



## Report of the Scientific Committee of the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN) on the conditions of use of certain substances to be used in food supplements-3

### Section of Food Safety and Nutrition

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## Abstract

Food supplements are foods, the purpose of which is to supplement the normal diet and which consist of concentrated nutrient sources (vitamins and minerals) or other substances with a nutritional or physiological effect, alone or in combination. The supplements are marketed in dosage form and are only supplied to the end consumer prepacked. In no event should they replace the use of medicines without suitable medical supervision. They should only be used to supplement the diet and, on the whole, their usage is not required if the individual has a varied and balanced diet, which cannot be replaced.

In Spain, food supplements are regulated by Royal Decree 1487/2009, which transposed Directive 2002/46/EC on the approximation of the laws of the Member States relating to food supplements into Spanish law. However, only the use of vitamins and minerals is currently regulated. Therefore the Scientific Committee has been asked to make an assessment of the proposal to authorise certain substances other than vitamins and minerals in the manufacture of food supplements.

The six substances proposed by the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN) are betaine hydrochloride, phytosterols, lactase, melatonin, methylsulphonylmethane and polyphenols from olive oil, olive leaves and olives.

The Scientific Committee has assessed each proposal, analysing the characteristics and sources of each substance, and the nutrition, metabolism and safety and has concluded, in each case, whether that submitted by the AECOSAN is acceptable from a safety viewpoint for use as a food supplement. In no event is the assessment intended as a guarantee of the biological efficiency of the substances and the estimated doses.

The Scientific Committee states that, in any case individuals undergoing medical treatment consult with the doctor the opportunity or suitability of consuming food supplements, given the possibility of interactions in certain cases. In addition, in the case of food supplements with an antioxidant effect, it should be noted that in certain conditions and at high doses, these compounds may behave as pro-oxidants.

## Keywords

Food supplements, betaine, phytosterols, lactase, melatonin, methylsulphonylmethane, phenolic compounds.

## 1. Introduction

The Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN) has drawn up a new proposal to authorise certain substances other than vitamins and minerals for use in the manufacture of food supplements and their corresponding maximum daily quantities for inclusion in a new Annex III of Royal Decree 1487/2009 (BOE, 2009). In this respect, the Executive Director of the AECOSAN has asked the Scientific Committee to assess, as on previous occasions, the proposal to authorise the use of certain substances in the manufacture of food supplements both with respect to the maximum daily quantities proposed and as regards the appropriateness of the authorisation.

In accordance with that stated in previous reports, food supplements are foods, the purpose of which is to supplement the normal diet and which consist of concentrated nutrient sources (vitamins and minerals) or other substances with a nutritional or physiological effect, alone or in combination. Supplements are marketed in dose form in capsules, pastilles, tablets, pills, sachets of powder, ampoules of liquids, drop dispensing bottles and other similar forms of liquids and powders designed to be taken in small unit quantities.

As foods, they are subject to the legislation applicable to other food products, such as Regulation (EC) No 178/2002 (EU, 2002a) laying down procedures in matters of food safety, Regulation (EC) No 1924/2006 (EU, 2006a) on nutrition and health claims made on foods and Regulation (EC) No 258/1997 (EU, 1997) concerning novel foods. Prior authorisation is not required for their marketing, only a notification of their placement on the market, although in some Member States of the European Union including Austria, the Netherlands, Sweden or the United Kingdom, this notification is not obligatory (FVO, 2011).

Royal Decree 1487/2009, of 26 September, relating to food supplements transposed into Spanish Law Directive 2002/46/EC (EU, 2002b) on the approximation of the laws of the Member States relating to food supplements and established, among other aspects, the requirements for the marketing of food supplements, including their labelling, presentation and advertising. In addition, it established in Annex I which vitamins and minerals can be used in the manufacture of food supplements, specifying in Annex II the substances or salts that may be used as sources of vitamins or minerals so that these nutrients are available for the organism.

With respect to substances other than vitamins and minerals, the foreword to Royal Decree 1487/2009 establishes that until maximum levels of nutrients or other substances with a nutritional or physiological effect used as ingredients of food supplements are established in the European Union, the reports pertaining to the Scientific Committee on Food (SCF) will be considered together with those from other international bodies of recognised scientific standing.

Moreover, the foreword to Directive 2002/46/EC indicates that substances that have been approved by the Scientific Committee on Food, on the basis of the said criteria, for use in the manufacture of foods intended for infants and young children and other foods for particular nutritional uses can be used in the manufacture of food supplements.

In this respect, Regulation (EC) No 953/2009 (EU, 2009) establishes the substances that may be added for specific nutritional purposes in foods for particular nutritional purposes and Directive 2006/141/EC (EU, 2006b) on infant formulae and follow-on formulae and its transposition in Spain through Royal Decree 867/2008 (BOE, 2008) regulates the inclusion of certain substances in the basic composition of infant formulae.

At present, Royal Decree 1487/2009 only includes vitamins and minerals among the substances authorised for use in the manufacture of food supplements in Spain. Nevertheless, it indicates that the specific regulations relating to other nutrients and ingredients used in food supplements such as amino acids or essential fatty acids may be regulated at a later stage, and once adequate scientific data are available.

At the moment, the European Commission does not expect to regulate the use of substances other than vitamins and minerals in food supplements and therefore some Member States, including Belgium, Denmark and Italy, apply

the guidelines existing prior to Directive 2002/46/EC or have subsequently drawn up national provisions. Safety assessment reports are also available for certain substances, prepared by national assessment bodies, as is the case in France, or the European Food Safety Authority (EFSA).

In addition, the approval of a health claim for a particular substance in the framework of Regulation (EC) No 1924/2006 does not suppose a guarantee of its safety as the EFSA only assesses the cause-effect relation between the intake of a set quantity of a substance and the effect that it is alleged to have. Therefore, the authorisation of a health claim does not imply that its safety has been assessed and, as indicated in the Regulation which establishes a list of authorised health claims for foods other than those referring to the reduction of disease risk and to children's development and health (article 13.1), this claim authorisation is not an authorisation to market the substance which is the subject of the claim, nor is it a ruling on the possibility of using the substance in food products nor the classification of a certain product as food (EU, 2012).

At present, in Spain it is possible to market food supplements containing substances authorised in other Member States under the principle of mutual recognition in the European Union, which guarantees the free movement of goods and services without having to harmonise the national legislation of the Member States. Consequently, the sale of a product legally manufactured in a Member State cannot be banned in another Member State, even though the technical or qualitative conditions differ from those imposed on the products. The only exception is in those cases of general interest such as the protection of health, consumers or the environment, as is the case of food supplements that are considered as medicinal products by the competent authority of a Member State and which, consequently, cannot be marketed as food supplements although considered as such in another Member State.

The lack of regulation relating to the manufacture in Spain of food supplements containing substances other than vitamins and minerals has prevented their manufacture at national level, but not their marketing through the use of the authorisation obtained in another Member State and the corresponding mutual recognition. Moreover, this supposes a competitive disadvantage for Spanish companies: do not have a legal instrument that facilitates, in the event of discrepancy with the regulations of another Member State, the protection of the consumer using an appropriate legal instrument.

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## 2. Proposal

AECOSAN has prepared the following proposal for substances other than vitamins and minerals which may be authorised for use in the manufacture of food supplements (Table 1).

Proposed substance	Proposed maximum daily quantity
Betaine hydrochloride	1.5 g
Phytosterols	3 g
Lactase	4 500 units FCC minimum
Melatonin	1 mg
Methylsulphonylmethane	1 g
Polyphenols from olive oil and olive leaves and fruit	5 mg

## 3. Evaluation of the proposals

### 3.1 General considerations

For food supplements, as for all other foods, nutritional and/or health claims may not be made unless approved in accordance with Regulation (EC) No 1924/2006.

The assessment of the EFSA in the framework of Regulation (EC) No 924/2006 is solely based on the study of the cause and effect relation between the intake of a certain substance and the effect it is alleged to have (efficiency and dose at which the effect occurs) and in no event supposes the approval of said substance for its use in the food sector nor is it an evaluation of its safety.

Consequently, the request for the report by the Scientific Committee with respect to the substances to be included in a new Annex III concerning other substances which may be used in the manufacture of food supplements (Royal Decree 1487/2009) is confined to their safety in the doses proposed for use in the manufacture of food supplements, given that the efficiency of the same is assessed and regulated at European level in the scope of Regulation (EC) No 1924/2006.

Food supplements are intended to supplement the normal diet and provide an additional amount of vitamins, minerals or other substances with nutritional or physiological effect. The supply of a concentrated quantity of nutrients or other substances may suppose a risk if taken in excess by the population who consume them.

Furthermore, in the case of pregnant women or nursing mothers, children, the elderly and the sick, food supplements should only be used under medical control and if there are reasons to justify their use, as the safety assessment of their use refers to adults with a normal physiological situation.

In no event should they replace the use of medicines without suitable medical supervision. They should only be used to supplement the diet and, on the whole, their usage is not required if the individual has a varied and balanced diet, which cannot be replaced.

In the case of food supplements with an antioxidant effect, it should be remembered that in certain conditions including: the intake of high doses, changes to the pH or the presence of certain substances, these compounds may behave as pro-oxidants.

In any case, individuals undergoing medical treatment must seek medical advice as to the suitability of taking food supplements, given the possibility of interactions in certain cases.

In the preparation of this report, reports prepared by other Agencies and other subsequently published work or work referring to data existing in Spain have been considered. The conclusions given here may require revision in the future in the light of new scientific evidence.

## 4. Betaine hydrochloride

### 4.1 Proposal

The AECOSAN has proposed a maximum daily quantity of 1.5 g for betaine hydrochloride.

This proposal is based on the authorisation of a health claim that betaine contributes to the normal metabolism of homocysteine. This claim may only be used for food which contains a minimum of 500 mg of betaine per quantified portion. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 1.5 g of betaine (EU, 2012).

In Italy, a maximum daily quantity of 1.5 g of betaine is authorised in food supplements (Italy, 2013). In Belgium trimethylglycine hydrochloride is authorised in food supplements (Belgium, 2013).

### 4.2 Characteristics and sources

Trimethylglycine or (carboxymethyl) trimethylammonium is a natural compound known as betaine, that is present in a multitude of organisms where it performs different physiological functions, especially as a methylant agent or by modifying osmosis (Craig, 2004). Humans obtain it through their diet or by oxidising the physiological choline that is converted into betaine. In mammals, betaine has two main functions: its function as the above-mentioned osmolyte, which permits the volume of the cell to be regulated, and its function as a methyl donor, essentially for the remethylation of homocysteine to a methionine, converting it into *N,N*-dimethylglycine (Lever and Slow, 2010). The function as a betaine osmolyte means that the tissue concentrations are higher than the plasma concentrations, especially in the renal medulla where concentrations may be higher than 100 mM. In addition it has a compensatory and modulating effect, allowing protein stability to be improved, and is particularly effective in counteracting the denaturing effect of the urea, important in the renal medulla function (Lever and Slow, 2010).

Betaine deficiency in the body has been associated with different pathologies or physiological alterations, including metabolic syndrome, dyslipidemia and diabetes. Therefore, betaine is considered to be important for human development, from the embryo to infancy. Although studies have been carried out with betaine supplements on animals as an ergogenic aid in athletes, the long-term effects of supplementation on humans are not known.

### 4.3 Nutrition and metabolism

The average intake of betaine from unfortified foods is approximately 145 mg/day in adults and 100 mg/day in children, with maximum intakes of 439 and 317 mg/day for adults and children, respectively (AFSSA, 2008). Betaine

absorption in the duodenum and its distribution are fast, reaching maximum plasma peaks of 20-70  $\mu\text{M}$  after 1-2 h (Craig, 2004). It is possible to determine the body's potential need for betaine, as loss of the excess through urine can be detected in laboratory tests. Blood concentrations vary according to the individual, ranging from 20-60 mol/l in females and from 25-75 mol/l in males. Unmetabolised urinary elimination is minimal, even in individuals who receive high doses of the compound. The metabolite dimethylglycine appears at concentrations of less than 10 mol/l (Lever and Slow, 2010). The principal mechanism of elimination is catabolism via a transmethylation reaction in the methionine cycle, a mitochondrial process mainly carried out in the liver and kidney (Shwahn et al., 2003) (Craig, 2004).

The function as a methylant agent of homocysteine is performed by betaine-homocysteine methyltransferase (BHMT), an enzyme that is osmoregulated by the osmolytes present, including betaine itself. Consequently, these two functions cannot be considered in isolation, as the enzymatic activity is reduced when the osmolyte concentration decreases (Lever and Slow, 2010). High levels of homocysteine have been linked to a high risk of cardiovascular disease (Rajaie and Esmailzadeh, 2011). As methionine is an amino acid of vital physiological importance, the conversion of homocysteine to methionine is very important for the regulation of this amino acid, and the hepatic generation of *S*-adenosyl methionine produced by betaine in the liver has been linked to the detoxification of the organism (Craig, 2004) (Kharbanda, 2009).

Regulation (EU) No 432/2012 establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children's development and health, authorises a claim that betaine contributes to normal homocysteine metabolism, specifying that this claim may only be used for food which contains at least 500 mg of betaine per quantified portion. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 1.5 g of betaine, and that a daily intake in excess of 4 g may significantly increase blood cholesterol levels (EU, 2012).

#### 4.4 Safety

In subchronic toxicity studies conducted on rats for 90 days, with regimens of 0, 1, 2 and 5 % of betaine, corresponding to 0, 800, 1 600 and 4 000 mg/kg/day in male rats and 0, 900, 1 800 and 4 400 mg/kg/day in female rats, it has been observed that in all cases, hepatomegalia and microvascular damage occurred in the rats treated with betaine, although the effect was reversible and disappeared after the supplement was eliminated (EFSA, 2005a). The reduction of the mean corpuscular volume in red blood cells and the decrease in haemoglobin concentrations was also observed, together with a nephromegaly in the group that consumed the highest dose of betaine (5 %), therefore it was not possible to determine the safety of the supplement for any of the doses studied (AFSSA, 2008). However, other sub-acute and sub-chronic toxicity studies report the possible safety of the same doses studied, although modifications to the mean corpuscular volume were also observed (Hayes et al., 2003).

Tests carried out on humans focussed on establishing possible hepatic and renal damage, as the metabolism in these organs is essential for the elimination of betaine from the body. Clinical trials tested the hepatic and renal functions in different conditions, and the effects on the lipid metabolism, varying the protocols from 2 to 20 g/day, with time periods ranging from 18 weeks to 13 years, although in all the cases the number of patients was relatively small. In the studies conducted using 4 g/day (McGregor et al., 2002) and 6 g/day (Schwab et al., 2002), both lasting 3 months, an increase in the total cholesterol and LDL cholesterol was observed of 7 % and 10 % respectively (4 g/day) and of 12 % and 23 % (6 g/day). A subsequent meta-analysis confirmed this increase, demonstrating that supplementation with betaine (6 g/day) provokes the increase of blood triglycerides (13 %) and LDL cholesterol (10 %), possibly caused by the increase of the synthesis of phosphatidylcholine, favouring the hepatic production of

VLDL (Olthof et al., 2005). In view of this data, it was concluded that a daily intake in excess of 4 g of betaine may significantly increase blood cholesterol levels (EFSA, 2011) (EU, 2012).

In 2008 the French Agency AFFSA (*Agence Française de Sécurité Sanitaire des Aliments*) issued an adverse opinion against the use of betaine in food supplements as it was not possible to guarantee consumer safety with doses of 250 mg/day. The reasons given were that it was not possible to reject a risk of hepatic damage, in view of the data and the non-establishment of a NOAEL (no observed adverse effect level) in animals; the risk of an increase in blood cholesterol and LDL cholesterol levels in human and the absence of a potential risk assessment of the methionine deficiency (AFSSA, 2008).

Although some sources of betaine, such as the anhydrous and monohydrate forms received a negative report in their assessment in accordance with Regulation (EC) No 258/1997 (EU, 2005), the applicant established that, in hydrochloride form, betaine has been marketed since 1982 in the European food supplement market and in the United States since the 1960s.

#### 4.5 Conclusion

The subchronic toxicity studies conducted in rats over 90 days did not permit the establishment of a NOAEL and, moreover, in clinical trials with humans negative effects were observed on the lipid profile caused by daily quantities of betaine somewhat higher than those proposed. The Scientific Committee concludes that, based on the currently available information and taking into account the general considerations reflected in this report, at present there is insufficient information to support the safety of the AECOSAN proposal for a maximum daily quantity of betaine hydrochloride of 1.5 g in food supplements.

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## 5. Phytosterols

### 5.1 Proposal

The AECOSAN has proposed a maximum daily quantity of 3 g of phytosterols.

This proposal is based on Regulation (EC) No 608/2004 concerning the labelling of foods and food ingredients with added phytosterols, phytosterol esters, phytostanols and/or phytostanol esters which lays down that the labelling must indicate that an intake in excess of 3 g/day of added plant sterols or stanols must be avoided (EU, 2004).

In Italy, phytosterols are authorised in food supplements in a maximum daily quantity of 3 g (Italy, 2013).

### 5.2 Characteristics and sources

Phytosterols or plant sterols and their reduced forms, phytostanols or plant stanols, are sterols of plant origin, belonging to the family of triterpenes, with a structure and function very similar to that of the cholesterol present in animals (Moreau et al., 2002).

The term phytosterols refers to more than 250 different compounds. All have a steroid nucleus, a hydroxyl group at the carbon 3 position and a double bond located mainly between carbon atoms 5 and 6 of the B-ring.

The main differences are in the lateral alkyl chain, which may vary due to the absence or presence of a methyl or ethyl group in the C-24, the saturation and the position of a double bond, and the geometry of the substitution in the C-24. Plant stanols are the saturated forms of plant sterols, without the double bonds on the steroid nucleus and on the lateral alkyl chain (Piironen et al., 2000).

The most abundant plant sterols found in the human diet are beta-sitosterol (with an input of 56-79 %), campesterol (18 %), stigmasterol (9 %) and brassicasterol. Beta-sitostanol and campestanol, which correspond to the saturated forms of beta-sitosterol and campesterol, are the principal stanols, although they are found in smaller quantities than the sterols (Figure 1).

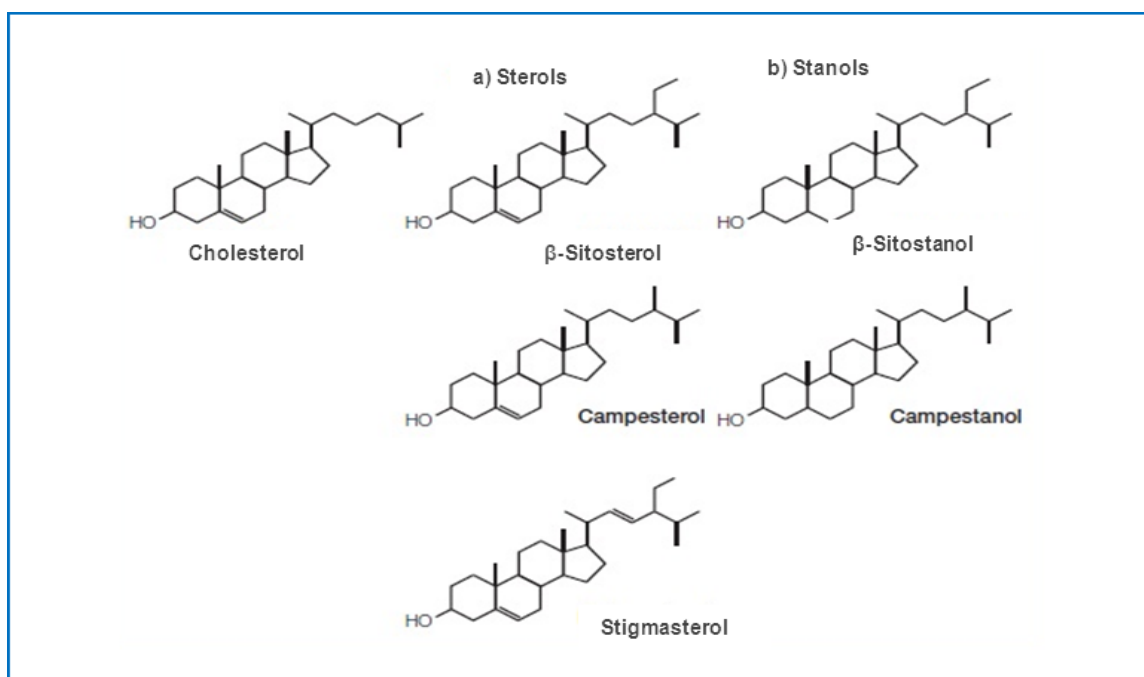


Figure 1. Structure of the cholesterol and of some of the more common plant sterols and stanols. Source: (Palou et al., 2005).

In addition to the free form, plant sterols may naturally be found as conjugated compounds, in which the hydroxyl group in position 3 of ring A of the sterol is esterified by a fatty acid, a ferulic acid or it is glycosylated (Moreau et al., 2002). Esters with fatty acids are present in the majority of the plants and form almost 50 % of the total plant sterols in some foods, such as corn oil. Ferulate esters are also found in significant quantities in many foods, whereas the glycosylated plant sterols are a minority component in plant foods, with certain exceptions, as they form 82 % of the total plant sterols of potatoes (Palou et al., 2005).

### 5.2.1 Sources

In general, the plant oils and derived products, such as margarine, are considered the richest natural sources in sterols (Piironen et al., 2000). Plant oils, such as corn, sunflower, soya, rapeseed and olive oil contain between 0.15 and 1.5 % of phytosterols. These concentrations are reduced in refined oil. Other foods that contribute to the daily intake of plant sterols are cereal grains and cereal-based products, nuts and seeds, pulses, and to a lesser extent vegetables and fruit. Stanols are also found in certain foods such as rye, maize and wheat, and in non-hydrogenated

plant oils. In Western diets, the daily intake of plant sterols is estimated to be 150-400 mg (approximately the same as the cholesterol intake), and is higher in certain vegetarian diets and in the Japanese diet, where it may reach as much as 300-500 mg/day. The daily intake of stanols is around 25 mg. (Ling and Jones, 1995).

### 5.3 Nutrition and metabolism

Both the phytosterols and the phytostanols have evoked great interest from a nutritional point of view in recent years, as their structural similarity to cholesterol gives them the capacity to reduce the absorption of this compound, thereby decreasing its total blood concentrations and the bond to LDL (Quilez et al., 2003) (Palou et al., 2005).

Although the chemical structure is similar to that of cholesterol, the absorption rate of phytosterols and phytostanols consumed is much lower than that of cholesterol. Whereas the absorption rate of cholesterol is from 40-60 %, that of phytosterols is in the region of 0.4-3.5 %, and is even lower for phytostanols (0.02-0.3 %), although this depends on the specific type of sterol (Ostlund et al., 2002). It would appear that the absorption rate does not increase linearly when the dosage of phytosterols is increased, indicating that it is a saturable process (Ostlund et al., 2002). Phytosterol absorption in females is described as being slightly higher than in males (Tilvis and Miettinen, 1986) and higher in children than in adults (Mellies et al., 1976).

Phytosterols are absorbed by the intestinal absorptive cells in the form of mixed micelles, which typically contain combinations of free cholesterol, mono- and diglycerides, fatty acids, phospholipids and bile acids.

Phytosterol esters, like sterified cholesterol needs to be hydrolysed by the pancreatic cholesterol esterase enzyme in order to be absorbed. The low absorption rate of phytosterols in comparison to that of cholesterol is partly explained by the flow from the intestinal cells to the intestinal lumen via the ABC transporters (*ATP-binding cassette*) G5 and G8 (Berge et al., 2000).

The absorbed phytosterols are transported by the chylomicrons and the VLDL, captured by the liver and then excreted in the bile. The circulating phytosterols are transported in the blood mainly in the LDL and HDL fractions. The tissues with LDL receptors such as the liver, the adrenal glands and the testicles are able to capture the phytosterols and convert them into steroid hormones (Ikeda and Sugano, 1978). As their concentration in these tissues is much lower than that of cholesterol, it would appear that they do not contribute significantly to hormone synthesis (SCF, 2000). Unabsorbed sitosterol and campesterol is converted by human colonic microflora into sitostanol/stigmastenone and campestanol/campestenone, respectively (Wilkins and Hackman, 1974).

It has been found that, in healthy adults, after an intake of 8.6 g/day of phytosterols, faecal concentrations of sterols and sterol metabolites increase from approximately 40 to 190 mg/g dry weight and from 30 to 50 mg/g dry weight, respectively (SCF, 2000). The principal sterol metabolites excreted are saturated metabolites in position 5,6 in  $\beta$  configuration or metabolites formed by oxidation in position 3. The faecal concentration of 4-cholesten-3-one increases slightly, but significantly (around 2 mg/g). The faecal concentration of secondary bile acids is reduced. The formation of small quantities of oxysterols can be excluded, but is considered to be unlikely (SCF, 2000).

#### 5.3.1 Effects of plant sterols and stanols on the intestinal absorption of cholesterol

Phytosterols and phytostanols are able to inhibit intestinal cholesterol absorption, both that from diet (approx. 300 mg/day) and the endogenous circulating cholesterol from the bile (approx. 1 000 mg/day). This results in a reduction of the total serum cholesterol and of LDL cholesterol (Ling and Jones, 1995) (Moreau et al., 2002), even in those individuals who are already following a low cholesterol diet (Moreau et al., 2002). HDL levels of cholesterol, on the whole, are not reduced with the intake of dietary phytosterol. Consequently, the faecal excretion of cholesterol and its intestinal degradation products are increased. Various studies on humans have measured the effect of the intake of plant sterols in the diet on the intestinal absorption of cholesterol (Katan et al., 2003) (Demonty et al., 2009). On the

whole, the studies demonstrate that an intake of 2 g/day of phytosterols reduces the absorption of cholesterol by 30-40 %, resulting in a lowering of LDL cholesterol of approximately 10 % (Katan et al., 2003). Therefore, they are considered to be useful for preventing cardiovascular disease, although there are no relevant clinical trials to prove this. It has been estimated that the intake of 2 g/day of sterols or stanols may reduce the risk of heart disease by some 25 % (Law, 2000). Other authors have also estimated that the intake of 3 g/day of sterols or stanols may reduce the risk of heart disease by some 15-40 %, depending on age and other dietary factors (Weststrate and Meijer, 1998). This effect is considered more significant with respect to hypocholesterolemia than the reduction of the intake of saturated fats.

The EFSA has approved the corresponding health claims regarding the effects of plant sterols (EFSA, 2008a) and stanols (EFSA, 2008b) in specific food matrices on the lowering of blood cholesterol, with the mention that the drop of blood cholesterol may reduce the risk of heart disease. In the case of plant sterols, the scientific panel has concluded that a daily intake of 2-2.4 g of phytosterols in a suitable food (for example, plant sterols added to fat based foods and low-fat foods, such as milk and yoghurt) may result in a clinically significant drop in the LDL cholesterol level of approximately 9 % (EFSA, 2008a). It has also indicated that the effect on the lowering of cholesterol may differ in other food matrices. Furthermore, it recommends that the products to which these compounds are added are only consumed by individuals wishing to reduce their blood cholesterol levels.

With respect to the effect of plant sterols in comparison to plant stanols, the scientific opinion issued by the Dietetic Products, Nutrition and Allergies-NDA Panel of the EFSA is that intakes of plant sterols and stanol esters in the range of 1.5 to 3.0 g in specific matrices (yellow fat spreads, dairy products, mayonnaise and salad dressings) are similarly efficient in lowering blood LDL cholesterol (EFSA, 2012). The opinion also concludes that a daily intake of plant sterols and stanol esters of 3 g (range of 2.6 to 3.4 g) in the above-mentioned matrices reduces LDL cholesterol by 11.3 % (IC 95 %: 10.0-12.5).

The exact mechanism by which the phytosterols reduce the circulating concentration of cholesterol has not been fully clarified, but various theories have been put forward. It has been considered that the principal mechanism is the displacement of cholesterol from the mixed micelles of bile salts and phospholipids, which are necessary for the cholesterol to be absorbed. That is, plant sterols, as they are more hydrophobic, have greater affinity for the micelles than cholesterol and, consequently, they displace them from the mixed micelles. The capacity of mixed micelles to incorporate sterols is limited, therefore the competition between the phytosterols and cholesterol reduces the cholesterol content of the micelles and consequently reduces their transportation towards the intestinal brush border membrane (Mel'nikov et al., 2004). Outside the micellar phase, the cholesterol is no longer soluble and may form co-crystals with the phytosterols and is then excreted together with the unabsorbed phytosterols. This results in a reduction of the intestinal absorption of cholesterol and increased faecal excretion of cholesterol and its metabolites. If this mechanism were solely responsible, this would mean that the plant sterols would have to be eaten at each meal containing cholesterol in order to achieve maximum efficiency. The fact that plant sterols also effectively reduce cholesterol when consumed once a day suggests that the reduced incorporation of cholesterol in mixed micelles is not the only mechanism involved (Plat and Mensink, 2005). It has also been suggested that plant sterols may reduce the cholesterol esterification rate in the enterocyte, by affecting the activity of the enzyme responsible for the esterification of the sterols in the intestine and the liver, the acyl coenzyme A: cholesterol acyltransferase (ACAT) (Child and Kuksis, 1983); consequently this would reduce the quantity of cholesterol exported to the blood. Moreover, it has been shown that sitosterol is not a suitable substrate for ACAT (Field and Mathur, 1983). This may also explain why the absorption of plant sterols is so low.

More recently, attention has also been focussed on the ABC transporters as the mechanism responsible for the reduced intestinal absorption of cholesterol by the plant sterols (Chen, 2001) (Kidambi and Patel, 2008). It would

appear that cholesterol and phytosterols share the same ABC transporters (ABCG5 and ABCG8) (Plat and Mensink, 2005) (von Bergmann et al., 2005). The ABCG5 and ABCG8 transporters are expressed in the cells of the intestinal mucosa and the canalicular membrane of the liver, and re-secrete sterols, especially the absorbed plant sterols, again to the intestinal lumen and from the liver to the bile. This also explains why plant sterols are absorbed less than cholesterol and also why they go more to the bile than cholesterol. If the plant sterols stimulate the flow of cholesterol to these transporters, this mechanism may explain the effect of the plant sterols on cholesterol absorption, and the fact that the intake of plant sterols once a day reduces LDL cholesterol levels in the blood to the same extent as the intake of these compounds three times a day (Plat and Mensink, 2005). It has been found that defects in these transporters lead to a rare hereditary disease called phytosterolemia or sitosterolemia, clinically defined by hyperabsorption and decreased biliary excretion of plant sterols (Belamarich et al., 1990). Another transporter has also been identified, NPC1L1 (Niemann-Pick C1 like 1), which is probably more involved in the transport of cholesterol and plant sterols in the intestinal mucosa (von Bergmann et al., 2005).

It should be noted that the hypocholesterolemic effect of plant sterols or stanols is the same in all individuals, and certain individuals have been described as “non-responders” (Rudkowska et al., 2008). Genetic factors have been suggested as one of the main causes of these differences (Quilez et al., 2003).

### 5.3.2 Effects of plant sterols on the bioavailability of carotenoids and liposoluble vitamins

Phytosterols, by interfering with the intestinal absorption of cholesterol, may also lead to an unwanted reduction in the absorption of carotenoids and liposoluble vitamins, as these share the same absorption path as cholesterol. In fact, the majority of studies made in this respect reveal a drop in the absolute levels of alpha- and beta-carotene, although the changes do not always reach significant differences (Plat and Mensink, 2001) (Richelle et al., 2004). Levels of oxygenated carotenoids (lutein/zeaxanthin and  $\beta$ -cryptoxanthin) are also generally reduced, whereas levels of retinol are not affected. Some studies also reveal a drop in the absolute concentrations of tocopherol (Mensink et al., 2002) (Richelle et al., 2004), although it is not known if this may be due to the food matrix. As liposoluble antioxidants are transported in the blood by lipoproteins, a reduction in plasma lipids may simply be the cause of a fall in the concentrations of liposoluble antioxidants. Therefore, concentrations of these antioxidants are generally normalised by a plasma lipid fraction, although there is no uniformity in the studies (Weststrate and Meijer, 1998) (Plat et al., 2000) (Plat and Mensink, 2001) (Mensink et al., 2002). In general, with the exception of beta-carotene, the drop in the concentrations of liposoluble antioxidants is parallel to the drop in total and LDL cholesterol levels. For example, in a study in which the diet was supplemented by plant stanol esters (3 g/day) for 4 weeks, a drop was observed in the plasma concentration of various carotenoids, whereas the absolute concentration of different isomers of tocopherol and of retinol remained unchanged (Mensink et al., 2002). In fact, the LDL particles were enriched in tocopherols, whereas the titers of various carotenoids did not reveal any changes and those of beta-carotene decreased. This suggests that the variations in the blood concentrations of antioxidants, described in several papers, cannot simply be explained by a reduction in the number of circulating LDL particles (Mensink et al., 2002).

In another study which studied the effect of the intake over one year of margarine enriched with sitostanol esters (Gylling et al., 1999), a reduction in the plasma concentration of beta-carotene of 33.3 % was observed with respect to the controls. When the concentration of beta-carotene was normalised by that of cholesterol, the difference was lower, but remained statistically significant. This study did not find any significant effects on vitamin D or retinol concentration, nor for the concentration quotients of alpha-tocopherol/cholesterol and alpha-carotene/cholesterol. Moreover, it showed that the serum concentrations of alpha-tocopherols and of carotenes (but not of retinol or vitamin D) were closely linked to the cholesterol absorption indicators (Gylling et al., 1999).

Other long-term studies produced similar results (Palou et al., 2005). Apart from the reduction in the plasma concentrations of carotenoids and liposoluble nutrients, no other relevant nutritional changes have been described. Analysing the data available in the literature, it has been calculated that the reduction of plasma carotenoids reaches a plateau when the dose of plant sterols reaches about 2.2 g/day (Plat et al., 2000) (Palou et al., 2005).

The alterations in the concentrations of beta-carotene associated with the excess intake of plant sterols has been generally confirmed in a number of studies (Plat and Mensink, 2001) (Richelle et al., 2004). The SCF estimated that the intake of 20 g/day for one year of products containing 8 % of free phytosterols reduced plasma concentrations of beta-carotene by 20 % (SCF, 2000). Although the concentration of beta-carotene is still within the broad normal range and the normal seasonal variations, this reduction in the plasma titers may be relevant in individuals with a non-optimum state of vitamin A (SCF, 2000).

A study by Noakes et al. (2002) demonstrated that when plant sterols are consumed (2.3-25 g/day) in margarines or yellow fat spreads, the dietary advice of an additional daily portion of vegetables or fruit (with a high content of carotenoids) may be efficient in maintaining the plasma concentrations of the carotenoids. Other studies have shown that the effects of plant sterols on carotenoid concentrations are slight or virtually undetectable if a balanced diet is maintained (Raeini-Sarjaz et al., 2002). In any case, during the excessive long-term consumption of foods enriched with plant sterols, an intake of beta-carotene from natural sources, that is, vegetables and fruit rich in carotenoids should be recommended to compensate for the expected reduction in beta-carotene titers and with other liposoluble nutrients (SCF, 2000).

## 5.4 Safety

### 5.4.1 Adverse collateral effects

Few adverse effects have been described linked to the excess intake of plant sterols or stanols either short- or long-term. As described in the previous paragraph, the main concern is that an excess intake is accompanied by a fall in the plasma concentrations of alpha- and beta-carotene, alpha-tocopherol, and/or lycopene (Plat and Mensink, 2001) (Richelle et al., 2004). On the whole, with the exception of beta-carotene, these decreases are often in parallel to the decreases in total and LDL cholesterol. Therefore, the intake of plant sterols should be accompanied by an increased intake of carotenoid-rich fruit and vegetables (SCF, 2000).

In addition, it has been found that an excess intake of plant sterols/stanols does not contribute, or only minimally, to an increase in the plasma concentrations of said compounds (Lichtenstein and Deckelbaum, 2001). Nevertheless, there may be individuals in the population with an abnormally high absorption of plant sterols. This is the case of individuals suffering from the afore-mentioned genetic disorder (phytosterolemia). These individuals absorb significant quantities of sitosterol (and cholesterol), and the high circulating levels lead to the development of xanthomas (Belamarich et al., 1990).

### 5.4.2 Toxicological studies

With respect to the potential toxic effects of plant sterols, in 2000 the SCF assessed a specific formulation of plant sterols in margarines and considered it acceptable, assuming an average intake of 20-30 g/day of new margarines/yellow fat spreads with a maximum of 8 % of phytosterols (SCF, 2000). This committee concluded that there were no evident safety risks from the consumption of these specific plant sterols (SCF, 2000). The toxicological information available for this assessment covered studies on the absorption, distribution, metabolism and excretion of these sterols (Sanders et al., 2000), studies on sub chronic toxicity (Hepburn et al., 1999), genotoxicity (Wolfreys and Hepburn, 2002), reproductive function and reproductive toxicity (Waalkens-Berendsen et al., 1999), potential

oestrogenic activity (Baker et al., 1999), in addition to studies on humans on microflora and faecal composition, and other parameters (Ayesh et al., 1999) (Weststrate et al., 1999).

In particular, sub-chronic studies on animals using a specific composition of phytosterol esters containing 62 % of total sterols, mainly beta-sitosterol (48.7 %), campesterol (25.8 %) and stigmasterol (21.6 %) and with only 1.1 % of brassicasterol, did not find any relevant toxic effects with the highest tested dose of up to 6.6 g/kg body weight /day (equivalent to 4.1 g of phytosterols/kg body weight/day) (Hepburn et al., 1999). Nor were any toxic effects observed in a two-generation reproduction toxicity study of rats, and using the same composition as above. A NOAEL value was established at 8.1 %, equivalent to a dose of 2.5-9.1 g/kg body weight /day (Waalkens-Berendsen et al., 1999).

The mutagenic potential of plant sterols (47.9 % beta-sitosterol, 28.8 % campesterol, 23.3 % stigmasterol) and of phytosterol esters (47.3 % beta-sitosterol, 28.1 % campesterol, 24.1 % stigmasterol) was also assessed in an analysis of mutagenicity in bacteria and in an *in vitro* analysis of chromosome aberrations. In addition, using the mixture containing 0.3 % cholesterol, 3.0 % brassicasterol, 28.1 % campesterol, 0.8 % campestanol, 18.7 % stigmasterol, 45.5 % beta-sitosterol, 2.6 % beta-sitostanol, 1.1 % D5-avenasterol and 1.9 % other compounds, an *in vitro* analysis of gene mutation was conducted on mammal cells, in addition to two *in vivo* studies of mutagenicity (analysis of micronuclei in rat bone marrow and analysis of the unprogrammed DNA synthesis in the liver), only with the phytosterol esters (Wolfreys and Hepburn, 2002). The phytosterols and the phytosterol esters did not reveal any evidence of mutagenic activity in any of the analyses. In the same study, in two toxicological *in vitro* analyses (analysis of mutagenicity in bacteria and *in vitro* analysis of chromosome aberrations), a decomposition product of the cholesterol, the 4-cholesten-3-one, and an important faecal sub product, 5-beta-cholestan-3-one were analysed, and no evidence of mutagenic activity was found in these tests (Wolfreys and Hepburn, 2002). In conclusion, the intake of phytosterols is considered to be safe according to that deduced from studies using animal models, without relevant toxicological effects.

With respect to the oestrogenic capacity, various studies on animals indicate that, when used at high levels or when administered by subcutaneous routes, plant sterols, especially sitosterol, may have oestrogenic activity. Certain oestrogenic effects have been described in fish (MacLatchy and Van Der Kraak, 1995) (Mellanen et al., 1996). Some controversial data also exists for rats with beta-sitosterol administered orally (Rosenblum et al., 1993), which was not confirmed by other studies on the same species (Baker et al., 1999). The SCF, after revising the studies available in this respect, including a two-generation reproduction study of rats mentioned above, considered that there was sufficient evidence in relation to the absence of endocrine effects when administered orally (SCF, 2002a).

With respect to plant stanols, there are several similar studies available which also do not show any adverse results (Palou et al., 2005): genotoxicity in the doses of the solubility limit, corresponding to dietary concentrations of 0.2 % to 1 % (expressed as free stanols, equivalent to approximately 0.5 g of stanols /kg body weight/day) (Turnbull et al., 1999b); two-generation toxicity reproduction study on rats at concentrations of up to 4.4 % of plant stanol esters (equivalent to 2.5 % of total stanols in the diet) (Whittaker et al., 1999); subchronic oral toxicity (Turnbull et al., 1999b) or toxicity for development (Slesinski et al., 1999) in rats; although at dietary concentrations of 5 %, the sub chronic intake of these substances reduced the plasma concentrations of the liposoluble vitamins E and K and, to a lesser extent, those of vitamin D. This effect was also observed in the hepatic titers of the liposoluble vitamins, except vitamin K which was not analysed (Turnbull et al., 1999b).

In an *in vitro* study on the potential oestrogenic activity of plant stanols, different mixtures of stanols derived from plant oils (58.3-67.1 % of sitostanol, 29.3-31.6 % of campestanol, 0.7-2.6 % of sitosterol, 0.2-1.1 % of campesterol, and 0.4-8.7 % of other sterol compounds) did not result in the proliferation of cells from a human breast adenocarcinoma (MCF-7) that responds to the oestrogens (Turnbull et al., 1999a). In an uterotrophic assay with

immature female rats, stanol esters, obtained from plants and wood, did not cause any significant changes in the uterus weight when the rats were fed with concentrations of 8.3 % in the diet for 4 days.

The majority of the studies conducted in humans were aimed at determining the efficiency of plant sterols, in the typical doses, with respect to their capacity to reduce blood cholesterol levels, particularly of LDL-cholesterol, and without affecting other parameters, such as HDL-cholesterol, triglycerides, etc. No secondary effects have been described (with the exception of the fall in the bioavailability of beta-carotene and other liposoluble compounds) or adverse reactions, which complements the results of the toxicological studies on animals and other studies in different systems and in *in vitro* models (Palou et al., 2005).

The number of studies carried out on children with hypercholesterolemia is significant (Amundsen et al., 2002) (Amundsen et al., 2004) (Ketomaki et al., 2004) and reveals similar effectiveness results as those tests conducted on adults in the lowering of cholesterol levels, without any adverse effects described, although the lowering effect of the plant sterols on the blood titers of provitamin A should be considered.

#### 5.4.3 Opinions of the Scientific Committee on Food and the NDA Panel of the European Food Safety Authority on the safety of plant sterols

The Scientific Committee on Food for the European Commission (SCF) and from here on the NDA Panel of the EFSA, have issued numerous opinions on the safety of including phytosterols and/or phytostanols in various foods (EFSA, 2003, 2006, 2007) (SCF, 2000, 2002a, 2002b, 2003a, 2003b, 2003c). In the SCF report on the long-term effects of the intake of high quantities of phytosterols from various food sources, with special attention given to the effects on beta-carotene (SCF, 2002a), it was noted that it was advisable to avoid intakes of plant sterols above a range of 1 to 3 g/day, as there was no evidence that additional benefits were obtained at higher doses. In later rulings relating to different foods with phytosterols, the SCF and the NDA Panel of the EFSA concluded that the addition of phytosterols was safe, provided that adequate management measures are taken to prevent the excessive intake of phytosterols. For these reasons, Regulation (EC) No 608/2004 concerning the labelling of foods and food ingredients with added phytosterols (EU, 2004), established that foods with added phytosterols must be presented such that they can easily be divided into portions containing a maximum of 1 g (in the case of 3 portions/day) or 3 g (in the case of 1 portion/day) of added phytosterols. It also established that a drink must not contain more than 3 g of phytosterols.

#### 5.5 Conclusion

Studies available to date have not been able to establish with sufficient accuracy a maximum tolerable daily intake for plant sterols as there is insufficient data in humans at doses above 6 to 9 g per day, taken regularly and over a prolonged period of time. The Scientific Committee of the AECOSAN shares the opinion of the SCF that the available data does not provide sufficient bases for numerically establishing maximum daily intake levels for plant sterols. However, considering that the maximum benefits in the lowering of blood cholesterol levels are reached at doses of 1.5 to 3 g per day, depending on the type of food, the formulation of specific sterols, dietary habits and other factors, in the absence of evidence of additional benefits at higher doses, the Scientific Committee also considers that it is advisable to avoid intakes of sterols in excess of this margin.

Therefore, the Scientific Committee concludes that, based on the information available to date and taking into account the general considerations reflected in this report, the AECOSAN proposal of a maximum daily quantity of 3 g for phytosterols is acceptable from the safety point of view for use as a food supplement, provided that only authorised phytosterols are used.



In addition, given that phytosterols may reduce the plasma levels of beta-carotene and other liposoluble vitamins, the Scientific Committee shares the opinion of the SCF that information about these effects on the plasma concentrations of beta-carotene should be provided to the consumer, together with the corresponding dietary advice regarding the regular consumption of fruits and vegetables. The lowering effect of beta-carotene is particularly important in individuals with non-optimum levels of vitamin A, and for individuals with specific vitamin A requirements and special care should be taken during pregnancy, lactation and infancy. It is also important to note that the intake of food supplements with plant sterols is in no way recommended for individuals affected by a rare inborn error of metabolism (phytosterolemia).

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## 6. Lactase

### 6.1 Proposal

The AECOSAN has proposed the inclusion of lactase in Royal Decree 1487/2009 without specifying a maximum daily quantity.

This proposal is based on the authorisation of a health claim that lactase is an enzyme that improves the digestion of lactose in individuals who have trouble digesting lactose. This claim can only be used for food supplements with a minimum dose of 4 500 units of the FCC (Food Chemicals Codex), together with instructions to the target population that the supplement is taken with each food that contains lactose (EU, 2012).

In Italy, lactase is authorised in food supplements without the establishment of a maximum daily quantity (Italy, 2013).

### 6.2 Characteristics and sources

The lactase or  $\beta$ -D-galactosidase enzyme is naturally secreted in the intestinal microvilli of mammals. The  $\beta$ -galactosidases, principally derived from various strains of *Kluyveromyces* and *Aspergillus*, have had industrial applications for many years. The use of fungal lactases is prevalent due, largely, to their extracellular nature, their high levels of production, stability and their consideration as GRAS substances. The  $\beta$ -galactosidases from various strains of *Aspergillus niger* and *A. oryzae* are commercially exploited for the hydrolysis of lactose in whey, to improve the symptoms of lactose intolerance and for the production of galacto-oligosaccharides. In some cases, exogenous administration has been tested with good results in humans (O'Connell and Walsh, 2008).

### 6.3 Nutrition and metabolism

Lactase or  $\beta$ -D-galactosidase hydrolyses the lactose in galactose and glucose.

### 6.4 Safety

Information has been available for some decades that the use of lactases in the food industry is safe and, although studies with animals or humans have been aimed at efficiency, some studies that infer their safety can be indicated.

The toxicity of the lactase preparation Neutralact® was assessed on rats (Coenen et al., 2000). The administration of the enzyme in doses of 500, 3 000 and 10 000 mg/kg body weight/day for 28 days does not cause a significant toxicity effect. The no observed adverse effect level (NOAEL) of the enzyme preparation was 10 000 mg/kg body weight/day. The authors concluded that there was no risk of toxicity with this lactase preparation.

Flood and Kondo (2004) studied tilactase, an enzyme preparation of  $\beta$ -galactosidase with lactase activity produced from the fungus *Penicillium multicolor*. The safety of tilactase was investigated in rats, dogs and rabbits.

Adult and young rats were catheter-fed 0, 500, 1 000, or 4 000 mg/kg body weight/day of the enzyme preparation for 35 days, and the dogs received 0, 200, 500 or 1 000 mg/kg body weight/day in capsule form for 30 days, without revealing significant changes related to the dose in the body weight, food intake, organ weight, urine analysis, blood

profiles, clinical chemistry, or histopathological profiles. The rats who received the same dose for 6 months did not reveal any doses related to adverse effects either, with the exception of a small increase in the weight of the large intestine, an effect considered as a physiological reaction to the passage of a large quantity of a non-absorbable substance. The NOAEL was 4 000 mg/kg body weight/day for rats and 1 000 mg/kg body weight/day for dogs. In reproduction toxicity tests, the NOAEL was set at 4 000 mg/kg body weight/day for rats and 1 000 for dogs and rabbits. The strain of *P. multicolor* used to prepare tilactase is not considered pathogenic. From this study an ADI of 40 mg/kg/day was inferred, that is that an ADI for an adult weighing 60 kg of 2 400 mg/day is adequate.

The toxicity studies suggest that the use of these enzymes in the food industry is safe (Flood and Kondo, 2004).

It is known that there is lactic activity in several fermented milk products which are not heat treated (AFSSA, 2008).

## 6.5 Conclusion

Lactase is considered by the NDA panel of the EFSA as sufficiently characterised and recommends a dose of 4 500 FCC (*Food Chemical Codex*) with each meal containing lactose although the dose should be adjusted to each individual depending on the meals taken with lactase (EFSA, 2009).

From the safety point of view, this consideration could be admitted as scientific evidence does not reveal that it could produce observable adverse effects.

As indicated in Regulation (EU) No 432/2012, establishing a list of permitted health claims made on foods, the target population should be informed that lactose tolerance is variable and advice should be sought regarding the role this substance may play in their diet (EU, 2012).

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## 7. Melatonin

### 7.1 Proposal

The AECOSAN has proposed a maximum daily quantity of 1 mg of melatonin. This proposal is based on the authorisation of two health claims, that melatonin contributes to the alleviation of subjective feelings of jet lag and that melatonin contributes to the reduction of sleep onset latency. The first claim can only be used with foods that contain a minimum of 0.5 mg of melatonin per quantified portion. In order to bear this claim, the consumer will be informed that the beneficial effect is obtained with a minimum intake of 0.5 mg that should be taken close to bedtime on the first day of travel and on the following few days after arrival at the destination (EU, 2012).

The second claim can only be used with foods that contain a minimum of 1 mg of melatonin per quantified portion. In order for a product to carry this claim, the consumer will be informed that the beneficial effect is obtained with an intake of 1 mg of melatonin taken close to bedtime (EU, 2012).

In Italy, a maximum daily quantity of 1 mg of melatonin is authorised in food supplements (Italy, 2013).

### 7.2 Characteristics and sources

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurohormone produced by the pineal gland in mammals and by other organs, especially the enterochromaphinic cells of the gastrointestinal tract and the retina (Hardeland et al., 1993) (Huether, 1993), whose principal physiological function is the regulation or control of circadian and seasonal rhythms (Armstrong et al., 1986). It was discovered in the nineteen-fifties (Lerner et al., 1958) (Lerner et al., 1959).

In recent years it has been demonstrated that melatonin also has several other additional functions, it is produced and acts in numerous tissues or cells that express melatonin receptors, at much lower levels (Hardeland, 2009). MT<sub>1</sub> and MT<sub>2</sub> melatonin receptors (membrane receptors) have been detected in numerous tissues of the CNS, in peripheral organs such the gastrointestinal tract, liver, lung, skin, adrenal gland, gonads, male organs, breast tissue, kidney, heart, blood vessels, adipose tissue, neutrophils, lymphocytes and lymphoid tissue (Ishii et al., 2009). Both receptors involve signalling through the inhibition of cAMP, protein-kinase A activity, effects on phospholipase A<sub>2</sub> and C, and effects on the potassium and calcium channels (Mathes, 2010). A third receptor, MT<sub>3</sub> which is an enzyme identified as quinone reductase 2 has also been identified.

The deficiencies in the production of melatonin or in the expression of the receptors or the falls in the levels of melatonin (as occur with age) result in numerous dysfunctions. In these cases, with insufficient levels of melatonin or poor melatonergic signalling, there may be numerous pathophysiological alterations, reflecting the pleiotropy of the molecule. Therefore, for example, a fall in night-time melatonin levels has been repeatedly observed in patients with neurodegenerative disorders and sleep disturbances (Uchida et al., 1996).

### 7.3 Nutrition and metabolism

Tryptophan and serotonin are the precursors for melatonin synthesis, and the enzymes that catalyse the formation are the *N*-acetyltransferase, which converts serotonin to *N*-acetyl-serotonin, and the enzyme hydroxyindole-*O*-methyltransferase, which converts *N*-acetyl-serotonin to melatonin (Figure 2). The melatonin present in blood circulation is principally metabolised via cytochrome P450 to 6-hydroxymelatonin, followed by conjugation, and excreted in urine as 6-sulfatoxymelatonin. This metabolite is not formed in the CNS where the oxidative rupture of the pyrrole ring predominates. The plasma half-life of melatonin is in a range of 30-50 minutes (Lane and Moss, 1985).

The regulation of melatonin synthesis is controlled by the light/dark cycle that acts through the anterior hypothalamic neural activation, via retinal ganglion cell axons through the optic nerve. The capacity for melatonin

synthesis varies with the individual. In humans, there is evidence that melatonin levels decrease with age. Serum levels of melatonin are very low in the first weeks of postnatal life, without diurnal variation. At the age of 6 months, the synthesis follows a circadian rhythm, reaching the maximum between 3 and 6 years old. At 40-50 years old, a decrease in melatonin synthesis is observed and after the age of 70 the circadian rhythm in the secretion of melatonin virtually disappears in the majority of individuals (Dziegiel et al., 2008). A seasonal difference also appears to be present in melatonin synthesis in humans, with higher levels in winter than in summer (Vijayalaxmi Reiter et al., 2004).

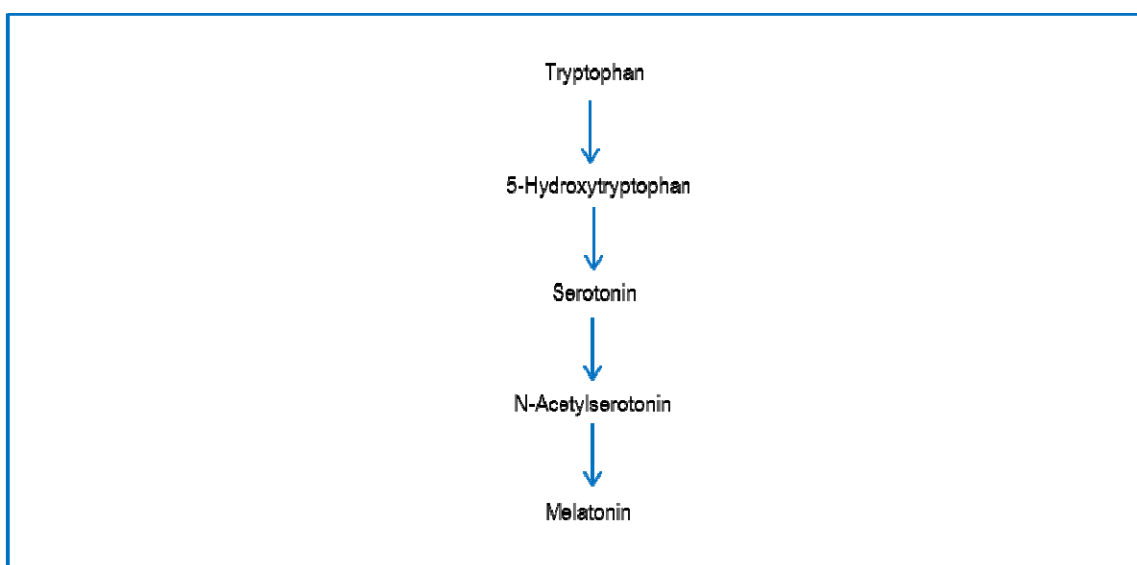


Figure 2. Metabolic phase of melatonin synthesis.

The endogenous production of melatonin in humans has been estimated at values of 25-30  $\mu\text{g/day}$  (Peters, 1992) or approximately 0.4  $\mu\text{g/kg/day}$  in a person with a body weight of 70 kg. Circulating levels of melatonin follow a daily pattern, with higher plasma levels at night than during the daytime, even in nocturnal animal species such as the rat (Reiter, 1991), levels that may vary with the stage of pregnancy (Pang et al., 1987). Melatonin is a hydrophobic molecule which diffuses through biological membranes, and is present in the biological fluids or tissues in concentrations similar to those observed in the plasma (Hardeland et al., 1993), (Menendez-Peláez and Reiter, 1993). Up to 70 % melatonin is bound to albumin in plasma, implying that the remaining 30 % in free form is diffused to the tissues (Hardeland et al., 2006).

Melatonin may be present in plants in higher quantities than those observed in vertebrate tissue (except in the pineal gland) and its function appears to be similar to that of vertebrates, "sweeper of free radicals", and it may also act as a coordinator of photoperiodic responses. Melatonin reduces the oxidative damage to macromolecules such as lipids, proteins and DNA (Reiter, 1991). Melatonin, thanks to its lipophilic property, is able to reach the intracellular compartments and protect all the parts of the plant from oxidative damage, especially the reproductive tissue and germ found in the seeds and flowers. The first papers identifying melatonin in plants and fruits such as tomato, rice, cabbage, oranges, apples and banana were published in 1995 (Dubbels et al., 1995) (Hattori et al., 1995). More recently, melatonin concentrations have been determined in seeds from 15 edible plants, reaching a range of 2 to



200 ng/g dry weight (Manchester et al., 2000), and in cherries in a range of 2-14 ng/g fresh weight (Burkhardt et al., 2001). The highest concentrations are found in black and white mustard (Table 2).

Table 2. Melatonin concentrations in the seeds of 15 edible plants	
Name	Melatonin content (ng/g seed)
Milk thistle ( <i>Silybum marianum</i> )	2
Poppy ( <i>Papaver somniferum</i> )	6
Anise ( <i>Pimpinella anisum</i> )	7
Coriander ( <i>Coriandrum sativum</i> )	7
Celery ( <i>Apium graveolens</i> )	7
Flaxseed ( <i>Linum usitatissimum</i> )	12
Cardamom ( <i>Elettaria cardamomum</i> )	15
Alfalfa ( <i>Medicago sativum</i> )	16
Fennel ( <i>Foeniculum vulgare</i> )	28
Sunflower ( <i>Helianthus annuus</i> )	29
Almond ( <i>Prunus amygdalus</i> )	39
Fenugreek ( <i>Trigonella foenum-graecum</i> )	43
Goji berry ( <i>Lycium barbarum</i> )	103
Black mustard ( <i>Brassica nigra</i> )	129
White mustard ( <i>Brassica hirta</i> )	189

#### 7.4 Safety

In the last 20 years, numerous clinical trials have examined the use of intakes of exogenous melatonin in different fields of medicine. The effects of melatonin have been assessed in a wide range of doses as an adjuvant medication with other therapeutic medicines in various diseases (Sánchez-Barceló et al., 2010).

In the different clinical trials conducted on humans, the doses of melatonin used range from 0.1 to 300 mg, which partly explains the low toxicity of melatonin and, on the other hand, the lack of knowledge about an optimum dose for each case of disorder for which melatonin may be effective. What is clear however is that in all the clinical trials carried out, the appearance of adverse effects due to melatonin is non-existent.

The opinions of the EFSA on the basis of various publications (meta-analysis of controlled clinical trials on subjects with sleep disorders) conclude that melatonin has beneficial physiological effects as it contributes to the alleviation of subjective feelings of jet lag and contributes to the reduction of sleep onset latency (EFSA 2010, 2011).

At experimental level using laboratory animals (rodents) the possible effect of melatonin on reproductive development and fertility was questioned. However, Jahnke et al. (1999) did not observe embryo/foetal toxicity in rats treated orally with melatonin (50, 100 and 200 mg/kg/day, between 6 to 19 days of gestation). Maternal toxicity was only observed at doses of 200 mg/kg/day at the “transient reduction in body weight gain” critical point. Melatonin did not effect prenatal survival or fetal weight and there were no incidents or effects of fetal malformation. The NOAEL and LOAEL (Lowest Observed Adverse Effect Level) of maternal toxicity were 100 and 200 mg/kg/day, respectively. In humans, there is evidence that melatonin administered orally is associated with sleep induction at a wide range of

doses from 0.2 mg (Attenburrow et al., 1996) (Herxheimer and Petrie, 2002), and therefore the NOAEL calculated in the development toxicity trials was 500 times the doses that produce sleep induction.

The benchmark dose (BMD) is applied to define the threshold for the pharmacological action of a substance. The BMD is the point on the dose-response curve that characterises a specific effect, the so-called benchmark response. The values are based on data from the dose-response curve and the variability of the data for the critical effect. The European Food Safety Authority suggests that the BMD approach is applicable to all substances present in foods, irrespective of their category or origin, especially when the identification of a NOAEL is uncertain (EFSA, 2009). In the case of melatonin, Lachenmeier et al. (2012) calculated the BMD from data provided by Attenburrow et al. (1996) who determined various parameters related to time and effectiveness in melatonin-induced sleep, at a low range of doses, where melatonin demonstrated a highly significant effect ( $p=0.001$ ). The calculated BMD was 0.4 mg/day, which is consistent with literature that demonstrates that melatonin is efficient at low doses (Herxheimer and Petrie, 2002). A threshold was calculated for the pharmacological action of 0.04 mg/day from the BMD with an uncertainty factor of 10, considering the differences among individuals. It is extremely important to clearly differentiate a medicinal product from a food supplement. Therefore, in the case of melatonin, given that the action of melatonin on receptors in the human brain is well-defined (Reppert et al., 1995), an intake of melatonin above 1 mg/day should be classified as a medicinal product (Lachenmeier et al., 2012).

## 7.5 Conclusion

With the information currently available, the Scientific Committee considers that there is no scientific evidence to link the consumption of melatonin with the development of adverse effects, and therefore establishes that melatonin may be considered as a safe food supplement for contributing to the alleviation of subjective feelings of jet lag and to the reduction of sleep onset latency, provided that this consumption is made within reasonable intake limits.

The Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AECOSAN proposal of a maximum daily quantity of 1 mg of melatonin is acceptable from the safety point of view for use as a food supplement.

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## 8. Methylsulphonylmethane

### 8.1 Proposal

The AECOSAN has recommended a maximum daily quantity of 1 g of methylsulphonylmethane.

Different health claims have been submitted to the EFSA for quantities ranging between 0.5 and 1.5 g/day of methylsulphonylmethane, for the general population, which include: (1) contribution to collagen synthesis in cartilage and to the normal maintenance of bone structure, articulations, teeth, hair and nails; (2) cartilage regeneration; (3) maintenance of normal acid-base balance in the body; (4) strengthening of the immune system function; (5) strengthening of the gastrointestinal system function; (6) contribution to cysteine synthesis and (7) vitamin production (C, H, B5, B13) needed for the correct function of metabolism, although none of these proposals demonstrated a cause and effect relationship between the alleged effect and the proposed dose (EFSA, 2010).

In Italy, methylsulphonylmethane is authorised in food supplements without the establishment of a maximum daily quantity (Italy, 2013). In Belgium methylsulphonylmethane is authorised in food supplements (Belgium, 2013).

### 8.2 Characteristics and sources

Methylsulphonylmethane (MSM) (Figure 3), also known as dimethyl sulfone (DMSO<sub>2</sub>) and methyl sulfone, is an organic compound containing sulphur (34 %), highly stable even at high temperatures (275 °C), present in small quantities in a large variety of fruits, vegetables, grains, meat, eggs and fish (Pearson et al., 1981).

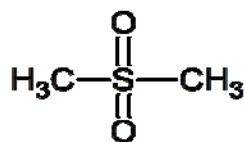


Figure 3. Chemical structure of methylsulphonylmethane.

Cow's milk is the principal source of MSM containing 6-8 µg/g (Williams et al., 1966) (Imanaka et al., 1985). Other foods containing MSM include coffee (1.6 µg/g), tomato (0.86 µg/g), tea (0.3 µg/g), maize (0.11 µg/g) and alfalfa (0.07 µg/g).

In humans, MSM levels have been detected in the range of 0-25 µmol/l in the plasma and cerebrospinal fluid from healthy individuals (Engelke et al., 2005). The MSM was detected using a magnetic resonance spectroscopy on the brain tissue of individuals who ate MSM as a supplement, but not on those who did not eat it, therefore suggesting that the MSM is able to cross the blood-brain barrier (Rose et al., 2000) (Lin et al., 2001) (Cecil et al., 2002). In humans, approximately 5-10 mg/day of MSM is excreted in the urine.

The kinetic behaviour of MSM in rats has been studied, demonstrating that MSM, after a single oral dose of 500 mg/kg body weight, is rapidly absorbed, distributed all around the body and mainly eliminated via renal excretion. Of note among the kinetic parameters described is a plasma half-life of 12 hours; approximately 85.8 % of the dose is excreted through urine within 120 hours after administration (Magnuson et al., 2007a).

The biological role of MSM is not altogether explained, it is considered a bioactive compound and a source of sulphur for the production of sulphur-containing amino acids such as cysteine and methionine (Parcell, 2002).

### 8.3 Nutrition and metabolism

MSM is a metabolite of dimethylsulfoxide (DMSO). In the troposphere, DMSO is a biotransformation product formed from phytoplankton and algae. In commercial production, MSM is synthesised from the reaction of DMSO with hydrogen peroxide, producing MSM and water. In humans, approximately 15 % of the dose taken of DMSO is metabolised into MSM (Hucker et al., 1967). Both compounds are able to act through their capacity as membrane stabilisers and barriers to free hydroxyl radicals; it has been suggested that the sulphur that forms part of its molecule may participate in the formation of cartilage (Parcell, 2002).

### 8.4 Safety

Scientific literature contains several clinical trials (double-blind and placebo controlled, multi-centre) to assess the efficiency and safety of MSM in patients with osteoarthritis (disorder characterised by the progressive rupture of the articular cartilage). In the case of mild to moderate osteoarthritis of the knee, Usha and Naidu (2004) assessed the effect of the MSM on pain relief through movement in 118 patients at doses of 1.5 g/day for 12 weeks. The only adverse effect observed, diarrhoea, was only observed in 5 % of the patients treated. Kim et al. (2006) also assessed in 29 patients with mild to moderate osteoarthritis of the knee, the effect of MSM (oral dose of 2 to 5 g/day, for 12 weeks) on the pain on movement, observing a statistically significantly palliative effect. The adverse effects were

minor and mainly related to the gastrointestinal tract. Subsequently, Brien et al. (2008) assessed six studies with 681 patients with osteoarthritis of the knee (treated with DMSO) and 168 treated with MSM, where they observed significantly less pain on movement, but the optimum dose and dosage period were not concluded in these clinical trials. In all the clinical trials carried out, the appearance of adverse effects due to MSM is non-existent or minimal and is linked to the gastrointestinal tract.

With respect to the safety of MSM at experimental level, acute toxicity trials were conducted on rats, oral dose constraint of 2.0 g/kg body weight, and oral dose of 1.5 g/kg body weight in sub chronic toxicity trials for 90 days (doses 15 times the maximum recommended dose in humans of 6 g/day equivalent to 0.1 g/kg body weight (60 kg) as a therapy (Horvath et al., 2002) and doses of 60 times the maximum recommended dose in humans of 1.5 g/day equivalent to 0.025 g/kg body weight (60 kg) as a supplement). No adverse effects or mortality, clinical toxicity effects, weight gain, alterations in the blood and biochemical parameters were observed in either of the trials, nor did the necropsy reveal any pathological lesions, nor were alterations observed in the weight of the organs or in the histopathology of the organs. A sub chronic NOAEL was established for the MSM of 1.5 g/kg/day (Horvath et al., 2002). Based on this study, extrapolating this NOAEL to humans (60 kg body weight) and taking a safety factor of 100, we would have a maximum daily quantity of 0.9 g≈1 g for MSM as a supplement. Another study at experimental level is that developed by Magnuson et al. (2007b) in which the potential toxicity was determined in the development of MSM in female pregnant rats treated during the period of organogenesis and histogenesis. The MSM was administered orally by gavage; oral doses of 50, 250, 500 and 1 000 mg/kg/day, during days 6 to 20 of gestation, not producing any maternal toxicity, nor were any anomalies observed in the foetal formation, or malformations in the skeletal tissues. The NOAEL for the maternal and development toxicity for MSM was 1 g/kg/day (Magnuson et al., 2007b). Based on this study, extrapolating this NOAEL to humans (60 kg body weight) and taking a safety factor of 100, we would have a maximum daily quantity of 0.6 g for MSM as a supplement.

The genotoxicity of MSM has also been assessed in *in vitro* and *in vivo* tests (Lee et al., 2006). The MSM did not induce reversion in *Salmonella typhimurium* TA98, TA100, TA1535 or TA1538 at concentrations of 10 000 µg/plate with and without S9 fractions. The MSM (with concentrations up to 5 000 µg/ml) was not observed to have any capacity for inducing chromosome damage in the *in vitro* trials of chromosome aberration in CHL cells with and without S9 fractions. Similarly, genotoxicity was not observed in bone marrow of mice treated with 5 000 mg MSM/kg.

All these studies at experimental level and those carried out in clinical studies on humans, support the evidence for the safety of MSM used at the recommended doses.

## 8.5 Conclusion

With the information currently available, the Scientific Committee considers that there is no scientific evidence to link the consumption of methylsulphonylmethane to the development of adverse effects.

Therefore, the Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AECOSAN proposal of a maximum daily quantity of 1 g of methylsulphonylmethane is acceptable from the safety point of view for use as a food supplement.

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## 9. Polyphenols from olive oil and olive leaves and fruit

### 9.1 Proposal

The AECOSAN has proposed a maximum daily quantity for polyphenols of olive oil and olive leaves and fruit (hereinafter, polyphenols in olive) of 5 mg.

This proposal is based on the dose which is effective on health of polyphenols in olive (fruit, olive mill waste waters or "alperujo"<sup>1</sup>, olive oil, leaf extracts of *Olea europea* L.). Specifically this dose is recommended for the protection of LDL particles from oxidative damage (EFSA, 2011).

### 9.2 Characteristics and sources

The phenolic compounds of the olive are compounds from the secondary metabolism of the plant which are synthesised in response to different situations of stress, pathogen attacks and damage caused by insects. The name of phenolic compounds is more appropriate, as not all the compounds to be considered are polyphenolic compounds and it will be used in this report.

The main phenolic compounds from the olive are oleuropein-glycoside, hydroxytyrosol and tyrosol. Oleuropein is formed from hydroxytyrosol and elenolic acid. There are other minority phenols such as ligstroside, tannins, etc. (Tuck and Hayball, 2002) (Bendini et al., 2007).

Oleuropein belongs to a group of phenolic compounds, the secoiridoids, only found in abundance in the *Oleaceae* family, which includes the species *Olea europea* L., or the olive. They are produced from the plant secondary metabolism of terpenes and are normally glycosylated. In general, they are formed from a phenylethyl alcohol (hydroxytyrosol and tyrosol), elenolic acid and eventually a glucosidic residue. Oleuropein in particular is an ester of hydroxytyrosol and the elenolic acid glucoside (Bendini et al., 2007) (Taamalli et al., 2012).

The sources of phenolic compounds of the olive are the leaf extracts, the "alperujo" which is the aqueous fraction obtained in the process to obtain virgin olive oil; and virgin olive oil itself (Biagi et al., 2014). There are minor differences in the profile although the main ones in all of them are the secoiridoids, and specifically oleuropein with its two phenylethyl alcohols, hydroxytyrosol and tyrosol to a lesser degree.

The secoiridoids (oleuropein) in aglyconic forms present in virgin olive oil come from the glycosides present in the fruit by hydrolysis of endogenous  $\beta$ -galactosidases during the process to obtain oil (milling and beating). They are amphiphilic and are partitioned between the oily layer and the vegetation water, and are more concentrated in the latter fraction due to the polar nature of their functional groups. During storage of virgin olive oil, hydrolysis of oleuropein takes place, increasing the content of simple phenols such as hydroxytyrosol (mainly) and aglycone ligstroside, which contains tyrosol and elenolic acid (Taamalli et al., 2012) (Biagi et al., 2014).

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<sup>1</sup> *Alperujo*: Also known as alpeorujo, this is a sub-product obtained from the continuous extraction of two phases of virgin olive oil. It is the mixture of: vegetation water or alpechines; solid parts of the olive, including the stone, the mesocarp and the skin; and fatty waste.



However there are clear differences in the quantitative aspect as oleuropein makes up between 0.005 and 0.12 % of virgin olive oil, 0.87 % in the “alperujo” and between 1 and 4 % in the leaves. Ordered from highest to lowest content of phenolic compounds we have the leaves, the “alperujo” and virgin olive oil (El and Karakaya, 2009) (Biagi et al., 2014).

The total content of phenolic compounds in the three sources obtained from the olive is variable and depends on numerous factors including the variety of olive, climate and humidity. In the leaves, the total content of phenolic compounds is 2 058 mg GAE (Gallic Acid Equivalence)/kg. In virgin olive oil, the phenolic content ranges between 100 and 800 mg/kg depending on numerous factors intrinsic to the olive or external. In fruit extracts, after the oily phase extraction process, the concentration ranges between 2 000 and 8 000 mg of GAE/kg of aqueous extract (Taamalli et al., 2012) (Biagi et al., 2014).

It should be made clear that the majority of the data obtained about phenolic compounds, especially that obtained in humans, refers to studies in which the dietary source was virgin olive oil, where data about the phenolic compounds from “alperujo” and other extracts of olive pulp and leaves is in the minority as these are not included in the normal diet of the population and are only used in food supplements or to enrich other foods. In any case, the majority molecules, from the qualitative view point, contained in the three sources mentioned above are similar with variations in the quantity and proportion due to the technological processes used (Tuck and Hayball, 2002) (El and Karakaya, 2009) (Taamalli et al., 2012) (Biagi et al., 2014).

Phenolic compounds of the olive, and especially those from virgin olive oil play a relevant role in stability and sensorial attributes (mainly bitterness and pungency). Nevertheless, the properties in greatest demand and currently under study are the role as natural antioxidising molecules acting on the oxidative stress of the body caused by the generation of free radicals of oxygen, they are also able to inhibit the proliferation of many pathogenic microorganisms. Therefore, for example, their positive role in the prevention of cardiovascular disease has been proved thanks to their action in preventing the oxidation of the LDL, they also inhibit platelet clumping, have an antieoplastic action thanks to the apoptotic properties and have an effect on the growth of respiratory and gastrointestinal pathogenic microorganisms (Cicerale et al., 2010) (Sabatini, 2010) (Hu et al., 2014).

### 9.3 Nutrition and metabolism

The main sources of food intake of phenolic compounds of the olive are virgin olive oil and table olives. Due to high levels of virgin olive oil consumption, the Mediterranean populations in Greece, Italy and Spain have higher intakes, with 18, 13 and 11 kg of virgin olive oil /year/person. If we consider an average intake of virgin olive oil of between 30 and 50 g/day (dressing and cooking) and an average content of 180 mg/kg, the intake of these food components would be 9; 7.5 and 5.5 mg/day. In the PREDIMED cohort, the intake of total phenols in the Spanish population was 820±323 mg/day of which 22±11 mg/day came from olive oil and 68.5±104.0 mg/day from olives. In total, phenols from the olive amounted to 11.0 % of the total phenols consumed (Tuck and Hayball, 2002) (Vissiers et al., 2004) (Tresserra et al., 2013).

Studies on animals and humans show that the phenolic compounds of the olive (from virgin olive oil, from the aqueous extracts from the pulp and leaf extracts) are well absorbed in the small intestine both in animals use din research and in humans as they appear in plasma and urine after their intake. The available data about their absorption and metabolism refer to the phenols mainly found in the olive, oleuropein, glycosylated or aglycone ligstroside (secoiridoids), hydroxytyrosol and tyrosol.

Their content and the proportions in the different olive products vary as a consequence of the processes to which they are subjected and mentioned above in the section of characteristics and sources.

Data on the plasma levels after the intake of virgin olive oil are variable among individuals and among studies due to the product consumed (virgin olive oil, leaf extracts, etc.), the treatment of the samples and the analytical techniques used. Thus, Rubió et al. (2012) observed, in humans, that after the intake of 30 ml of different virgin olive oils with different phenol contents (250, 500 and 750 mg of total phenols/kg), corresponding to an intake of 6.9, 13.8 and 20.7 g, different metabolites were detected in plasma between 0 and 6 hours after the intake including: hydroxytyrosol sulphate (HyTy), HyTy acetate sulphate, homovanillic acid and homovanillic acid sulphate. These have a T<sub>max</sub> between 1 and 2 hours (between 45 and 120 minutes for all the metabolites) and therefore absorption is fast. The conjugated forms with glucuronic were not detected. Both for the area under the curve (AUC) and for C<sub>max</sub> there is a lineal dose-response relation between the oils, although there are no significant differences in these two variables between the medium and the high concentrations. This is attributed to the high inter-individual variability and to the modulation of Phase II enzymes (SNPs, epigenetics or genetics) (Rubio et al., 2012). In previous studies, always using virgin olive oil, it was observed that on increasing the quantity of olive oil phenols consumed, the increase in the concentration of HyTy conjugated with glucuronide was dose-dependent (Vissiers et al., 2004).

The fraction of HyTy metabolites in plasma with respect to that consumed is similar in the oils with a low, medium and high phenol content and varies between 17 and 23 % (Rubio et al., 2012). Other authors indicate a figure for the urine recovery of the phenols consumed ranging between 5 and 72 %, the majority in the form of glucuronide-conjugates (Vissiers et al., 2004). The studies of Visioli et al. (2003) administering HyTy present urine recovery data for this simple phenol and for its metabolite, homovanillic acid, of 44 % of the total HyTy administered and 234 % of the free HyTy administered. In humans the high excretion rate of free HyTy (more than 100 %) suggests the hydrolysis of the OE. It also concluded that the excretion of HyTy in humans is higher when it is administered as a natural component of virgin olive oil (44 % of the HyTy) than after administration added to refined olive oil (23 % of the HyTy) or added to yogurt (5.8 % of the dose). This huge variability may also be linked to the different approximations in the determination of the urinary excretion and the different analytical techniques used, as mentioned above.

The presence in plasma and urine of homovanillic acid and its conjugates with sulphate indicate the action of the Catechol-o-methyl transferase (COMT), an enzyme that also intervenes in the metabolism of the catecholamines.

In the study by García-Villalba et al. (2013), humans were given 250 mg of an extract of olive leaves rich in oleuropein. These authors state that during the absorption of the oleuropein, that is, almost totally hydrolysed in the gastrointestinal tract, the principal product of hydrolysis is HyTy in blood appearing as conjugated glucuronide in the first 30 minutes after the intake of the extract. These authors detect 15 metabolites in plasma, the majority conjugated with glucuronide which is in contrast to other studies in which these compounds were not detected but conjugates were present with sulphate (Rubio et al., 2012).

The absorption pattern of the different phenolic compounds in plasma was very similar. The absorption mechanism is not clear and there are different hypotheses for different classes of phenolic compounds. Two-way passive dissemination mechanisms have been proposed according to some authors in *in vitro* tests with Caco-2 cells (Manna et al., 2000) and transcellular, paracellular or glucose transporters for the HyTy and OE, respectively. Based on the results of Manna et al. (2000) it might be thought that, in humans, HyTy is completely absorbed (100 %). Bonanome et al. (2000), administering 100 g of virgin olive oil, concluded that its absorption did not take place through the formation of chylomicrons, and there may be an antioxidant effect *in vivo* probably in the postprandial period.

Following the administration of marked HyTy, 90 % of the radioactivity administered was detected in urine within the next 5 hours and only 5 % in faeces. The sulfo-conjugates are a significant fraction of the total radioactivity and in addition are the principal products of urinary excretion. Based on these results, the authors suggest that COMT,

alcohol deshydrogenase, aldehyde deshydrogenase and phenolsulfotransferase take part in the exogenous metabolism of HyTy (D'Angelo et al., 2001).

The urinary excretion kinetics was also similar for the majority of compounds. The maximum rate of urinary excretion was reached in the first 4 hours, and then a rapid drop in the base values was observed with the exception of the sulphated metabolites, the excretion of which was not completed 24 hours after the intake of an extract of olive leaf (García-Villaba et al. 2013). Miró-Casas et al. (2003a) and Miró-Casas et al. (2003b) in studies with a single dose and short-term consumption of virgin olive oil, obtain maximum plasma levels of HyTy and 3-*O*-methyl HyTy at 32 and 53 minutes after their intake and an elimination half-life of 2.43 hours with a maximum concentration of 26 µg/l. Given that HyTy appears in plasma and urine 98 % of the time in conjugated forms, a first step is suggested that includes intestinal and hepatic metabolism of the HyTy consumed.

Some authors have described differences in the absorption and metabolism of the phenolic compounds of the leaf extracts between genus and hormonal state, the latter in a study on pre- and post-menopause females (De Bock et al., 2013).

#### 9.4 Safety

A recent publication (Martin and Apple, 2010) advised of the potential harmful effect of an excess intake of antioxidants after intake as concentrated or purified products or enriched with different antioxidant molecules forming part of food supplements. In certain conditions, for example high concentrations of phenolic compounds, high pH or the presence of Fe, these compounds may enter an autooxidation process and behave as pro-oxidants. Evidence of this behaviour exists, for example, in phenolic compounds of green tea such as epigallocatechin galate. In certain conditions this phenolic compound generates hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and induces oxidation processes, and a certain hepatic toxicity is observed.

In this context, different studies have been made on the toxicity of different phenols obtained and purified from different parts of the olive (especially HyTy) and from extracts of olive pulp (aqueous extracts) and from the olive leaf.

The most complete assessment of safety was carried out using an aqueous extract of olive pulp (Soni et al., 2006). In acute toxicity no deleterious effects were observed for doses between 1 000 and 2 000 mg/kg of the extract, where LD<sub>50</sub>>2 000. With doses higher than 5 000 mg/kg administered for 29 days, micronucleus tests did not produce negative results where LD<sub>50</sub>>5g/kg. Nor were pathological changes observed in organs at these doses administered for 14 days. Sub chronic toxicity studies established a NOAEL of 2 000 mg/kg of the extract which corresponds to 120 mg/kg/day of total phenols consumed. Certain incidents were observed but cannot be attributed to the extract or were without importance. With respect to reproduction and development toxicity, nor was damage found up to doses of 2000 mg/kg of the extract, establishing a NOAEL>2 000 mg/kg (Soni et al., 2006).

Lastly, *in vitro* genotoxicity tests using various strains of *S. tiphymurium* and *E. coli* and with doses of up to 5 000 µg/plate found some cases that offer equivocal evidence of mutagenic activity in two strains of *S. tiphymurium*. In another study on chromosomal aberrations with Chinese Hamsters and doses of up to 1 000 µg/ml, the extracts revealed positive results in the induction of chromosomal aberrations at the maximum dose.

*In vivo* tests using micronucleus assays on Crl rats: CD® *Sprague Dawley* did not reveal significant effects at doses between 1 000 and 500 mg/kg/day for 28 days and between 1 000 and 2 000 mg/kg in single doses (Soni et al., 2006).

In a study in 2003 on *in vitro* cytotoxicity in normal and cancerous cells from the mouth and saliva glands of certain phenolic compounds from olive oil (Babich and Visioli, 2003), the authors concluded that at the concentrations at which the phenolic compounds tested are found in olive oil, there is no evidence of cytotoxic effects, even at far higher doses.

In a recent publication, the toxicity of HyTy was assessed at doses of 5, 50 and 500 mg/kg/day, one of the main phenolic compounds in olive products (virgin olive oil, olives, leaf, *alperujo*) (Auñon et al., 2013). Following the analysis of the functional, histopathological, hematological and clinical effects, the authors recommend a NOAEL of 500 mg/kg/day. *In vitro* genotoxicity studies reveal that HyTy is not genotoxic at the usual physiological doses. No data is available for the *in vivo* genotoxicity. A study carried out using *Drosophila* rejects the genotoxicity of the phenolic compounds of virgin olive oil (Anter et al., 2010). Studies on safety for leaf extracts are scarce. Although no serious adverse effects have been described, certain minor disorders (cough, vertigo, headache and cough) and allergic reactions have been observed. A report from the European Medicines Agency advises against its use in individuals with gallstones as it could precipitate the appearance of biliary colic. It also reports that there are no studies regarding its administration to children and pregnant and nursing women due to lack of data in these populations (European Medicines Agency, 2011).

A study carried out using *alpechin* (vegetable water) and the phenolic compounds contained within (El Hajjouji et al., 2007) reveals genotoxic effects of *alpechin*, oleuropein and gallic acid in tests on chromosomal aberrations of *Vicia faba* (micronuclei).

## 9.5 Conclusion

Altogether, the studies reveal low toxicity levels and therefore high safety levels for the phenolic compounds from the olive although these studies warn of their possible pro-oxidant role at high concentrations and in certain ambient conditions (high pH and presence of Fe). In addition, the intake of antioxidant compounds in our normal diet may be high, in particular in a Mediterranean diet with a high intake of antioxidant-rich food (fruit and vegetables, etc.). We believe that the proposed dose of 5 mg/day, which coincides with that listed in the EFSA report on virgin olive oil biophenols and their role in preventing the oxidation of LDL particles and, therefore, the development of cardiovascular disease, is adequate for their intake as a food supplement.

Therefore, the Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AECOSAN proposal of a maximum daily quantity of 5 mg of phenolic compounds from olive oil and olive leaves and fruit is acceptable from the safety point of view for use as a food supplement.

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