterols from all of their conjugates for a final chromatographic analysis. When the analytical focus is on oxidation products of plant sterols, some important factors must be taken into consideration, eg the avoidance of artefacts during the sample preparation procedure.

A common approach for the analysis of plant sterols includes a total lipid extraction with chloroform/methanol/water solvent systems[141,142] prior to hydrolysis. Several modifications have been used as alternatives to the original methods mentioned above, eg chloroform has been replaced by dichloromethane[143] and isopropanol has been chosen instead of methanol.[144] Hubbard *et al*[145] compared several extraction methods and reported that a chloroform/methanol extraction procedure[141] gave higher recovery for sterols than other procedures tested, including a method by Bligh and Dyer.[142] However, lipid extracts of intact tissue may not include sterols occurring as polar conjugates, ie steryl glycosides with many carbohydrate units, because these are not soluble in the non-polar lipid phase.[146,147]

Alkaline hydrolysis liberates sterols from their esters, and after this saponification step, free sterols can be extracted as part of the unsaponifiable matter. This approach has been applied by Thompson and Merola,[148] who developed a simplified alternative method to the AOAC official method[149] to determine cholesterol in multicomponent foods by shortening the saponification step. The authors reported this method to be capable of determining major plant sterols. Recently, Phillips *et al*[68] used an application of this method to determine plant sterol contents in experimental diets.

Alternatively, samples are directly saponified and then the non-saponifiables are extracted as described by Klatt *et al*,[151] who proposed a direct saponification method for the determination of cholesterol in various foods. Their method was a modification of the AOAC official method and it was adopted first action by the AOAC.[152] Also, it has been recommended that direct saponification can be used to determine plant sterols in pasta products[153] and in diet composite samples.[154]

In the determination of plant sterols levels, free sterols as well as esterified sterols are included in the total sterol yield when methods involving alkaline hydrolysis are used. However, steryl glycosides are totally overlooked, because the acetal bond between the sterol and the carbohydrate moiety (Fig 3) cannot be hydrolysed in alkaline conditions. This leads to underestimation of the total plant sterol concentration in the sample material. This problem does not occur when only cholesterol is to be determined, because it is not present as glycosides.

Inclusion of an acid hydrolysis step prior to alkaline hydrolysis has been suggested as one alternative to release sterols from their glycosides.[2,72,74] According to Jonker *et al*[72] and Toivo *et al*,[74] this approach increases the total sterol concentration obtained by up to tens of per cent, depending on the food matrix (ie its concentration of steryl glycosides), compared to a parallel method without the acid hydrolysis step. However, it has been reported that Δ^5-avenasterol and fucosterol are unstable in acidic conditions, which may make the use of acid hydrolysis problematic for certain food matrices rich in these sterols.[82]

The unsaponifiable matter represents up to 1% of the total material in food lipids and contains, in addition to sterols, a number of other compounds, including tocopherols, carotenoids and other hydrocarbons. The unsaponifiables are usually extracted with a non-polar organic solvent (eg cyclohexane, hexane or hexane/diethyl ether). Many authors have found it useful to further clean up the extract in edible oils. This has been carried out by thin layer chromatography (TLC)[84,155] or column chromatography.[140,157] When the conventional approach is applied for the extraction of the unsaponifiable matter and the sample clean-up process, the analysis time needed is problematic: these techniques are time-consuming, laborious and have little potential for automation. Also, several hundred millilitres of organic solvents per sample are needed.

Solid phase extraction (SPE) methods have been suggested as an alternative to the clean-up step required in sample preparation. Both the normal phase mode and reverse phase mode have been used for the isolation of the sterol fraction. Silica gel cartridges[158] as well as a non-polar C18 adsorbent material[83] have been used to clean up the fraction of plant sterols in vegetable oils and fats, while Oles *et al*[159] used aminopropyl cartridges to clean up the fraction of cholesterol in several food matrices. Bello[158] compared a proposed SPE clean-up method to a conventional TLC method and reported that plant sterols could be reliably quantified using both methods. Toivo *et al*[74] compared silica and C18 adsorbent materials in the clean-up of the sterol fraction of whole wheat flour and concluded that both adsorbents can be applied equally. The main advantages of SPE methods are a short preparation time, partly because of the possibility of the simultaneous handling of several samples, and a reduced need for solvents as compared to column chromatography clean-up procedures.

Recently, Lechner *et al*[160] presented a different approach to sample preparation in the determination of plant sterols in vegetable oils. SPE with silica gel was applied to isolate the non-glyceridic components, when prior to SPE the free hydroxyl groups had been silylated. Then sterols and tocopherols could be analysed by GC without further sample preparation.

Although current food composition data on plant sterols have not been widely categorised among their various chemical forms, many methods have been presented to study plant sterols and their conjugates separately. A common approach in these methods has been the fractionation of different classes of total lipid extracts (obtained eg using a chloroform/methanol type of extraction) using column chromatography on silicic acid[113] or modern SPE on silica gel cartridges,[161] and then further fractionation performed with TLC[162,163] or SPE on silica gel.[113] Glass[164] used silica gel SPE cartridges to fractionate lipid classes, including sterol classes, without the need for further fractionation. Neutral alumina SPE has been used to separate free and esterified sterols in edible oils[89] and in cereal lipids.[94] GC methods have been used in the detection and quantitation of sterols from different fractions, mainly after hydrolysis of steryl conjugates.[114,162]

Chromatographic analysis

Capillary gas chromatography of trimethylsilyl (TMS) ether derivatives of sterols is currently recognised as the most suitable method for sterol analysis. Fenton[137] has widely reviewed the chromatographic analysis of cholesterol. The same basics can be used to evaluate the analysis of plant sterols.

Packed columns have been replaced by capillary columns in most laboratories,[137] since the former could not offer the effective separation needed in plant sterol analysis. Many common sterols were not separable using them. For example, sitosterol and lanosterol co-eluted,[165] while they are well separated by modern capillary columns.[150] Also, new capillary GC methods are able to separate stanols from their corresponding sterols.[68,150,166] Excellent separation occurs with the commonly used stationary phases, which include 14% cyanopropyl-phenyl-methylpolysiloxane (eg DB-1701) and 5% diphenyl-95% dimethylpolysiloxane (eg OV-5).[68,166]

Plant sterols are very often derivatised before GC analysis in order to make them more stable and to improve analytical sensitivity. According to Fenton,[137] the most common derivatisation procedure used for plant sterols is to derivatise them to their trimethylsilyl (TMS) ethers, which offers higher thermostability, lower polarity and improved peak shape. On the other hand, several methods which do not involve derivatisation have been suggested.[153,158,167]

Plant sterol analysis methods normally include the use of an internal standard. Internal standards proposed in the literature include 5α-cholestane, cholesterol, cholestanol and betulin. As Fenton[137] points out, the internal standard should be added to the sample at the earliest possible step to compensate for losses which occur during extraction, transfers, evaporations, and derivatisation. The right choice of internal standard is very important, but may be problematic. For example, 5α-cholestane is effectively extracted with sterols, but is not derivatisable, whereas the reliable use of cholesterol and cholestanol depends on the food matrix, because they may be naturally present in the foods to be analysed.

The most frequently used technique for detecting plant sterols is flame ionisation detection (FID)[137] However, mass spectrometry combined with GC has become an important technique for the identification of sterols and evaluating peak purities of the eluting sterols. Characteristic mass spectral data for different plant sterols are available in the literature.[84,140,166,168]

High-performance liquid chromatography (HPLC) with UV detection is also used to determine major plant sterols in foods. HPLC methods offer a non-destructive alternative to GC methods.

Also, plant sterols can be analysed without clean-up and derivatisation. However, HPLC methods are not able to exhibit sufficient selectivity for separation of sterols and corresponding stanols. Indyk[169] and Holen[170] have optimised many factors, including columns, mobile phases and column temperatures, for reverse phase HPLC with short-wave UV detection (206–214 nm), while Warner and Mounts[171] used evaporative light-scattering detection.

HPLC is a very reliable method to determine ergosterol, because it has a strong UV absorption at a specific wavelength (282 nm) which makes it easy to determine without interference from other sterols.[172]

HPLC methods have also been used to analyse steryl esters of phenolic acids.[101,173] These compounds can be detected in the long-wave UV region, and several steryl esters of phenolic acids such as cinnamic acid, ferulic acid and coumaric acid have been separated using reverse phase columns.

Analysis of plant sterol oxidation products

Oxidised plant sterols are formed as minor constituents from plant sterols. A lot of research has been focused on identifying cholesterol oxidation products and their analysis in foods of animal origin. However, many analytical problems still exist.[174,175] More than 30 different cholesterol oxidation products have been identified.[175] Since in plant materials a number of plant sterols are present, the profile of oxidation products is more complicated, which makes the analytical separation even more challenging. However, methods usually used for studying plant sterol oxides are based on those developed for studying cholesterol oxides.

Analysis of plant sterol oxides consists of extraction of total lipids from plant materials, saponification of lipids, purification and enrichment of sterol oxides and chromatographic analysis. During the sample preparation procedure, special care must be taken to minimise artefact formation and further reactions of sterol oxides, because sterol oxides are less stable than sterols. Thus high temperatures, light and oxygen availability should be avoided. Based on the high variation in sterol oxide contents reported, Smith[175] stated that as analysis methods improve, the levels of sterol oxides reported in foods tend to diminish.

Although saponification at room temperature requires a lot of time, it is considered to be necessary, because epoxy and keto compounds are destroyed and epimerisation of hydroxy and keto compounds occurs in hot alkaline solutions.[17,176] As an alternative to alkaline hydrolysis, a method based on enzymatic hydrolysis has been suggested.[135] Since most oxidation products are more polar than native sterols, extraction of unsaponifiables after saponification should be performed with more polar solvents than when unoxidised sterols are analysed, eg diethyl ether is used rather than hexane.[17,122,176]

Preparative TLC and SPE have been used to clean up sterol oxides from the excess of native sterols. Relatively polar eluent mixtures, eg heptane/ethyl acetate (1:1) and diethyl ether/cyclohexane (9:1), have been used to develop silica gel plates.[132,177,178] SPE methods with silica and aminopropyl phases have been shown to be efficient and reproducible in isolating sterol oxides. Separation is achieved by stepwise elution with increasing solvent polarity, eg elution with a combination of hexane and diethyl ether is used to remove sterols, while acetone is used to elute polar sterol oxides.[132,179] However, the advantage of TLC over SPE is that less polar sterol oxides can be isolated as their own fraction.

Qualitative and quantitative analysis of extracted and purified plant sterol oxides is performed by capillary GC after derivatisation to trimethylsilyl ethers.[131,132,180] To avoid discrimination and alteration of analytes, on-column injection at low temperatures is recommended.[17] Detailed mass spectral and retention data on sterol oxides are available to support identification.[132,178] HPLC, widely used in the analysis of cholesterol oxides,[181,182] is not effective enough to separate the large number of plant sterol products.

BIOAVAILABILITY AND PHYSIOLOGICAL EFFECTS

Overview of methods

Methods used to study the metabolism of plant sterols and their metabolic effects include: analysis and measurement of dietary plant sterols (see previous section); their intestinal hydrolysis and distribution to intestinal oil and micellar phases; absorption and serum (lipoprotein) contents; distribution to vascular and extravascular cells (tissues); biliary secretion; and qualitative and quantitative analysis in faeces.

A major metabolic effect of dietary plant sterols is the inhibition of absorption and subsequent compensatory stimulation of the synthesis of cholesterol. The ultimate effect is a lowering of serum cholesterol owing to the enhanced elimination of cholesterol in stools. Thus several aspects of cholesterol metabolism need to be studied, including methods of fat-soluble vitamin absorption.

Hydrolysis of conjugated plant sterols in the human intestine has been incompletely studied, especially for the glucosides. Intubation studies of small intestinal contents have shown that esterified plant sterols are hydrolysed effectively in the upper intestine and transferred to a micellar phase.[4] Thus about a half of the esterified sterols and stanols are hydrolysed during passage of the first 0.5 m of the upper small intestine. Free and less esterified sterols are transferred to the micellar phase. The higher the free sterol content in the micellar phase, the more cholesterol remains in the oil phase, retarding its transfer to the micellar phase and final absorption. This appears to be the mechanism for the inhibition of cholesterol absorption by plant stanols, but it apparently is also applicable to plant sterols. Thus feeding of sitosterol-rich plant sterols lowers the serum content of campesterol in man, but increases the serum content of sitosterol, whilst the feeding of stanols lowers the serum content of both campesterol and sitosterol.[183] Sitosterol in the first case and the stanols in the second case apparently prevent the absorption of other sterols.

In general, competition of sterol transfer to the micellar phase may regulate the absorption of different sterols from their mixtures in the intestinal lumen. For instance, large amounts of free stanols in the micellar phase apparently prevent absorption not only of cholesterol but also of plant sterols, resulting in a lowering of the respective serum levels. Regulation of absorption may be even more important when micelles or vesicles are transferred from the brush border membrane to the inside of the enterocyte. Assuming that the SR-B1 (scavenger receptor B1) of the enterocytes actually regulates cholesterol absorption, the receptor might select sterols from micelles on the basis of their concentration and chemical structure, being capable of picking up free and esterified sterols.[184]

The absorption efficiency of plant sterols can be measured by the intestinal perfusion technique, in which the concentrations of plant sterols are related to that of the unabsorbed marker sitostanol in the upper and lower sites of the contents of the small intestine.[185,186] Plant sterol absorption can also be measured after a single dose[187] or the chronic feeding of labelled plant sterols.[188] The daily biliary secretion of campesterol and sitosterol, or their stanols, (defined by faecal output) can also be used for the measurement of plant sterol absorption.[189,190] The absorption of sitosterol appears to be less than that of campesterol. Thus the percentage absorption of campesterol is around 10% that of sitosterol (roughly 5%), whilst that of campestanol is around 2%, and sitostanol 1%. With high plant sterol intakes the respective values are lower, despite higher mass absorption. Thus the serum concentration of plant sterols, especially their ratios to the levels of cholesterol, reflects plant sterol and cholesterol absorption.[191,192] Changes in serum ratios of plant sterols to cholesterol actually reflect changes in cholesterol absorption.[193]

The absorption of plant sterols can also be quantified from vascular walls and tissues, where their contents are, as in serum, less than 1% of respective cholesterol values.[194] Elimination of plant sterols from

the body takes place into the bile, apparently mostly unchanged, indicating that, in the steady state, absorbed plant sterols can be recovered in bile. Biliary plant sterol secretion can be determined indirectly by multiplying their ratio to cholesterol by the daily biliary cholesterol secretion. The latter can be determined by dividing faecal cholesterol elimination (cholesterol plus coprostanol and coprostanone) by (1−absorption%) and subtracting the unabsorbed dietary cholesterol.

Unabsorbed plant sterols can be determined in stools as unchanged plant sterols and their bacterial conversion products methyl and ethyl coprostanones and coprostanols.[195] In general, most of them are in an unconjugated form, and even in colectomised patients less than 10% are conjugated, indicating that hydrolysis in the small intestine appears to be effective. Colonic bacteria give rise to additional hydrolysis. Analysis of diets showed that from 5 to 40% of the plant sterols were in an alkaline-resistant form, probably owing to the presence of glycosides, and could be quantified after additional acid hydrolysis.[196] Faecal analysis showed that, after saponification, only traces of acid-hydrolysable sterols were available, a sign of effective intestinal hydrolysis of plant sterol glycosides.[196] Colonic bacteria convert plant sterols to 5β-coprostane products, while 5α-stanols are hardly formed. Less than 10% of faecal plant sterols are stanols of dietary origin.[4,197] Colonic bacteria can also apparently conjugate faecal sterols in some conditions, but the sterol nucleus is hardly modified.[198] Thus faecal recovery of dietary plant sterols can be considered to be complete, and this method has been used to measure dietary plant sterol intake.

Review of available data

Human data

Plant sterol consumption. As already noted, the quantity of plant sterols in the normal diet is negatively correlated with cholesterol absorption and serum total and LDL cholesterol. Intakes, especially that of sitosterol, are increased two- to threefold in vegetarians. Serum sterol levels of around 350 vs 270 $\mu g\,dl^{-1}$ in non-vegetarians have been observed. The serum and biliary clearances of vegetarians were higher than in non-vegetarians.[199]

Cholesterol absorption and serum cholesterol were decreased and cholesterol synthesis was increased in a negative correlation ($P < 0.01$) with cholesterol absorption. Serum total and LDL cholesterol concentrations were positively related to cholesterol absorption ($P < 0.05$) and negatively related to dietary plant sterols ($P < 0.05$) and cholesterol synthesis ($P < 0.05$). Serum plant sterol ratios to cholesterol were positively related to their dietary intake and biliary secretion ($P < 0.001$) and to cholesterol synthesis ($P < 0.001$) and negatively related to cholesterol absorption ($P < 0.05$). Respective sitosterol and campesterol absorptions (measured from biliary secretion and faecal output) were 3.1 and 12.4% in the non-vegetarians, but, despite higher dietary plant sterol intake, quite similar, 3.7 and 9.4% respectively, in the vegetarians. Cholesterol feeding to the vegetarians, even though it increased cholesterol absorption and serum LDL and cholesterol levels, had no effect on serum or biliary plant sterols. Thus, despite the markedly enlarged daily influx of cholesterol into the intestinal pool (+69%), no actual reduction could be detected in plant sterol absorption.

Phytosterol-rich diets in children have been reported to increase the serum concentration of different plant sterols.[200] Formula diets containing 300–400 $mg\,day^{-1}$ of phytosterols increased serum sitosterol and campesterol concentrations three to five times compared with children on low-plant-sterol milk diets. The highest values were 17.6 $mg\,dl^{-1}$ for campesterol and 9.8 $mg\,dl^{-1}$ for sitosterol. These are similar to the concentrations seen in subjects with phytosterolemia.[201] The values are very much higher than in adult vegetarians on an even higher phytosterol intake, suggesting that in children the absorption of plant sterols might be high.

Phytosterol feeding. From the early 1950s, large amounts of plant sterols (up to 50 $g\,day^{-1}$) were fed in a poorly soluble form to patients with hypercholesterolemia to lower their serum cholesterol level.[5]

Demonstration of the prevention of hypercholesterolemia in rabbits and the lowering of plasma cholesterol in man[202] resulted in a clinical study being undertaken to lower serum cholesterol in 26 mainly hypercholesterolemic patients.[203] A large number of clinical studies were performed subsequently. However, interest in their use declined owing to concerns over the potential for developing phytosterolemia due to the increase in serum plant sterols,[200,204,205] especially of campestrol, and attention was focused on the subsequent introduction of hypolipidemic agents. A further reason why interest waned was due to the difficulties in measuring effects following the consumption of poorly soluble plant sterols.

The clinical entity of hereditary sitosterolemia was actually discovered in 1974 by Bhattacharyya and Connor.[187] The disorder was shown to be strongly atheromatotic, most likely owing to markedly increased serum sitosterol and campesterol concentrations, such that myocardial infarctions could occur already at a relatively young age.[206]

Introduction of plant stanols in animal experiments in the 1970s showed that they were virtually unabsorbable, inhibited cholesterol absorption and lowered serum cholesterol more effectively than plant sterols. They also reduced serum and tissue plant sterols and arterial atheromata.[207–209]

Application of the free plant stanols for human studies[210] revealed their potential to lower plasma cholesterol in relatively small (1.5 $g\,day^{-1}$) and safe doses, resulting in the development of fat-soluble plant stanol esters in the early 1990s.[211–214] They have been marketed in Finland since 1995 as the plant stanol

ester margarine Benecol*. Fat solubility of plant stanols was considered important for its effective cholesterol lowering. In fact, a relatively large dose of homogenised sitostanol had virtually no effect on serum total or LDL cholesterol in mildly hypercholesterolemic men,[242] suggesting that poor solubility might have contributed to negative results.

The popularity of stanol esters for cholesterol lowering has renewed the interest in using plant sterols for that purpose. Phytosterols added to a butter-containing diet lowered serum cholesterol quite effectively even in normal cholesterolemic humans.[215] In combination treatment, sitosterol consumption significantly increased the hypocholesterolemic effect of lovastatin[216] and bezafibrate.[217] Corn oil phytosterols added to olive oil decreased LDL cholesterol and increased campesterol (both insignificantly) in normal cholesterolemic subjects, but increased cholesterol synthesis.[218] On the other hand, free tallow oil sterols containing mainly sitosterol, sitostanol and campesterol significantly lowered serum cholesterol in a short-term study (−6%). There was no effect on serum plant sterols, but increased cholesterol synthesis was found in individuals with a low baseline cholesterol level.[219] In another study, phytosterols significantly reduced both total and LDL cholesterol, but had no effect on serum campesterol level or cholesterol synthesis.[220]

In addition to the initial free sitostanol-feeding experiment in man,[210] intestinal infusion and long-term treatment studies showed that sitostanol was virtually unabsorbable. Plant sterol and cholesterol absorption is inhibited and serum cholesterol is more effectively reduced than sitosterol.[185,186,210,221] In children, reductions of serum cholesterol were 24 vs 17% respectively, and those of sitosterol 14 vs 51%. Faecal elimination of cholesterol is also higher following sitostanol ingestion compared with sitosterol.

Functional phytosterol foods

The above term includes any food items with added free or esterified plant sterols or stanols. A general recommendation is that the phytosterols should be in a fat-soluble form. Only plant stanol esters (Benecol*) and plant sterol esters (Take Control*) fulfil this criterion.

In the early 1990s, sitostanol ester was introduced to lower serum cholesterol in small groups of mildly hypercholesterolemic non-diabetic[211] and diabetic[213] subjects. Since then, the stanol ester margarine has been reported to lower serum total cholesterol by 10–15% and LDL cholesterol by up to 20% in several studies of patients with (1) mild or familial hypercholesterolemia (children and adults), (2) type 2 diabetes mellitus, (3) postmenopausal myocardial infarction and (4) colectomy, either alone or in combination with statins, neomycin and cholestyramine.[6,190,222,223] The effects are also seen in normal cholesterolemic subjects.[224] Similar results have been reported more recently by several other investigators.[225–231] Consumption of stanol ester margarine has a consistent and specific effect on lowering total and LDL cholesterol. An increase in HDL cholesterol is observed only occasionally, and triglyceride or VLDL values usually remain unchanged.

A cholesterol-lowering effect can also be observed on a low dietary intake of cholesterol, indicating that stanol esters inhibit not only absorption of dietary but also that of biliary cholesterol. In fact, measurements of cholesterol absorption and faecal elimination of cholesterol as bile acids, and cholesterol itself, have shown that faecal elimination of cholesterol is increased, even if it is assumed that dietary cholesterol is not absorbed. Thus elimination of biliary cholesterol is increased by stanol ester consumption. In addition, the stanols are also effective in a butter-rich diet. The effects of a variable dietary fat composition have not been fully investigated. The decrease in cholesterol is usually highest in subjects with high baseline cholesterol levels or with high baseline cholesterol absorption, when the decrements of absorption efficiency for serum plant sterols and cholestanol (sterols inhibiting cholesterol absorption) tend to be highest. The plant sterol and cholestanol ratios to cholesterol are decreased in serum as long as stanol ester consumption continues, while the increase in sitostanol or campestanol, even during long-term consumption, is detectable, but minimal, in most studies. This is probably because of the rapid biliary secretion of stanols.[4,190]

Harmful clinical or chemical adverse events have not been observed in a careful review of clinical studies.[230,231] Fat-soluble vitamin concentrations are unchanged after consumption for a year, even though β-carotene content can decrease.

Cholesterol absorption efficiency was decreased by 45% in a group of 52 patients studied for sterol balance[4] on a scheduled sitostanol intake of 0.8–3 g day^{-1}. The respective increase in faecal sitostanol was 2.3 g day^{-1}, a value considered to indicate total recovery of the dietary intake. Faecal fat and bile acids were not changed, but the reduced absorption efficiency increased faecal output of cholesterol by 32%, cholesterol synthesis by 15% and turnover by 10% (all $P < 0.01$). Precursor sterol (cholestenol, desmosterol and lathosterol) ratios to cholesterol (indicators of cholesterol synthesis) were increased in serum proportionately to synthesis values of sterol balance data, and inversely to the ratios of cholestanol and plant sterols and cholesterol absorption. The higher the decrease in LDL cholesterol, the higher were the increments of the precursor sterol ratios and cholesterol synthesis, and the higher were the decrements of the sterol ratios reflecting cholesterol absorption or the cholesterol absorption percentage.

Results using sterol ester margarine have only been reported in two studies.[232,233] Serum cholesterol was significantly decreased by both sterol and stanol ester consumption, and no difference was seen between the two phytosterol groups. However, serum plant sterol concentrations were increased by sterol esters and

decreased by stanol esters. Increase of plant sterols from 0.83 to 1.61 and 3.24 g day^{-1} indicated that serum cholesterol lowering ranged from 4.9 to 9.9%, with no significant difference between the doses. It is calculated that an intake of 1.6 g day^{-1} will beneficially affect plasma cholesterol concentrations without seriously affecting plasma carotenoid concentrations.

Preliminary additional data from Maki *et al*[234] show that in a group of 224 subjects on a National Education Program Step 1 diet, randomised to consume low-fat control spread or 1.1 and 2.2 g day^{-1} plant sterols (as esters) in a fat-reduced spread, LDL cholesterol was reduced by 5.9 and 6.9% respectively in relation to controls. Corresponding apo B levels were decreased by 5.1 and 6.4%, and serum plant sterol concentrations increased by 33 and 48%. Fat-soluble vitamins were not affected by plant sterols, but cholesterol-related *trans-β*-carotene levels were significantly decreased. Other preliminary observations of 3 weeks duration in groups of 15 subjects showed similar reductions of 13.4 and 10.2% in cholesterol concentrations by the consumption of 1.8 g of sterol or stanol (both as esters) respectively, compared with 6% in controls. Respective cholesterol absorption efficiencies were decreased by 36 and 26%, and synthesis increased by 53 and 38% without any change in cholesterol turnover.[248]

Animals

Earlier studies of plant sterol effects in experimental animals have been extensively reviewed by Pollak and Kritchevsky.[5] Only more recent, mainly stanol studies are referred to here. Plant sterols fed to rats have been reported to reduce plasma total, LDL and VLDL cholesterol and triglyceride and liver cholesterol concentrations and to increase markedly (up four to nine times) liver campesterol and sitosterol contents.[235,236] In hamsters, sitostanol feeding lowered serum and hepatic cholesterol but increased serum campesterol concentration in low-sitostanol or tallow oil sterol groups, while the hepatic sitosterol level was increased by feeding a synthetic sitostanol mixture.[237] In rats, sitostanol feeding in the form of tallow oil sterols lowered LDL cholesterol but increased HDL cholesterol proportionately to LDL cholesterol. Plasma campesterol and sitosterol contents were also increased, but plasma sitostanol levels remained negligible. An increased lathosterol/cholesterol ratio in plasma and hepatic cholesterol fractional synthesis rate indicated that cholesterol synthesis was increased compensatorily by feeding of a stanol mixture.[238] Sitostanol fed to cholesterol-fed hamsters and rabbits showed that serum cholesterol lowering was associated with enhanced faecal elimination of cholesterol due to reduced cholesterol absorption (significant for hamsters) and a compensatorily increased (significant for hamsters) fractional synthesis rate of cholesterol.[239]

That phytosterol-induced lipid lowering may have preventive effects on development of atherosclerosis was indicated by a marked reduction of aortic atheromatous lesions in apo E-deficient mice treated with tallow oil sterols.[240] Dietary sitostanol feeding to cholesterol-fed rabbits reduced plasma total and VLDL cholesterol concentrations and also depressed plaque accretion in coronary arteries and ascending aorta. Plasma plant sterol ratios to cholesterol were unchanged. Sitostanol was found in plasma only occasionally, hepatic campesterol levels were related to hepatic cholesterol (significantly decreased), and virtually no plant sterols were found in arterial walls or plaques.[241]

Modulating factors

The dose of plant sterols seems to be important, such that about 2 g day^{-1} of stanols or sterols seems to offer an ideal dose for lowering cholesterol. Higher doses may not improve efficacy and may produce adverse effects, except in the case of stanols with their negligible absorption. Intake of phytosterol esters has been recommended to be taken once with each major meal, because the inhibition of biliary cholesterol absorption has been assumed to be potentiated during the food-induced emptying of the gall bladder. On the other hand, a single daily dose seems to produce similar effects to those seen when several daily doses are taken.[230] The earlier animal studies and some human studies have clearly shown more beneficial effects from free stanols versus sterols, while for esterified phytosterols there are no significant differences between the unsaturated and saturated compounds. Free plant sterols are poorly soluble. If consumed in a crystalline or homogenised form during food digestion, they may enter the micellar phase less effectively than fat-soluble sterols released from sterol esters after their hydrolysis by intestinal contents. However, no comparable studies have been performed, except that in the study by Denke[242] homogenised sitostanol was ineffective, and in infusion studies a more pronounced effect on cholesterol was shown by a micellar solution of sitosterol than by a sitosterol suspension.[243]

Stanol ester margarine consumption enhances cholesterol synthesis, which might limit the reduction of serum cholesterol. Thus a combination of cholesterol synthesis inhibitors with stanols could further enhance cholesterol lowering. In fact, combination of the two potentiates additionally their effect on serum cholesterol.[244,245] Accordingly, treatment of hypercholesterolemia should be started with dietary measures, including the use of stanol ester margarine, and in resistant cases additional hypocholesterolemic drugs, usually statins, should be used. This treatment is also advisable to combat hyperlipidemia, but may be less effective in pure hypertriglyceridemia. In this condition only statins are effective to some extent, as opposed to stanols.

Plant sterol esters could also be included in the treatment with clofibrate. The latter drug apparently enhances biliary secretion of cholesterol[193,246] such that the enhanced intestinal cholesterol pool could

potentiate plant sterol-induced cholesterol malabsorption. A combination of sitosterol with bezafibrate significantly enhances the hypocholesterolemic potential.[217] Sitostanol ester reduced cholesterol absorption by up to 65%,[247] but its combination with neomycin further lowered cholesterol absorption efficiency, frequently by close to 100%.[250] Thus lowering of cholesterol absorption to zero would lower LDL cholesterol only by about 37%, because cholesterol synthesis is correspondingly doubled. Since plant sterols are not synthesised within the human body, their respective reduction is higher than that of cholesterol, close to 60%.

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