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Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) on the use conditions for certain substances other than vitamins, minerals and plants in food supplements

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Abstract

Food supplements are foods whose purpose is to supplement the normal diet and which consist of concentrated sources of nutrients (vitamins and minerals) or other substances with a nutritional or physiological effect, alone or in combination. The supplements are marketed in dose form and, in no event, should they replace the use of medication 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 49 substances proposed by the AESAN belong to different groups: fatty acids, amino acids, peptides, enzymes, flavonoids, carotenoids, nucleotides, polysaccharides, oligosaccharides and others.

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

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the AESAN is acceptable from a safety viewpoint for use as a food supplement. In no event is the assessment intended as a guarantee of the efficiency of the substances and the estimated doses.

Key words

Food supplements, fatty acids, amino acids, peptides, enzymes, flavonoids, carotenoids, nucleotides, polysaccharides, oligosaccharides.

Abbreviations

ADI: Acceptable Daily Intake.
AI: Adequate Intake.
AR: Average Requirement.
b.w.: body weight.
DRI: Dietary Reference Intakes.
EAR: Estimated Average Requirements.
GRAS: Generally Recognized As Safe.
HOI: Highest Observed Intake.
LD_{50}: Lethal Dose 50.
LOAEL: Lowest Observed Adverse Effect Level.
NDA: Dietetic Products, Nutrition and Allergies.
NOAEL: No Observed Adverse Effect Level.
NOEL: No Observed effect Level.
OSL: Observed Safe Level.
RDA: Recommended Dietary Allowances.
UF: Uncertainty Factor.
UL: Upper Level.
ULS: Upper Levels for Supplements.

1. Introduction

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 liquid, 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 (BOE, 2009) 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, who 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.

The Spanish Agency for Food Safety and Nutrition (AESAN) has drawn up a 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. For this purpose, they have used different documentary sources such as the authorisation existing in other Member States, the legislation on other substances that may be added for specific nutritional purposes in foods for particular nutritional purposes and reports from different European assessment bodies.

Therefore, the Executive Directore of the AESAN has asked the Scientific Committee to assess the proposal to authorise the use of certain substances other than vitamins and minerals in the manufacture of food supplements both with respect to the maximum daily quantities proposed and as regards the appropriateness of the authorisation.

2. Proposal

The Sub-Directorate General of Food Risk Management for the AESAN 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).

Table 1. Substances and maximum quantities proposed by the AESAN for their use in the manufacture of food supplements

Group	Proposed substance	Proposed maximum daily amount	Proposed warning
Fatty acids	Arachidonic acid	-	-
	Linoleic acid and alpha-linolenic acid	1,000 mg of alpha-linolenic acid. Linoleic/alpha-linolenic acid ratio: maximum 5	-
	Oleic acid	-	-
	Omega-3 fatty acids (DHA + EPA)	3,000 mg	-
Amino acids (and their salts of Na, K, Ca, Mg and HCl) and other nitrogen compounds	Branched-chain amino acids (L-isoleucine + L-leucine + L-valine)	Total 5,000 mg	Must not be consumed by pregnant women, children, or for extended lengths of time without medical control
	L-glutamic acid	-	-

	L-alanine	-	-
	Beta-alanine	-	-
	L-arginine	3,000 mg	-
	L-carnitine	L-carnitine or L-carnitine hydrochloride 2,000 mg	-
		L-carnitine tartrate 3,000 mg	-
	L-cysteine	300 mg	-
	L-glutamine	2,000 mg	-
	L-histidine	750 mg	-
	L-isoleucine	1,500 mg	-
	L-leucine	2,950 mg	-
	L-lysine	2,250 mg	-
	L-methionine + L-cysteine	Total: maximum 1,100 mg (L-methionine: maximum 800 mg and L-cysteine: maximum 300 mg)	-
	L-ornithine-alpha-	2,000 mg	-
	Taurine	1,000 mg	-
	L-tyrosine + L-phenylalanine	1,900 mg	-
	L-threonine	1,150 mg	-
	L-tryptophan (obtained by protein hydrolysis)	300 mg	Not recommended for persons receiving treatment with antidepressants
	L-valine	1,950 mg	-
Dipeptides and peptides	Glutathione	50 mg	-
	Lactoferrin	200 mg	-
Enzymes	Coenzyme Q-10 or Ubiquinone	200 mg	-
Flavonoids, Carotenoids	Astaxanthin from crustaceans and fish	4 mg	-
	Lycopene	15 mg	-
	All *trans* Lutein/Zeaxanthin	20 mg	
	Quercetin	75 mg	Not recommended for pregnant women

	Rutin	150 mg	Not recommended for pregnant women
Nucleotides	Adenosine 5-monophosphate and its sodium salts	450 mg as sum total of Adenosine, Cytidine, Guanosine, Inosine and Uridine	-
	Cytidine 5-monophosphate and its sodium salts	450 mg as sum total of Adenosine, Cytidine, Guanosine, Inosine and Uridine	-
	Guanosine 5-monophosphate and its sodium salts	450 mg as sum total of Adenosine, Cytidine, Guanosine, Inosine and Uridine	-
	Inosine 5-monophosphate and its sodium salts	450 mg as sum total of Adenosine, Cytidine, Guanosine, Inosine and Uridine	-
	Uridine 5-monophosphate and its sodium salts	450 mg as sum total of Adenosine, Cytidine, Guanosine, Inosine and Uridine	-
Polysaccharides and Oligosaccharides	Beta-glucans	4,000 mg	-
	Chitosan obtained from shells of crustaceans	3,000 mg	Excessive intake may cause intestinal upset
	Fructo-oligosaccharides (FOS)	9 g FOS or 9 g of FOS + Inulin	Excessive intake may cause intestinal upset
	Galacto-oligosaccharides	-	-
	Konjac glucomannan (*Amorphophallus konjac* K.	4,000 mg	Do not use in dehydrated foods
	Guar gum	10 g	Do not use in dehydrated foods
	Inulin	9 g Inulin or 9 g of FOS + Inulin	Excessive intake may cause intestinal upset
	Pectins	10 g	-
Other substances	Choline (as choline, choline	1,500 mg	-
	Chondroitin sulphate	500 mg	-
	Creatine monohydrate	3,000 mg	-
	Glucosamine (as sulphate or	500 mg	-
	Inositol hexaphosphate	2,000 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 1924/2006 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 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 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.

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4. Fatty acids

4.1 Arachidonic acid

4.1.1 Proposal

The AESAN has recommended the inclusion of arachidonic acid in Royal Decree 1487/2009 without specifying a maximum daily amount. The proposal is based on the scientific opinion of the EFSA on the dietary reference values for the fats, which confirm that a dietary reference value is not required to be established for this fatty acid (EFSA, 2010).

In Belgium and in Italy (legislative proposal) it is authorised in food supplements without the establishment of a maximum daily amount (Belgium, 1992) (Italy, 2012).

4.1.2 Characteristics and sources

Arachidonic acid (*cis*-5, *cis*-8, *cis*-11, *cis*-14-eicosatetraenoic acid) is an omega-6 long-chain polyunsaturated fatty acid (PUFA), consisting of a chain of 20 carbon atoms with 4 double *cis* bonds in positions 5, 8, 11 and 14.

Arachidonic acid (AA) is present in low quantities in meat, eggs, fish, algae and other aquatic plants.

4.1.3 Nutrition and metabolism

Function

In general, long-chain PUFAs in our body may be used as a source of energy, although their preferred destination is as membrane phospholipids, as they are essential for multiple properties and functions of the biological membranes, such as fluidity, permeability, enzyme activity and membrane receptors, and signal transduction. In addition, the AA and the eicosapentaenoic acid (EPA) are mobilised from the membrane phospholipids in response to different stimuli for their transformation into eicosanoids, a group of bioactive compounds that take part in the regulation of multiple physiological functions, including blood pressure, blood coagulation, kidney function and immunological and inflammatory reactions, and they also play a very important role in the nervous system. Specifically, AA is the precursor of the series 2 prostanoids and the series 4 leukotrienes (Palou et al., 2008). Some of these are proinflammatory, vasoconstrictor, and/or proaggregatory compounds, such as prostaglandin E2, thromboxane A2, and leukotriene B4. However, others have antiinflammatory and/or antiaggregatory properties such as prostacyclin, lipoxin A4, and the epoxyeicosatrienoic acids (Harris et al., 2009). Epoxyeicosatrienoic acids also have significant vasodilatory properties. It should be noted that the eicosanoids produced from AA and EPA are different, as those produced from EPA are less inflammatory or are even antiinflammatory.

AA and EPA, as substrates for the synthesis of eicosanoids, compete for the same enzymes, and consequently, the relative concentrations of the products formed depend on the concentrations of said fatty acids in the cellular membrane. The cellular membranes typically contain a high proportion of AA and a low proportion of EPA and DHA (docosahexaenoic acid), and therefore the AA is the dominant substrate for the synthesis of eicosanoids. However, a high intake of EPA and/or DHA may inhibit the production of eicosanoids derived from AA (Culp et al., 1979) (Corey et al., 1983).

In our body, the AA comes partly from the intake and partly from the endogenous synthesis of linoleic acid. In fact, AA is not considered essential for a healthy adult whose usual diet provides linoleic acid in quantities of more than 2.5 % of dietary energy (FAO, 2010). The degree of conversion of linoleic acid from diet into AA is

estimated to be around 0.2 %. However, this process is closely regulated and mainly depends on the intake of AA (Liou and Innis, 2009). This means, when the linoleic acid is the only n-6 PUFA provided by the diet, all of the AA present in the tissues comes from linoleic aid (Whelan et al., 1993). When the intake of AA in the diet increases, the conversion of linoleic acid into AA decreases (Li et al., 1994). Variations in the intake of linoleic acid (above the essential minimum quantities), do not materially alter the content of AA in the tissues, nor have they been described as increasing the formation of proinflammatory mediators (Adam et al., 2003).

Normal consumption

AA is usually a minority component of the total of the PUFAs consumed in the diet. In Western diets, the intake of AA is estimated to be between 0.17 and 0.22 g/day, approximately 100 times lower than the average intake of its metabolic precursor, linoleic acid (Li et al., 1994).

In Spain, the mean intake of AA is 0.34 and 0.22 g/day in men and women respectively, where the supply of linoleic acid is 17.3 and 14.6 g/day in men and women respectively (Palou et al., 2008).

Recommended intake

AA is synthesised by our body from linoleic acid and, therefore, it is not strictly speaking an essential fatty acid in spite of its important role in the maintenance of metabolic integrity. In this respect, the opinion issued by the NDA Panel (Panel on Dietetic Products, Nutrition and Allergies) of the EFSA is to establish a dietary reference value for this fatty acid (EFSA, 2010). In addition, as at present there is no consistent evidence to show that the intake of any of the n-6 PUFAs (polyunsaturated fatty acids) has adverse health effects (for example, in the promotion of diet-related diseases), the NDA Panel of the EFSA has recommended not establishing maximum intake levels (Upper Level, UL) for all or any of the n-6 PUFAs (EFSA, 2010).

4.1.4 Safety

No adverse effects derived from the intake of AA have been described.

Nevertheless, there is some scientific debate as to the convenience of reducing the intake of AA, and, in general, of n-6 PUFAs. Arguments in favour of this reduction are based on the fact that these fatty acids are precursors to a wide range of proinflammatory molecules. Therefore, a reduction in the intake of AA or its precursor, linoleic acid, might reduce the inflammatory potential and, therefore, reduce the risk of cardiovascular disease (Harris et al., 2009). However, the fact that AA metabolises into both proinflammatory and antiinflammatory molecules must be considered (Harris et al., 2009), and the majority of studies which have addressed these aspects have not been able to demonstrate that a higher intake of AA leads to an increase in inflammation in normal metabolic conditions.

Specifically, studies conducted on humans reveal that high plasma levels of n-6 PUFAs, principally AA, are associated with reduced plasma levels of proinflammatory markers, in particular interleukin-6 and the interleukin-1 receptor antagonist, and higher levels of antiinflammatory markers, particularly the transforming growth factor-beta (Ferrucci et al., 2006). In a meta-analysis of 25 case-control studies (including 1,998 cases and 6,913 controls) in which the content of n-6 PUFAs in blood and/or tissues and the incidence of cardiovascular events were assessed, the content of linoleic acid was inversely associated with the risk of coronary cardiopathy, while no links were observed between the AA content and the risk of coronary disease (Harris et al., 2007). In

relation to these results, in observational studies a higher intake of n-6 PUFAs has been associated with unaltered or lower levels of inflammatory markers (Pischon et al., 2003). In addition, in a controlled study over 7 weeks, in which healthy volunteers received 1.5 g/day of AA (dose equivalent to approximately seven times the usual intake of said fatty acid), no effects were observed on platelet aggregation, haemorrhage times, vasoactive metabolite equilibrium, serum lipid concentrations or the immune response (Ferretti et al., 1997) (Kelley et al., 1997) (Nelson et al., 1997a) (Nelson et al., 1997b). Similarly, in a study conducted in Japan, supplementation with AA (840 mg/day for 4 weeks) did not have any apparent effects on any of the metabolic parameters studied or the platelet function (Kusumoto et al., 2007).

In addition, studies on vascular endothelial cells have shown that n-6 PUFAs could have antiinflammatory properties, as the production of adhesion molecules, chemokines and interleukins (all of which are principal mediators of the atherosclerotic process) is suppressed (De Caterina et al., 2000).

However, it should be noted that supplementation with AA, although it does not appear to have proinflammatory effects on healthy individuals, may counteract the antiinflammatory effects of supplementation with n-3 PUFAs (Li et al., 1994).

4.1.5 Conclusion

With the information available, the Scientific Committee concludes that there is no scientific evidence to relate the intake of arachidonic acid to the development of adverse effects, and therefore considers that the proposal presented by the AESAN not to recommend a maximum amount is acceptable from the point of view of its safety for use as a food supplement.

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4.2 Linoleic acid and alpha-linolenic acid

4.2.1 Proposal

The AESAN proposes a maximum daily amount for alpha-linolenic acid of 1 g, with a linoleic acid/alpha-linolenic acid ratio of a maximum of 5. This proposal is based on the assessment made in France by the AFSSA (*Agence Française de Sécurité Sanitaire des Aliments*) for these substances (AFSSA, 2008).

In addition, linoleic acid is included among the substances controlled by Royal Decree 867/2008, of 23 May, approving the specific technical and sanitary standards regarding infant formulae and follow-on formulae (BOE, 2008). This establishes the quantities and proportion between said fatty acids (not less than 5 or more than 15). In Belgium and in Italy (legislative proposal) it is authorised in food supplements without the establishment of a maximum daily amount (Belgium, 1992) (Italy, 2012).

4.2.2 Characteristics and sources

Alpha-linolenic acid is an omega-3 polyunsaturated essential fatty acid, consisting of 18 carbon atoms and 3 degrees of unsaturation (18:3, n-3).

Linoleic acid is an omega-6 polyunsaturated essential fatty acid, consisting of 18 carbon atoms and 2 degrees of unsaturation (18:2, n-6).

In human beings, the lack of enzymes Δ12- and Δ15-desaturases, which permit the introduction of double bonds in the n-6 and n-3 positions respectively, determines that these fatty acids cannot be synthesised and that they are therefore essential, meaning that they must be provided in the diet.

Alpha-linolenic acid is mainly found in vegetable seed oils, in particular in the seeds of flax, rapeseed, hemp, in nuts (especially walnuts), pulses (including beans, lentils and chick peas) and green leaf vegetables (spinach, lettuce, etc.).

Linoleic acid is mainly found in sunflower oil (it represents approximately 70 % of the fatty acids of this oil), and in safflower, maize, soybean, evening primrose and pumpkin oils. Vegetables, nuts, cereals, seeds, eggs and fish are also a source of linoleic acid.

Claims approved by the EFSA

Different health claims or statements have been approved by the EFSA for said fatty acids (some of them refer to infants and children, although with respect to food supplements, we mainly refer to adults).

With respect to alpha-linolenic acid, the EFSA has approved the claim regarding its "contribution to brain development" in children aged 3 to 6 years old (EFSA, 2009c). This claim is mainly based on a study on humans and 20 studies in animals, considered relevant. The study on humans (Holman et al., 1982) was a case report of alpha-linolenic acid deficiency in a 6-year old girl, maintained on total parenteral nutrition containing safflower oil (devoid of alpha-linolenic acid, but with a high content of linoleic acid) for 5 months. The girl developed neurological and visual problems: episodes of numbness/lethargy, paresthesia, weakness, inability to walk, pain in the legs and blurred vision. After switching to another parenteral nutrition preparation containing soybean oil (with an adequate content of alpha-linolenic and linoleic acids), the neurological symptoms disappeared in a few months.

Based on this study on humans and on various studies in animals, the Panel considered that there was a cause-effect relation between alpha-linolenic acid and the "contribution to brain development". However, the deficiency in the diet of alpha-linolenic acid that leads to impaired brain development has not been demonstrated in humans under normal dietary conditions. The evidence provided does not establish a benefit for brain development in children with an intake of alpha-linolenic acid above 0.2 % of the total energy. This quantity is taken as part of a balanced diet.

EFSA has also accepted the claim for alpha-linolenic acid, "alpha-linolenic acid, an essential fatty acid, contributes to brain and nerve tissue development" (EFSA, 2011). The target population is infants and children from birth to the age of 3 years, ages at which food supplements cannot be digested. In order to use the claim, follow-on formulae must comply with the composition criteria established in Directive 2006/141/EC; other foodstuffs intended for infants and children must contain a minimum of 15 % of the adequate intake of the 0.5 % of the total dietary energy in order to be able to use this claim (EU, 2006a).

Another claim accepted by the EFSA for alpha-linolenic acid is that "alpha-linolenic acid contributes to the maintenance of normal blood cholesterol levels" (EFSA, 2009a). The target population is the general population. The NDA Panel of the EFSA considered that in order to bear the claim, a food should contain at least *15 % of the proposed labelling reference intake value of 2 g of alpha-linolenic acid per day*. Such amount can be easily consumed as part of a balanced diet. However, Regulation (EC) No 1924/2006 finally established that this claim can only be used with respect to foods that are, at minimum, a source of alpha-linolenic acid in accordance with the approved claim of *source of omega-3 acids* that can only be used if the product contains at least 0.3 g of alpha-linolenic acid per 100 g and 100 kcal, or at least 40 mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100 g and per 100 kcal (EU, 2006b).

As regards linoleic acid, there is also a claim approved by the EFSA (2009b) on the "maintenance of normal blood cholesterol levels". The NDA Panel of the EFSA considered that in order to bear the claim, a food should contain at least *15 % of the proposed labelling reference intake value of 10 g of linoleic acid per day*. The Panel also noted that such amount can be easily consumed as part of a balanced diet. The target population is the general population.

4.2.3 Nutrition and metabolism

Alpha-linolenic acid is essential in human nutrition as a precursor of long-chain n-3 fatty acids. Eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and, to a lesser degree, docosahexaenoic acid (DHA) are synthesised from alpha-linolenic acid through the sequential action of various desaturases and elongases in animal tissues, but not in plants. Estimations for the conversion of alpha-linolenic acid into EPA range from 8 to 12 %, whereas conversion to DHA may be less than 1 % (Goyens et al., 2006). Given this low conversion rate and the fact that the alpha-linolenic acids EPA and DHA may have a different biological function, many authorities make separate recommendations for alpha-linolenic acid, and for EPA and DHA. EPA is a precursor of the series-3 prostaglandins and the series-5 leukotrienes (Kinsella et al., 1990). DHA is a component of the structural membrane lipids, especially of the phospholipids in the nervous system and retina.

Linoleic acid is also essential, and is the precursor of arachidonic acid. Arachidonic acid is the precursor of the series-2 prostaglandins and the series-4 leukotrienes (Kinsella et al., 1990). Linoleic acid, when incorporated in skin ceramides, is essential for maintaining the water permeability barrier, preventing excessive trans-epidermal water loss, with the consequent loss in energy due to water evaporation. Linoleic acid also plays a role in the reproductive, platelet, inflammatory, and immune functions and in the regulation of lipemia.

Linoleic and alpha-linolenic acids are converted into their respective long-chain polyunsaturated fatty acids by the same enzymes. In fact, the conversion of alpha-linolenic acid to EPA and DHA decreases when the quantity of linoleic acid in the diet increases (and vice-versa). However, the conversion of alpha-linolenic acid into its long-chain derivatives does not depend on the n3/n6 proportion but on the absolute intake quantities of said fatty acids. A decrease in the intake of linoleic acid determines an increase in the proportion of alpha-linolenic acid that is converted into EPA. On the other hand, an increase in the intake of alpha-linolenic acid determines an increase in the absolute quantity of DHA that is synthesised (Goyens et al., 2006). Therefore, some of the diet recommendations also include directives for the n3/n6 ratio in the diet. This ratio, in particular between the essential n-6 and n-3, is an important determining factor for health; traditional diets are based on a ratio close to 1, whereas in our present diet this ratio is more than 9 (Hu et al., 2001). This characteristic has been associated with the promotion of the pathogenesis of numerous diseases, including cardiovascular diseases, cancer and inflammatory and immune diseases; whereas an increase in n-3 concentrations, together with a lower n-6/n-3 ratio, may have inhibiting consequences on said diseases (Simopoulos, 2006). Nevertheless, there is no established consensus on what the optimum ratio between both types of polyunsaturated fatty acids should be.

Normal consumption

The average intake of polyunsaturated fatty acids in European countries varies considerably in both children and adults. No overt signs of deficiency have been observed in the cases with the lowest intake of n-3 or n-6 polyunsaturated fatty acids in children, adolescents or adults in Europe. Although very infrequently, and in specific conditions, a deficiency has been observed in n-3 fatty acids in patients who receive enteral or parenteral nutrition without alpha-linolenic acid (Holman et al., 1982) (Bjerve, 1989). According to the NDA Panel of the EFSA, nor are there signs of deficiencies in pregnant or lactating women (EFSA, 2010).

In Spain, there is no individual data for the intake of alpha-linolenic and linoleic acids. There are figures for the total intake of polyunsaturated fatty acids, which represent, in adults aged between 19 and 64 years, 6.1 % of the total intake in men and 5.8 % in women (EFSA, 2010).

The Mediterranean diet is relatively rich in alpha-linolenic acid, as it is present in a wide variety of food of plant origin. Although the fat content of these foods is low, the relative abundance of alpha-linolenic acid, together with the relatively high intake of vegetables, contributes to making the intake of this acid appreciable in countries with this diet (Palou et al., 2008). For example, a portion of spinach contains approximately 475 mg of alpha-linolenic acid, equivalent to almost 30 % of the recommended daily intake (1-2 g). Similarly, a portion of 20 g of nuts (for example, walnuts) provides a significant intake of alpha-linolenic acid, and it has been observed that this intake maintained for a period of three weeks increases the circulating levels of alpha-linolenic acid and EPA in healthy individuals (Marangoni et al., 2007). It has been estimated that the alpha-linolenic acid in the Mediterranean diet may represent approximately 0.6 to 1 % of the total daily energy (or around 2 g per day) whereas the mean intake of linoleic acid does not exceed 7 g per day (De Lorgeril and Salen, 2006).

Recommended intake

The opinion of the NDA Panel of the EFSA (EFSA, 2010) is that there is insufficient scientific data to establish average requirement (AR) values, lower threshold intake, or the population reference intake. In the case of linoleic acid, a negative (beneficial) association has been demonstrated, depending on the dose, between the intake of linoleic acid and the blood LDL (Low Density Lipoprotein)-cholesterol concentrations, whereas this relation is positive for the HDL (High Density Lipoprotein)-cholesterol concentrations. In addition, linoleic acid

reduces fasting blood triglyceride concentrations, in comparison with the carbohydrates. There is also evidence that the replacement of saturated fatty acids with n-6 polyunsaturated fatty acids (without changing the total fat consumption) reduces the number of cardiovascular events in the population. However, the relation between the intake of linoleic acid and the lipid profile in the blood is continuous and a threshold value cannot be identified for the intake of linoleic acid below which the risk of cardiovascular events increases. Therefore, the Panel recommended establishing an adequate intake value (AI) of linoleic acid of 4 % of dietary energy, based on the lowest intake levels of different population groups from various European countries in which overt symptoms of linolenic acid deficiency have not been observed. With the same criteria, with respect to alpha-linolenic acid, the Panel recommended establishing an adequate intake of said fatty acid of 0.5 % of dietary energy.

The Joint FAO/WHO Expert Committee on fats and fatty acids in human nutrition (FAO, 2010) also concluded that a daily intake of alpha-linolenic acid of between 0.5 % and 0.6 % of the total dietary energy is sufficient for preventing deficiency symptoms. The total of n-3 fatty acids in the intake may range between 0.5 and 2 % of the total dietary energy value, whereas the minimum dietary requirement of alpha-linolenic acid of 0.5 % for adults prevents deficiencies. The higher value of 2 %, including alpha-linolenic acid, and its longer-chain derivatives (mainly EPA and DHA), may form part of a healthy diet.

With respect to linoleic acid, the FAO/WHO group of experts (FAO, 2010) recognises that there is limited data in humans to specify set requirements for said fatty acid in order to prevent deficiencies, and concludes that an acceptable range of variation should be established. Studies in animals and in humans show that the symptoms of deficiency (for example, in rats, a reduced growth rate, scaling of the skin or necrosis of the tail) are prevented when linolenic acid represents between 1 and 2 % of the total dietary energy value. Therefore, they recommend that an adequate intake of linoleic acid must represent between 2 and 3 % of the total dietary energy value. Considering that the acceptable upper value for the total of polyunsaturated fatty acids and for the total of n-3 is, respectively, 11 % and 2 % of the total dietary energy value, an acceptable range is obtained for the intake of n-6 (particularly linolenic acid) between 2.5 and 9 % of the total dietary energy value. The lower value or adequate intake value (AI) of 2.5 to 3.5 % would permit the prevention of deficiency symptoms, while the highest value, as part of a healthy diet, would contribute to long-term health with the reduction of LDL-cholesterol and total cholesterol levels, and consequently the risk of coronary cardiopathy. For infants aged between 6-24 months, an adequate intake range is recommended of 3 to 4.5 % of the total dietary energy value, with an acceptable upper nutrient distribution value of 10 %. They also conclude that there is insufficient evidence to establish a relation between the intake of n-6 polyunsaturated fatty acids and cancer.

Lastly, based on existing scientific evidence, the FAO/WHO Expert Committee (FAO, 2010) also concluded that there are no grounds for a specific recommendation for the ratio of n-6/n-3 fatty acids, nor for linoleic/alpha-linolenic acids if the intake of these fatty acids is within the recommendation established in the report.

For the labelling of these foods, the EFSA has recommended a reference value for alpha-linolenic acid of 2 g (EFSA, 2009d). This value corresponds to the upper end of the range of average intake observed in adults in some European countries (0.7-2.3 g/day or ~ 0.4-0.8 % of the total dietary energy value). This value is consistent with the recommended intakes of alpha-linolenic acid, based on considerations relating to cardiovascular and neurological health, of approximately 1 % of the total dietary energy value, corresponding to 2-3 g of alpha-linolenic acid/day for an energy intake of 1,800-2,700 kcal/day. The Panel considers therefore that the proposed labelling reference intake value for alpha-linolenic acid of 2 g is consistent with recommended intakes for individuals in the general population.

With respect to linoleic acid, the Panel has proposed a food labelling reference value of 10 g (EFSA, 2009d), which is consistent with recommended intakes for adult individuals in the general population in European countries based on considerations of cardiovascular health (4 % of total dietary energy, equivalent to 8-12 g/day

in adults). The mean intake of n-6 polyunsaturated fatty acids (including principally linoleic acid) in Europe is between 7 and 19 g/day.

4.2.4 Safety

There is no available data for the adverse effects from a high intake of alpha-linolenic acid and linoleic acid; the majority of the data refers to an excessive intake of EPA and DHA, which are biologically more powerful than their precursor, and that an excess in their intake in the form of supplements may increase the lipid peroxidation and reduce the production of cytokines (Meydani, 2000) (Vedin et al., 2008). However, the FAO/WHO Expert Committee (FAO, 2010) also recognised that high intakes of these n-3 fatty acids have not had short- or medium-term adverse effects in tests on humans, and that some individuals in populations with a high intake of shellfish, consume even higher quantities with no apparent evidence of damage. Experimental studies show that the risk of lipid peroxidation may increase when the intake of polyunsaturated fatty acids represents more than 11 % of the total dietary energy value, in particular when the intake of tocopherol is low (Elmadfa and Kornsteiner, 2009).

In addition, some studies have associated the intake of large amounts of fat, in particular linoleic acid, with a greater long-term increase in the risk of cancer. In this respect, the results of a meta-analysis do not suggest a direct relation between the intake of high quantities of linoleic acid and cancer, although, based on the data from some studies, the existence of a certain increase in the risk cannot be dismissed (Zock and Katan, 1998).

The opinion adopted by the NDA Panel of the EFSA is that there is no consistent evidence that the intake of alpha-linolenic acid or of any of the n-6 polyunsaturated fatty acids have adverse effects on health (for example, in the promotion of diet-related disease). The group of experts proposes not establishing an UL for either alpha-linolenic acid or for the total or any of the n-6 polyunsaturated fatty acids (EFSA, 2010).

4.2.5 Conclusion

After reviewing the available information, the Scientific Committee concluded that there is insufficient scientific evidence to relate a high consumption of alpha-linolenic acid with the development of adverse effects and, consequently, they share the opinion issued by the NDA

Panel of the EFSA not to establish total maximum intake values for either alpha-linolenic acid or for the total or any of the n-6 polyunsaturated fatty acids (EFSA, 2010).

Nevertheless, considering the scope of the request (maximum levels in relation to the formulation of food supplements), the Scientific Committee of the AESAN considers there are reasonable grounds to establish prudent limits which are within the reference intake value of 2 g/day for alpha-linolenic acid, established by the EFSA.

Similarly and although there is no established consensus on the optimum ratio of n-6 and n-3 polyunsaturated fatty acids, we consider the maintenance of the ratio of reference intake values established by the EFSA for both fatty acids (ratio of 5-10 g/day for linoleic acid and 2 g/day for alpha-linolenic acid) to be adequate.

Consequently, this Committee considers that, based on the information currently available and considering the general considerations reflected in this report, the proposal of a maximum quantity of 1 g/day of alpha-linolenic acid with a linoleic acid/alpha-linolenic acid ratio of a maximum of 5 presented by the AESAN, is acceptable from the point of view of its safety for use as ingredients in food supplements.

In addition, fatty acids from the n-3 family are susceptible to oxidation and therefore the stability of the final product must be ensured.

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4.3 Oleic acid

4.3.1 Proposal

The AESAN has proposed the inclusion of oleic acid in Royal Decree 1487/2009 without specifying a maximum daily amount. The industry has been consulted about the proposal as, at European level, there is a large number of supplements containing it.

4.3.2 Characteristics and sources

Oleic acid is a monounsaturated fatty acid (MUFA) with 18 carbon atoms and a double bond in position 9-*cis*.

It is the most common of the MUFAs and is found in variable quantities in fats and oils in the diet. Olive oil contains approximately 71 % oleic acid, rapeseed oil contains about 60 %, and palm oil 40 %. High oleic varieties of sunflower oil and rapeseed oil contain around 75-85 % oleic acid. It is also found in significant quantity in ox tallow (around 43 %) and lard (about 44 %).

Claims approved by the EFSA

Maintenance of normal blood LDL-cholesterol concentrations. The health claim referring to the replacement of saturated fatty acids present in food with mixtures of MUFAs (for example, oleic acid) and/or mixtures of PUFAs (for example, linoleic acid and alpha-linolenic acid) and the maintenance of normal blood LDL-cholesterol levels has been accepted (EFSA, 2011). The target population is the general population.

4.3.3 Nutrition and metabolism

Function

As with other fatty acids, monounsaturated fatty acids (MUFAs) are absorbed almost completely in the intestine. These fatty acids may be oxidised (to obtain energy), converted into other fatty acids, or incorporated into tissue lipids. Human being is able to synthesise MUFAs and, consequently, they are not required as such in the diet. MUFAs can be obtained from diet, but can also be synthesised in our body. The synthesis process occurs from stearic acid due to the action of the enzyme $\Delta 9$ desaturase. This enzyme, which is very active in the tissue of mammals, catalyses the introduction of double bonds in position 9-10 of the fatty acid chain, mainly forming oleic acid. The products of the *de novo* synthesis are esterified with glycerol to form triglycerides. In the liver, these triglycerides are incorporated in the VLDLs (Very Low Density Lipoproteins) and transported through the circulation to the target tissues.

Normal consumption

The mean intake of MUFAs in the adult European population ranges between 11 and 18 % of the total dietary energy. The highest average was observed in Greece, where it represented 22 to 23 % of dietary energy (EFSA, 2010).

In babies, the mean intake of MUFAs ranges between 8 and 11 % of total dietary energy, and in children and teenagers between 10 and 13 %. In Portugal and Spain, the highest intake averages have been observed (EFSA, 2010).

Recommended intake

The NDA Panel of the EFSA has proposed not establishing a reference dietary value for MUFAs (EFSA, 2010). This is because MUFAs are not essential from a nutritional point of view, as they can be synthesised in our body, and they do not have a specific known role in the prevention or promotion of diet-related diseases. Consequently, they are not considered as essential constituents of diet.

The Institute of Medicine in the United States (IoM, 2005) did not establish adequate intake values (AI), average requirement values (AR) or recommended dietary allowances (RDA) either, as there is no evidence to indicate that MUFAs are essential in diet and they do not have any known role in the prevention of chronic diseases.

The WHO/FAO (2003) has calculated the MUFA intake targets as "total fat less all saturated fatty acids + polyunsaturated fatty acids + *trans* fatty acids" and has not established a definitive value.

However, in Europe, several organisations have made intake recommendations for MUFAs which range, broadly speaking, between values of up to 10 and 20 % of total dietary energy. Specifically, recommendations from the German-speaking countries, including Germany, Austria and Switzerland (D-A-CH, 2008), indicate that the MUFAs can be consumed up to 10 % of the total dietary energy. In those cases in which a greater amount of fat is consumed, a MUFA intake of more than 10 % of dietary energy is acceptable. Nutritional recommendations in Nordic countries, including the five Scandinavian countries (Denmark, Finland, Iceland, Norway and Sweden) (NNR, 2004), establish the intake of *cis* unsaturated fatty acids in adults and children over two years of age at around 20 % of total dietary energy, and from those, MUFAs must meet approximately 10 to 15 %, and PUFAs around 5 to 10 %. The COMA Committee of the United Kingdom (DoH, 1991) recommends that MUFAs (mainly oleic acid) provide an average 12 % of total dietary energy (including alcohol). The French Agency (AFSSA, 2001) recommends an intake of MUFAs of 20 % of total dietary energy in adults, including pregnant and lactating mothers. The Health Council of the Netherlands (GR, 2001) does not formulate adequate intake values (AI) for MUFAs individually, but does recommend ranges for MUFAs and PUFAs together. These have been calculated on the basis of the reference dietary values for fats, saturated fatty acids and *trans* fatty acids. For a total fat intake of 20 % of total dietary energy, the intake of MUFAs and PUFAs must not be less than 8 % of total energy and more than 19 %. With a fat intake of 35 % of dietary energy, the optimum consumption of MUFAs and PUFAs must represent between 22 and 33 % of dietary energy. As the intake of PUFAs must be between 3 and 12 % of dietary energy, the upper and lower limits for the intake of MUFAs would be between 10 and 30 % (GR, 2001).

No specific recommendations have been made for children.

4.3.4 Safety

No adverse effects derived from a greater intake of oleic acid have been described.

Nevertheless, it should be noted that some publications link oleic acid to the incidence of cancer or to processes related to the progression of said disease. Specifically, high levels of oleic acid in erythrocytes have been linked to a higher incidence of breast cancer in postmenopausal women (Pala et al., 2001). However, this study does not establish whether the oleic acid comes from diet, and it must be remembered that although tissue oleic acid comes partly from diet, the majority is usually from endogenous synthesis from stearic acid. In addition, it has been observed in cell lines derived from a mammary tumour that oleic acid induces migration, proliferation and invasion, and prolongs the survival of these cells, however this effect has not been observed in non-tumour epithelial cells (Soto-Guzman et al., 2010).

Nevertheless, the results associating oleic acid with cancer are controversial. In fact, potentially protective effects of oleic acid against cancer have been described, as it has been demonstrated that said fatty acid represses the transcriptional activity of the Her-2/neu oncogene, which is associated with breast, ovary and stomach carcinomas (Menendez et al., 2006).

Moreover, although the analytic studies have provided some inconsistent results, in general the majority of case-control and cohort studies have shown that oleic acid and olive oil are associated with a reduction in the risk of cancer (mainly breast cancer, colorectal cancer and prostate cancer), in comparison with diets rich in total fats and in linoleic acid or saturated fatty acids (Binukumar and Mathew, 2005) (López-Miranda et al., 2008). On the other hand, in a meta-analysis of observational studies up to July 2003 (Boyd et al., 2003), the intake of MUFAs was not significantly linked to the risk of breast cancer.

4.3.5 Conclusions

With the available information, the Scientific Committee concluded that there is no scientific evidence to link the intake of oleic acid with the development of adverse effects, and consequently considers that oleic acid may safely be consumed as a food supplement provided that this consumption is within reasonable intake limits.

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4.4 Omega-3 fatty acids (DHA + EPA)

4.4.1 Proposal

The AESAN has proposed a maximum daily amount for the sum of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) of 3 g. This proposal is based on the scientific opinion of the EFSA which claims that supplemental intakes of DHA and EPA combined in doses of up to 5 g do not raise safety concerns (EFSA 2012).

In Belgium and in Italy (legislative proposal) they are authorised in food supplements without the establishment of a maximum daily amount (Belgium, 1992) (Italy, 2012).

They are also authorised in Denmark. In the case of DHA, the total maximum amount used must not exceed 1,050 mg per recommended daily dose and in the case of EPA, the total maximum amount used must not exceed 1,500 mg per recommended daily dose (Denmark, 2011).

4.4.2 Characteristics and sources

Eicosapentaenoic acid (EPA; 20:5Δ5c,8c,11c,14c,17c) and docosahexaenoic acid (DHA; 22:6Δ4c,7c,10c,13c,16c,19c) are long-chain omega 3 polyunsaturated fatty acids (n-3 LCPUFAs).

The principal source of EPA and DHA is fish and crustaceans, mainly Antarctic krill (*Euphausia superba*). Other natural sources are human milk, cultivated marine algae and marine mammals. These fatty acids may also be provided by foods and supplements enriched with n-3 LCPUFAs.

n-3 LCPUFAs are present in foods and food supplements mainly as triglycerides, and, in smaller quantities, as free fatty acids or as phospholipids. They are also found in the form of ethyl esters in concentrated supplements produced synthetically. Fatty acids linked to phospholipids have been observed to present a higher rate of absorption and incorporation in the cellular membranes than those consumed in the form of triglycerides. Those consumed in the form of ethyl esters present the lowest rate of absorption and incorporation in cellular membranes (EFSA, 2012). In any case, these fatty acids are almost completely absorbed irrespective of the source, and therefore this factor has not been considered by the NDA Panel of the EFSA in the safety assessment of EPA and DHA in the long-term (EFSA, 2012).

The EPA and DPA acids also come in the body from the endogenous synthesis of alpha-linolenic acid (ALA) through the sequential action of various desaturases and elongases in animal tissue, but not in plant tissue. Estimations indicate a low conversion of ALA in EPA and DHA, which is lower if the daily intake of these n-3 LCPUFAs is high (EFSA, 2010a). In fact, the endogenous production of EPA and DHA from ALA may be insignificant in comparison to the doses used in the studies considered for the safety assessment of said fatty acids (EFSA, 2012).

n-3 LCPUFAs may experience a process of peroxidation during the processing and storage of foods and food supplements rich in these fatty acids in the absence of adequate quantities of antioxidants (for example, vitamin E) (EFSA, 2012).

Claims approved by the EFSA

The EFSA has approved the following health claims or statements as regards EPA and DHA (EFSA, 2009, 2010c):

- *Maintenance of the normal cardiac function*. The target population is the general population. The Panel considers the intake of 250 mg of EPA and DHA necessary in order to obtain the claimed effect. Such amount can be consumed as part of a balanced diet.
- *Maintenance of normal blood pressure*. The target population is the general population. The Panel considers the intake of 3 g/day of EPA and DHA necessary in order to obtain the claimed effect.
- *Maintenance of normal blood concentrations of triglycerides*. The target population is adult men and women. The Panel considers the intake of 2 g/day of EPA and DHA necessary in order to obtain the claimed effect. Such amount can be consumed as part of a balanced diet.

In addition, with respect to DHA, the EFSA has also approved the following health claims (EFSA, 2010b):

- *Maintenance of the normal brain function*. The target population is the general population. The Panel considers the intake of 250 mg of DHA necessary, in one or two doses, in order to obtain the claimed effect. Such amount can be consumed as part of a balanced diet.
- *Maintenance of normal vision*. The target population is the general population. The Panel considers the intake of 250 mg of DHA necessary, in one or two doses, in order to obtain the claimed effect. Such amount can be consumed as part of a balanced diet.

4.4.3 Nutrition and metabolism

Function

The n-3 LCPUFAs in our body may be used as a source of energy (i.e. oxidised to carbon dioxide and water), incorporated in tissue lipids (these are important structural components of the cellular membranes), or used in the synthesis of eicosanoids. Small quantities are lost during the scaling of skin and other epithelial cells (IoM, 2005).

Specifically, DHA is an important component of the structural membrane lipids, especially of the phospholipids in the nervous tissue and retina. EPA can be transformed into eicosanoids, a group of biologically active substances that include prostaglandins,

prostacyclins and leukotrienes, that take part in the control of blood pressure, kidney function, blood coagulation, inflammatory and immune reactions and other functions in the tissues (Kinsella et al., 1990) (Miles and Calder, 2012). Other metabolytes of EPA and DHA (resolvins, protectins) are believed to take part in the inflammatory response (Kohli and Levy, 2009).

High intakes of EPA and DHA result in a drop in the tissue levels of arachidonic acid (AA) and an increase in the concentrations of EPA and DHA. Supplementation with DHA is also accompanied by an increase of EPA, which may be explained by the retroconversion of DHA to EPA or by the inhibition of the EPA metabolism. These effects of supplementation with DHA induce changes in the metabolism of AA and in the equilibrium of the eicosanoids synthesised from n-6 and n-3 fatty acids, and they may therefore affect the functions regulated by the above eicosanoids (IoM, 2005).

Although the endogenous interconversion of EPA and DHA may occur *in vivo*, particularly when the fatty acid is administered alone and in high doses, this process is considered insignificant when both fatty acids are administered in combination, in the doses used in the different studies considered for the safety assessment (EFSA, 2012). In this respect, the NDA Panel of the EFSA highlights that the effects of these n-3 LCPUFAs may depend on the method of administration (for example, whether they are administered alone or in combination) (EFSA, 2012).

Normal consumption

The mean intake of n-3 LCPUFAs in European countries varies significantly according to gender, age-group, eating habits (for example a high or scarce intake of fish) and supplement intake habits (EFSA, 2012). In addition, it should be noted that there is considerable diversity in the methodology used to assess the individual intakes, making direct comparisons difficult.

As regards EPA, studies conducted in different European countries reveal that its intake from food only in adults varies between relatively low values of 50 mg/day (Spain, both sexes, occasional fish consumers, 35-65 years old) or 150 mg/day (France, men, ≥ 45 years old) up to higher values of 308 mg/day (France, women, 35 years old) and 428 mg/day (Belgium, women, 18-39 years old). The data from the surveys which also consider food supplements show a slightly higher intake of EPA (up to 330 mg/day, Norway, 16-79 years old). In consumers of large quantities of fish or shellfish, the average daily intake varies between 320 mg/day (Spain, 35-65 years old) and 991 mg/day (France, 18 years old), although there are no data for those individuals which also consider the intake through supplements (EFSA, 2012).

In the case of DHA, the average daily intake from food of said fatty acid ranges between 131 mg/day (Belgium, women, 18-39 years old) and 273 mg/day (France, men, ≥ 45 years old), up to higher values of 574 mg/day (France, women, 35 years old) and 668 mg/day (France, men, 45 years old). The data from the surveys considering food and food supplements together reveal higher values for the average daily intake of DHA in the adult population in general (up to 490 mg/day, Norway, 16-79 years old). The average daily intake in consumers of large quantities of fish or shellfish ranges between 600 mg/day (Finnish women, 18 years old) and 1,709 mg/day (France, 18 years old). Nor are there any studies that have assessed the intake of DHA from both food and food supplements for consumers of large quantities of fish or shellfish (EFSA, 2012).

In short, the average daily intake of n-3 LCPUFAs from food only in adults is generally < 1,200 mg/day, and < 1,300 mg/day if food supplements are also considered. For consumers of large quantities of fish or shellfish, the mean intake of n-3 LCPUFAs from food is generally < 2.7 g/day. There are no studies on the intake of EPA and DHA from food and food supplements for consumers of large quantities of fish or shellfish (EFSA, 2012). In Spain, a study of the Basque population reveals that the intake of EPA and DHA only from food is also usually < 1,200 mg/day, varying between 250 mg/day (in occasional consumers of fish) to 1,170 mg/day in consumers of large quantities of fish (Amiano et al., 2001).

Recommended intake

The recommendations of national and international bodies for the intake of n-3 LCPUFAs (mainly as EPA and DHA) range between 200 and 600 mg/day for adults, and between 40 and 250 mg/day for children over 6 months and for children and teenagers (EFSA, 2012). Specifically, the EFSA recommends an intake of 250 mg/day in adults for the sum of EPA and DHA (EFSA, 2010a). These recommendations have been generally based on the inverse relation observed between the intake of these n-3 LCPUFAs (especially from fish oils and fish) and a reduced risk of coronary artery disease.

Some bodies, including the EFSA (2010a), have also made specific DHA recommendations for infants and children aged between 6 and 24 months ranging from 70 to 100 mg/day, based on its build-up in the central nervous system and its effects on the visual function, and an additional quantity of DHA (100-200 mg/day) for pregnant and lactating women.

4.4.4 Safety

The adverse effects which have been described in humans in relation to the intake of EPA and DHA are bleeding episodes, alteration in the immune function, alterations in the lipid and glucose metabolisms, and an increase of lipid peroxidation.

Bleeding episodes

The possible effects of n-3 LCPUFAs on bleeding episodes are compatible with the antithrombotic function of the n-3 PUFAs (Vanschoonbeek et al., 2003). A study of the inuit in Greenland with high consumption of fatty fish (mean intake of n-3 LCPUFAs of around 6.5 g/day) (Bang et al., 1971) reveals an increase in the tendency to bleed through the nose and the urinary tract, and an increase in the mortality rate due to hemorrhagic stroke. An increase in the bleeding time and a reduced *in vitro* platelet aggregation are also shown. The NDA Panel of the EFSA highlights that other factors other than the intake of n-3 LCPUFAs in the diet which could have been responsible or partly responsible for these effects were not controlled in these studies (EFSA, 2012).

Various controlled human intervention studies have addressed the hypothesis that supplementation with n-3 LCPUFAs might modify the platelet function, and increase the haemorrhage time and the risk of spontaneous bleeding and hemorrhagic stroke. For example, a human intervention study (Yokoyama et al., 2007) which investigated the effects of 1.8 g/day of EPA in the form of ethyl esters consumed for five years in combination with statins (n = 9,326), compared with statins alone (n = 9,319), in hypercholesterolemic individuals and consumers of large quantities of fish in primary and secondary prevention of coronary heart disease, also assessed its possible effects on the risk of cerebrovascular accident (Tanaka et al., 2008). No significant differences were observed in the total incidence of cerebrovascular accidents, or in the incidence of brain or subarachnoid haemorrhage. However, bleeding (nose bleeds, subcutaneous haemorrhage, etc.), evaluated using self-reporting as a possible secondary effect, was observed to be more frequent in the EPA group than in the controls. With respect to these results, the NDA Panel of the EFSA indicates that as the information is self-reported and the data are not objective, it may be subject to bias. Therefore, the Panel concludes that the intake of EPA alone in doses of up to 1.8 g/day for two years does not increase the risk of haemorrhages (EFSA, 2012).

It is noticeable that among the different prospective cohort studies published to date on the relation between the dietary intake of n-3 LCPUFAs and the risk of cerebrovascular accident, none has revealed an increase in the risk of haemorrhagic stroke (He et al., 2002) (Skerrett and Hennekens, 2003) (IoM, 2005). The mean daily intake of n-3 LCPUFAs in the highest intake quintiles in these studies was < 1 g/day. The NDA Panel of the EFSA concludes that there is no evidence of an increased risk of haemorrhagic stroke with the dose of n-3 LCPUFAs that is normally consumed in western diets, or in the supplementary doses of EPA of up to 1.8 g/day (EFSA, 2012).

A review that includes 48 random controlled tests on subjects at high risk of cardiovascular event (Hooper et al., 2004) has assessed the effects of n-3 LCPUFAs in doses of 0.4 to 7 g/day (in comparison with a placebo or a control oil) for at least 6 (and up to 47) months, on events related to cardiovascular disease. The majority of the subjects were taking antithrombotics for the prevention or treatment of cardiovascular disease. Seven of the studies, which used EPA and DHA in doses of 1.8 to 6.9 g/day for 6 to 24 months, reported bleeding episodes. No differences were observed in the risk of bleeding between the intervention group and the control group (or placebo). The study that used the highest dose of n-3 LCPUFAs (6.9 g/day) lasted six months and the longest lasting study (24 months) used 5.1 g/day of EPA and DHA.

After revising these and other published studies, the NDA Panel of the EFSA considers that the supplementary intake of EPA and DHA combined of up to approximately 5 g/day for a maximum of 2 years and up to approximately 7 g/day for a maximum of 6 months, does not increase the risk of spontaneous hemorrhagic episodes or haemorrhagic complications, even in subjects with a high risk of bleeding (for example, those taking acetylsalicylic acid or anti-coagulants). The Panel indicates that the data available are insufficient to conclude whether the same doses administered mainly as EPA or DHA, separately, would have different effects. The NDA Panel of the EFSA also considers that the intake of EPA alone in doses of up to 1.8 g/day for two years does not increase the risk of haemorrhagic complications (EFSA, 2012).

Platelet function

As regards the possible effects of n-3 LCPUFAs on platelet function, Violi et al. (2010) have reviewed different published studies that analyse these effects. Of the 21 studies identified, only seven were controlled. Of these, three were conducted on healthy subjects and four on subjects with hypercholesterolemia, high blood pressure, type 2 diabetes, or a combination of these pathologies. Doses of n-3 LCPUFAs varied between 1 and 4 g/day and the studies lasted from 30 days to 1 year. No effects from the intake of n-3 LCPUFAs on platelet aggregation were observed in the shortest and longest studies respectively. However, in five studies an inhibition of the platelet function or a prolongation of platelet survival were observed (length of study: 4-16 weeks). The effect on the platelet function does not seem to be dose-dependent. The dose-response relation between the intake of EPA and DHA and platelet aggregation, the Von Willebrand factor (vWF), coagulations factors VII and VIII, antithrombin III (AT III) activity, the activity of protein C, fibrinogen, fibronectin and fibrinolysis (plasminogen activator inhibitor 1, PAI-1; tissue plasminogen activator, t-PA) was specifically assessed in one of the studies on ten healthy men supplemented with doses of 1.3, 4 or 9 g of n-3 LCPUFAs per day for periods of 6 weeks each (Schmidt et al., 1990). No significant effects on platelet aggregation were observed from supplementation with these fatty acids. The plasmatic fibrinogen decreased depending on the dose after the intake of 1.3 and 9 g of n-3 LCPUFAs. The vWF decreased after the highest dose, whereas the plasmatic concentrations of the factor VII, factor VIII, and AT III activity, protein C and fibronectin activity were not altered by n-3 LCPUFAs. At rest, the PAI and t-PA increased after the intake of 9 g of n-3 LCPUFAs, and the PAI increased as a result of the intake of n-3 LCPUFAs according to the dose. In short, no clear relation was observed between the intake of these fatty acids and the majority of variables relating to blood coagulation. The NDA Panel of the EFSA concludes that the changes in the platelet function observed with supplementary doses of EPA and DHA (either separately or in combination) of up to approximately 4 g/day are not considered adverse, as they are not associated with an increased risk of complications (EFSA, 2012).

Glucose homeostasis

As regards the effects on glucose homeostasis, human intervention studies, the majority uncontrolled, have described adverse effects of the supplementation of n-3 LCPUFAs (≥ 10 g/day) on glucose homeostasis, such as the increase of insulin requirements, the increase of glycosylated haemoglobin (HbA1c), and an increase in fasting and postprandial glycemia in patients with type 1 and 2 diabetes (De Caterina et al., 2007). In 2005, the IoM (Institute of Medicine) declared that individuals with impaired glucose tolerance or conditions of diabetes requiring higher doses of glucose-lowering treatments should exercise caution in taking supplements of EPA and DHA (IoM, 2005).

After revising these and other studies, the NDA Panel of the EFSA indicates that the human intervention studies carried out with their respective fat intake controls do not, on the whole, reveal a differential effect of the

supplementary vegetable oils and fish oil, in doses of up to 5 g/day of EPA and DHA taken for 12 weeks, on the control of blood glucose levels in diabetic subjects, or on insulin sensitivity in healthy or diabetic subjects. The Panel considers that the intake of EPA and DHA combined supplements of up to 5 g/day taken for a maximum of 12 weeks does not significantly affect glucose homeostasis in healthy subjects or in diabetics. The Panel also highlights that the available data are insufficient to conclude whether the same doses administered mainly in the form of EPA or DHA would have different effects (EFSA, 2012).

Cholesterolemia

With respect to the effects of n-3 LCPUFAs on cholesterolemia, a meta-analysis (Balk et al., 2006) which collected 21 random controlled tests (on approximately 8,000 individuals) conducted on healthy individuals, individuals with diabetes, hypertension or dyslipidemia, or in individuals with cardiovascular disease, revealed that supplementation with EPA + DHA led to a significant increase in LDL-cholesterol concentrations of +0.155 mmol/l compared to the controls. In the majority of the studies, the changes in LDL-cholesterol concentrations associated with the intake of EPA and DHA were < 5 %. In this meta-analysis, studies that used > 6 g/day of EPA + DHA or that lasted less than 4 weeks were excluded. The doses of EPA and DHA varied between 0.9 and 5.9 g/day (from fish oil and food) and the intervention lasted between 4 weeks and 2 years. Changes in LDL-cholesterol concentrations were accompanied by a significant drop in the concentration of triglycerides (−0.31 mmol/l) and a significant increase in HDL-cholesterol concentrations (+0.041 mmol/l), without significant changes in the total cholesterol. The doses of EPA and DHA were also observed to have an accumulative effect on the magnitude of the changes in the triglycerides, whereas the changes in the levels of LDL-cholesterol and HDL-cholesterol appeared to be dose-independent.

In another meta-analysis conducted by Hartweg et al. (2008, 2009) the effect of supplementation with EPA and DHA was assessed on cholesterolemia in individuals with type 2 diabetes. These individuals presented a higher risk of cardiovascular disease, and therefore an increase in LDL-cholesterol concentrations could be critical. The majority of the studies provided EPA and DHA in combination in doses of up to 6 g/day. Compared with the placebo (mainly vegetable oils), supplementation with n-3 LCPUFAs significantly increased by 3 % LDL-cholesterol concentrations (mean increase of +0.08 mmol/l). This effect was only observed for doses of n-3 LCPUFAs higher than 2 g/day and was accompanied by a significant reduction in the concentration of circulating triglycerides of around 7 % (mean reduction of 0.17 mmol/l), whereas no changes were observed in other parameters related to circulating lipids, including total cholesterol.

In a systematic review and meta-analysis of random controlled tests published recently (Jacobson et al., 2011) (Wei and Jacobson, 2011), six of the studies compared EPA (ethyl esters, > 90 % EPA) with DHA (ethyl esters, > 90 % DHA) and used olive oil, sunflower oil, maize oil or ALA as fat control. These studies lasted 4-7 weeks and administered EPA and DHA in doses of between 2.3 and 4 g/day each one. Treatment with DHA resulted in a significant increase in LDL-cholesterol concentrations of 2.6 % compared with the control group, and 3.3 % compared with the EPA group, which did not result in significant changes in LDL-cholesterol concentrations (-0.7 %). It should be noted that the increase in LDL-cholesterol concentrations induced by supplementation with DHA compared to the controls, also resulted in a significant decrease in the concentration of triglycerides (-22.4 %) and a significant increase in the concentration of HDL-cholesterol (+7.3 %). In addition, supplementation with EPA, which did not have significant effects on LDL-cholesterol concentrations (-0.7%), nor did it have significant effects on HDL-cholesterol concentrations (+1.4 %), such that the n-3 LCPUFAs seem to have different effects on the plasmatic lipids.

Considering these and other studies, the opinion of the NDA Panel of the EFSA is that the intake of EPA and DHA combined supplements of 2-6 g/day, and the intake of supplements containing mainly DHA of 2-4 g/day result in an increase in LDL-cholesterol concentrations of around 3 %, and this increase is accompanied by a drop in the concentration of triglycerides, without changes in the concentration of total cholesterol (or non-HDL). It also notes that the intake of supplements containing mainly EPA in doses of up to 4 g/day does not have significant effects on LDL-cholesterol concentrations. According to the consideration of the NDA Panel of the EFSA, the small increase in LDL-cholesterol concentrations associated with the supplementation of EPA and DHA combined or with DHA alone in the above doses does not seem to be adverse in relation to cardiovascular risk (EFSA, 2012).

Lipid peroxidation and oxidative stress

With respect to the effects of EPA and DHA on lipid peroxidation and oxidative stress, some studies on laboratory animals have revealed confusing results due to the presence of primary and secondary oxidation products in the supplements administered without the presence of antioxidants. Nevertheless, this effect was not observed when administered in the presence of vitamin E (IoM, 2005) (VKM, 2011). In addition, the majority of human intervention studies used fish oil stabilised with antioxidants; however some studies have not detailed whether or not antioxidants were used. The presence of primary and secondary oxidation products in the supplements administered was only described in a few studies.

In 10 identified human intervention studies with oils rich in n-3 LCPUFAs and stabilised with antioxidants, using vegetable oils as a control (olive, maize, sunflower, safflower or soybean), plasmatic or urinary levels of F2-isoprostanes were measured, as a marker of lipid peroxidation (Mas et al., 2010) (VKM, 2011). These studies used DHA alone (800 mg-4 g/day), EPA alone (1.6-4 g/day) or EPA and DHA in combination as fish oil (2-4 g/day) for 3-6 weeks. In half of the studies, there was a fall in the plasmatic or urinary concentrations of F2-isoprostanes in the group supplemented with n-3 LCPUFAs compared to the control group (Higdon et al., 2000) (Mori et al., 2000) (Mori et al., 2003) (Barden et al., 2004) (Mas et al., 2010), whereas in the rest of the studies no significant differences were described between the groups (Stier et al., 2001) (Engler et al., 2004) (Tholstrup et al., 2004) (Wu et al., 2006) (Himmelfarb et al., 2007).

In addition, oxidation of the LDLs is also associated with increased cardiovascular risk. Some studies have analysed the effects of supplementation with EPA and DHA on oxidised LDL particles (measured directly in blood) or on LDL susceptibility to oxidation (measured *ex vivo)*. According to the opinion of the NDA Panel of the EFSA, the method employed to measure LDL susceptibility to oxidation is not an appropriate method for determining the peroxidation of the LDLs (EFSA, 2012).

Studies which have analysed the effects of supplementation with EPA and DHA in the form of fish oil or in the form of ethyl esters on LDL susceptibility to oxidation have produced varying results. Some short-term studies (4-6 weeks) have described an increase in LDL susceptibility to oxidation, whereas long-term interventions (6-16 weeks) in doses of up to 5 g/day did not reveal any effects on LDL susceptibility to oxidation in comparison with the control group (the majority were supplemented with vegetable oils) (VKM, 2011). In addition, two studies in which the diet was supplemented with salmon providing 1.5 g/day and 2.9 g/day of EPA and DHA, respectively, (Seierstad et al., 2005) or with herrings that provided 1.2 g/day of EPA and DHA (Lindqvist et al., 2009), did not show effects of the intervention on plasma oxidised LDL concentrations in comparison with the controls.

With respect to other possible markers of lipid oxidation, supplementation with EPA and DHA in doses of up to 4.5 g/day were not seen to affect various measures traditionally used to determine lipid peroxidation, such as

thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), conjugated dienes or lipid hydroperoxides (VKM, 2011). However the NDA Panel of the EFSA notes that these parameters are not reliable markers of lipid peroxidation *in vivo* (EFSA, 2011).

In conclusion, in accordance with the opinion of the NDA Panel of the EFSA, supplementary intakes of EPA and DHA consumed either alone or in combination, in doses of up to 5 g/day for a period of up to 16 weeks, do not result in changes in lipid peroxidation, which may cause concern in relation to the risk of CVD, provided the oxidative stability of these n-3 LCPUFAs is guaranteed (EFSA, 2012).

Immune function

With respect to the effects on the immune function, there are indications from *ex vivo* and *in vitro* studies on peripheral white blood cells taken from individuals consuming n-3 LCPUFAs, that these fatty acids may decrease cytokine expression and the proliferation of these blood cells in doses as low as 0.9 mg/day of EPA and 0.6 mg/day of DHA taken in the form of fish oil for 6-8 weeks (IoM, 2005). However, the relevance of these changes *in vivo* is not known. In addition, there are no available human intervention studies that have investigated the effects of n-3 LCPUFAs on the risk of infections *in vivo*. However, a recent meta-analysis of 18 random controlled tests reveals a significant fall in soluble intercellular adhesion molecule-1 (sICAM-1) concentrations as an effect of supplementation with n-3 LCPUFAs (doses of 0.272 to 6.6 g/day) but does not show effects on markers of vascular inflammation (Yang et al., 2012).

In view of these studies, the NDA Panel of the EFSA considers that a supplementary intake of EPA and DHA of up to 5 g/day is unlikely to induce changes to the immune function. The Panel also highlights that the available data are insufficient to conclude whether the same doses administered mainly in the form of EPA or DHA would have different effects (EFSA, 2012).

4.4.5 Conclusion

With the available information, the Scientific Committee concludes that there is insufficient scientific evidence to link a high intake of EPA and DHA to the development of adverse effects and, consequently, it shares the opinion issued by the NDA Scientific Panel of the EFSA (EFSA, 2012) of not establishing total maximum intake levels for EPA and DHA, neither in combined form or in individual form, and that the intake of supplements of EPA and DHA combined in doses of up to 5 g/day, or the intake of supplements of EPA alone up to 1.8 g/day, do not pose safety concerns for the adult population. It also considers that supplementary doses of DHA alone of up to approximately 1 g/day do not pose safety concerns for the population in general. It should be noted that the intake data for EPA and DHA from foods and food supplements in European populations, including Spain, are generally below these amounts.

The Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AESAN proposal of a maximum amount of 3 g/day of the combination of EPA and DHA is acceptable from the safety point of view for use as ingredients of food supplements.

In addition, considering that the family of n-3 fatty acids is susceptible to oxidation, the stability of the end product should be ensured.

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5. Amino acids and other nitrogen compounds

5.1 Branched-chain amino acids (L-isoleucine + L-leucine + L-valine)

5.1.1 Proposal

The AESAN proposes a maximum daily amount of the sum of L-isoleucine, L-leucine and L-valine of 5 g. This proposal is based on the authorisation in Italy of a maximum reference intake value of 5 g/day of the sum of L-leucine, L-isoleucine and L-valine, with the warning that it should not be consumed by pregnant women, children, or for prolonged periods of time without medical supervision (Italy, 2012).

5.1.2 Characteristics and sources

The branched-chain amino acids are L-leucine, L-isoleucine and L-valine. They are branched aliphatic amino acids, hydrophobic in nature, and very stable and are scarcely affected by the processes carried out in the food industry. They are essential amino acids, but as they make up almost 50 % of the total number of essential amino acids present in protein foods, they are never the limiting ones of the nutritional value of the proteins. Given that they make up 35 % of the essential amino acids of muscle proteins, any protein food of animal origin is a good source (Harper et al., 1984).

The EFSA has published a scientific opinion relating to the verification of health claims relative to the branched-chain amino acids: i) growth and maintenance of muscular mass; ii) faster recovery from muscle fatigue after exercise; iii) attenuation of the decrease in muscular strength that takes place after exercise at altitude; iv) improvement of the immune system; v) improvement of the cognitive function after exercise; and vi) reduction in tiredness after exercise. Based on the information presented, the NDA Panel of the EFSA has not been able to establish a cause and effect relation in any case (EFSA, 2010).

5.1.3 Nutrition and metabolism

The branched-chain amino acids are powerful regulators of protein turnover, allowing energy to be obtained in high intensity exercises in the absence of glycogen, reducing central fatigue syndrome in vigorous exercise, they are able to regulate the metabolism of glucose, are important for the maintenance of the immune function and have been used for improving cirrhosis of the liver, hepatic encephalopathy, the survival of liver transplants and in the improvement of sepsis and multiple trauma (Baker, 2005) (Cynober and Harris, 2006) (Zhang et al., 2011).

The metabolism of these amino acids mainly takes place in the muscle and involves transamination reactions and subsequent oxidative decarboxylation. Lastly, each one enters at different levels of the Krebs cycle.

In spite of the modern techniques used in the studies of balance and oxidation of these amino acids, the intake recommendations for healthy adults are still being debated. Another significant problem which hinders the establishment of recommendations is determining how the requirements of a certain branched-chain amino acid affect the requirements of the other two. Nevertheless, the joint report of the FAO/WHO/UNU establishes a daily requirement of L-leucine of 39 mg/kg b.w./day (WHO, 2007). Moreover, on the basis that the three branched-chain amino acids share common oxidative catabolic routes, that their daily requirements essentially reflect their basal catabolic levels and bases on the contents of the three amino acids in the composition of body proteins, it is possible to calculate the daily requirements of L-isoleucine and L-valine as a function of that established for L-leucine. These values are 20 and 26 mg/kg b.w./day, respectively (WHO, 2007). However, in spite of the above and the numerous studies existing on supplementation with branched-chain amino acids, in diverse physiological

and pathological situations, there is still no agreement on what are adequate doses of branched-chain amino acids taken as food supplements (Cynober and Harris, 2006).

5.1.4 Safety

Acute and sub-acute toxicity tests on rats using branched-chain amino acids (in the proportions 2.1:1:1.2 for L-leucine:L-isoleucine:L-valine) indicate that the mean acute lethal dose is more than 10 g of branched-chain amino acids/kg b.w. (Okazaki et al., 1989). Chronic toxicity studies, in rats, indicate that doses of 2.5 g/kg b.w./day for 3 months or 1.25 g/kg b.w./day for 1 year did not have any toxic effect (Okazaki et al., 1989).

In humans, the majority of the sports supplement studies have used doses of more than 5 g of total branched-chain amino acids, without detecting toxic effects (Shimomura et al., 2004). There is even a study in which up to a total of 14.4 g of branched-chain amino acids was administered for 30 days, without observing harmful effects on health (DeLorenzo et al., 2003). In addition, enteral administration, in doses of 240 mg/kg b.w./day for 6 months, in patients with hepatic encephalopathy, sepsis or multiple trauma, did not cause any toxicity or adverse effects (Baker, 2005). Nevertheless, there is one study conducted on five individuals, in which a single acute dose of 5 g of branched-chain amino acids (in the proportions 1:2.3:1.2 for L-isoleucine:L-leucine: L-valine) produced a reduction in the content of L-methionine and aromatic amino acids. In addition, a transitory increase in the plasma levels of insulin was observed, without this affecting the glycemia or the plasma levels of free fatty acids (Zhang et al., 2011).

Finally, the Scientific Committee of the Norwegian Food Safety Authority (VKM, 2011) established that branched-chain amino acids present a moderate risk. This is understood as when changes take place in the biomarkers, but without adverse effects to health. Moreover, there is no maximum tolerable intake level, as no toxicity studies in humans are available to provide these bases. In addition, the Italian Ministry of Labour, Health and Social Policies (Italy, 2012), based on the studies carried out by Zhang and his collaborators, establishes a maximum reference intake quantity of branched-chain amino acids of 5 g/day, with the warning that they should not be taken by pregnant women, children or for prolonged periods of time without medical supervision (Zhang et al., 2011).

5.1.5 Conclusion

The Scientific Committee considers that, although no toxicity studies in humans are available, the toxicity studies in other models of animals used for experimental purposes, and those carried out in humans with the administration of a single dose, indicate that intake levels of up to three times more than the intake recommendations are well-tolerated by healthy adult subjects. Therefore, the Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, a maximum daily amount of 5 g of the sum of L-isoleucine, L-leucine and L-valine is acceptable from a safety viewpoint for use as a food supplement.

In addition, it understands that just as in Italy, these amino acids must not be consumed by pregnant women, children or for prolonged periods of time without medical supervision.

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5.2 L-glutamic acid

5.2.1 Proposal

The AESAN has recommended the inclusion of L-glutamic acid in Royal Decree 1487/2009 without specifying a maximum daily amount. The proposal is based on the fact that Regulation (EC) No 953/2009 (EU, 2009) includes L-glutamic acid among the substances that may be added for specific nutritional purposes in foods for particular nutritional uses.

5.2.2 Characteristics and sources

L-glutamic acid is a dicarboxylic acid, also known as 2-aminopentanedioic acid or 2-aminoglutaric acid. The chemical formula is $C_5H_9NO_4$. It is a branched-chain amino acid, with an acid chain. The salts of L-glutamic acid, and its carboxylate anion, are known as glutamate and in this form a physiological pH is found.

In foods, it is found in free form or bonded to other amino acids forming proteins. The main food sources of L-glutamic acid include meat, poultry, fish, eggs, dairy products, some vegetables (tomatoes, mushrooms, peas and broccoli) and wheat. The free form is responsible for the increase in flavour of foods (Kulkarni et al., 2005). L-glutamic acid may also be consumed as a food additive in the form of monosodium glutamate, calcium glutamate, potassium glutamate and ammonium glutamate.

5.2.3 Nutrition and metabolism

L-glutamic acid is a non-essential amino acid, which can be obtained from glucose through the Krebs cycle. It can also be synthesised through the transamination of alpha-ketoglutarate which accepts amino groups from other amino acids (Pamela, 1994) (Hardman et al., 2001).

L-glutamic acid is absorbed in the small intestine through a specific active transport system for amino acids (Schultz et al., 1970). Subsequently it may undergo: i) a transamination to form alpha-ketoglutarate and aspartate; ii) an oxidative deamination in the liver with the release of free ammonium and its subsequent incorporation into the urea cycle; and iii) a decarboxylation to form GABA (gamma-aminobutyric acid) (Garattini, 2000).

L-glutamic acid plays a central role in the metabolism of nitrogen and is an important neurotransmitter. In addition, it is involved in the synthesis of proteins (proteinogenic amino acid) and in the physiology of taste.

The normal mean intake of L-glutamic acid in a male adult weighing 70 kg is calculated at 28 g per day. This value is found in the diet and the break-up of intestinal proteins. The daily turnover of L-glutamic acid is 48 g. Although the turnover is high, the average reserves of L-glutamic acid are around 20 mg. This is because it is used rapidly by different tissues, especially the liver and muscle (Munro, 1979).

The International Programme on Chemical Safety (IPCS) and in accordance with that published by the Joint FAO/WHO Expert Committee on Food Additives (WHO, 1974) establishes that the daily intake of L-glutamic acid should be between 0 and 120 mg/kg b.w. This recommendation is independent of the source of L-glutamic acid, including that present in food, monosodium glutamate, potassium glutamate, calcium glutamate and ammonium glutamate.

5.2.4 Safety

Mutagenicity tests did not reveal, in cell cultures, any effect when monosodium glutamate was added to the culture medium at 0.1 % for 72 hours (FDA, 1969). Oral administration in rats, from two weeks before the start of the gestation and through the lactating period, of monosodium glutamate (up to 7 g/kg b.w./day) did not have any effect on reproduction and embryonic development (McColl et al., 1965).

Acute toxicity studies conducted on animals used for experimental purposes (mouse, rat, guinea pig and rabbit) indicate that the LD_{50} is 6.9-15.0 g/kg b.w. when it is administered intraperitoneally (Yanigisawa et al., 1961), and 12.9-30.0 g/kg b.w. when it is administered orally (Izeki, 1964).

The administration of L-glutamic acid of up to 4 % in the diet, for one year, in mice and rats did not produce any changes (Little, 1953a, 1953b).

As regards the possible neurotoxic effects of L-glutamic acid, when supplied orally in high doses (0.7 g/kg b.w. in lactating mice and 1.2 g/kg b.w. in adult mice) and acutely, neural degeneration occurs (Takasaki et al., 1979). This study also indicates that neonatal mice are more susceptible to the intake of high doses of glutamate. Nevertheless, no neurotoxic effects were observed when the L-glutamic acid is supplied for long periods of time as part of the research animal feed in doses that are even higher than those that produce a neurotoxic effect when administered in isolation (Heywood et al., 1977). The neurotoxic effects do not exist in humans, as the administration of palatable maximum doses of monosodium glutamate cause an increase in the plasma levels of L-glutamic acid of up to 40 times less than that of rodents (Salmona et al., 1980). In addition, in rodents, increases of up to 10 times in the plasma levels of L-glutamic acid do not induce increases in the total content of L-glutamic acid in the brain, although this does not exclude that it may build up in specific parts of the same (Garattini, 2000). In this respect, oral administration to rats of 4 g of glutamate/kg b.w. leads to a rise in the hypothalamic level of L-glutamic acid of up to nine times. Nevertheless, this increase is one or two orders of magnitude lower than that necessary to induce neurotoxicity (Sohn et al., 1998).

At present, it is known that L-glutamic acid is safe for the general population (Kulkarni et al., 2005). Nevertheless, doses of between 1.5-12 g of L-glutamic acid, in a single meal, produce, 15-25 minutes after its intake, a series of adverse effects including a burning sensation, oppression and/or numbness of the chest, which may spread to the neck, face, shoulders and arms. Dizziness, headaches, nausea and vomiting may also appear (Kulkarni et al., 2005).

5.2.5 Conclusion

The Scientific Committee considers that L-glutamic acid is present in protein foods in the diet, has a low level of toxicity and adverse effects are only observed at doses of above 1.5 g. Therefore, the Committee concludes that a maximum amount of 1 g/day is acceptable from a safety point of view for use in food supplements.

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5.3 Beta-alanine

5.3.1 Proposal

The AESAN has proposed the inclusion of beta-alanine in Royal Decree 1487/2009 without specifying a maximum daily amount.

In Italy, beta-alanine is authorised in food supplements without the establishment of a maximum daily amount (Italy, 2012).

5.3.2 Characteristics and sources

Beta-alanine (β-ALA) is a non-proteinogenic amino acid whose amino group is in the beta position with respect to the carboxyl group. It is a component of the natural peptides, carnosine and anserine and of pantothenic acid. It is synthesised *in vivo* by the degradation of dihydrouracil and carnosine. β-ALA is a limiting precursor of the carnosine synthesis rate (EFSA, 2010).

β-ALA performs various functions in the nervous system, it may act as a neurotransmitter or neuromodulator and has anchor points on the hippocampus in receptors of NMDA, GABA-A, GABA-C and glycine to help with the learning of new information (Caruso et al., 2012).

The principal dietary source of β-ALA is the dipeptide, carnosine, which is found in the proteins of animal-origin food, meat and derivatives and fish and derivatives.

β-ALA is one of the new food supplements that causes enthusiasm among athletes, due to the many health and exercise benefits with which it has been accredited. It can be administered in solution or in powder form in gelatine capsules and it can be determined in foods by established methods

5.3.3 Nutrition and metabolism

β-ALA is a naturally occurring amino acid, and together with L-histidine is one of the precursors of carnosine, which is synthesised from β-ALA and L-histidine, the carnosine synthase acting as an enzyme. β-ALA is the limiting amino acid in the carnosine synthesis velocity. In turn, carnosinase, the enzyme present in cells and serum, hydrolyses the carnosine to β-ALA and L-histidine (Culberstone et al., 2010) (Spradley et al., 2012).

In the body, β-ALA is synthesised by the chemical decomposition of the pyrimidines, decarboxylation of L-aspartate by the microorganisms of the intestinal flora and transamination of 3-oxopropanoate by L-aspartate (Caruso et al., 2012). The endogenous synthesis of β-ALA takes place in the liver from irreversible degradation of thymine, cytosine and uracil. Once synthesised, β-ALA is carried to the muscle cells where it crosses the sarcolemma by a process that depends on the Na^+ and Cl^-. The intracellular uptake of β-ALA uses the same cotransporter as glycine, taurine and GABA and therefore supplementation with β-ALA affects, due to competitive inhibition, the uptake of these substances. However the doses of β-ALA which reduce the intracellular content of taurine far exceed those administered to humans, and therefore concerns that the administration of β-ALA compromises intramuscular concentrations of taurine are not founded (Caruso et al., 2012).

The EFSA has published a scientific opinion regarding the verification of health claims relating to β-ALA: i) increased physical performance during intense exercise in a short period of time; ii) increased time until

exhaustion; and iii) increase in the muscular reserves of carnosine. However, based on the information presented, the NDA Panel of the EFSA has not been able to establish a cause and effect relation in any case (EFSA, 2010).

5.3.4 Safety

Doses of β-ALA above 10 mg/kg b.w. have been observed to provoke a short period of paresthesia, the severity of which increases with the dose. Nevertheless paresthesia does not occur when a high dose, approximately 40 mg/kg b.w., is taken with the diet. This would seem to suggest that the paresthesia is the consequence of a rapid increase in the plasma levels of β-ALA when it is taken alone, as this does not occur when the β-ALA forms part of a diet containing meat derived products and consequently dipeptide carnosine with L-histidine (Harris et al., 2006).

The precautions required when β-ALA is used as a food supplement are due to its potential to induce paresthesia, which is characterised by an increase in the sensitivity of the nociceptive neurons, the transmitters of neuropathic pain, causing redness and a stinging sensation on the skin (Harrris et al., 2006) (Crozier et al., 2007) (Sale et al., 2010). The severity of episodes of paresthesia is dose-dependent, but in general it is maintained during the 60 minutes after ingestion (Harris et al., 2006). The severity of the paresthesia in some cases leads to the intake of β-ALA being reduced or even stopped (Harris et al., 2006). In order to detect the paresthesia resulting from an acute intake of β-ALA, doses of between 10 and 40 mg/kg b.w. were administered to six men. The acute intake of the lowest dose resulted in a mild reddening 20 minutes after intake and a significant paresthesia occurred with a dose of 20 mg/kg b.w. No secondary effects were observed with doses of 40 mg/kg b.w. when the β-ALA was taken with extract of chicken meat, whereas if the dose was administered orally as a food supplement it led to paresthesia in some individuals. This indicates that the episodes of paresthesia depend on the dose and the method of administration. The same article (Harris et al., 2006) includes the evaluation of the chronic intake of β-ALA by healthy men. Different methods of intake were tested. In one, 10 mg/kg b.w. was taken three times a day for two weeks, and few secondary effects, including mild occasional reddening, were observed. In the other two strategies, the intakes were 3.2 and 6.4 g/day, administered in doses of 400 and 800 mg to healthy men for 4 weeks. These protocols caused a few cases of slight paresthesia and throat pain in one individual. It was concluded that symptoms similar to paresthesia began to appear in doses of more than 10 mg/kg b.w. The prevalence and severity of the paresthesia is related to the blood peak levels of β-ALA, which leads to the recommendation to use delayed/gradual release supplements of β-ALA.

The plasma kinetics and the incidence of symptoms similar to paresthesia were monitored in a random simple blinded test that included the study of the gradual release over time of a β-ALA supplement (Decombaz et al., 2011). Healthy adults (n = 11) received three treatments: 1.6 g of gradually-released β-ALA orally, the same dose administered normally (bolus), and a placebo. Urine and plasma samples were taken during the 6 hours following the administration of each treatment and a questionnaire was given to the participants. The results showed that the gradually-released β-ALA caused a lower number of plasma peaks and led to a more prolonged lag-time, together with reduced loss through urine indicating a greater retention of β-ALA. The normal form of administering β-ALA led to symptoms similar to paresthesia, whereas the gradually-released dose and the placebo behaved similarly. To conclude, higher absolute doses of gradually-released β-ALA can be taken without the risk of paresthesia (Decombaz et al., 2011).

5.3.5 Conclusion

The Scientific Committee considers that, based on the available information to date and taking into account the general considerations reflected in this report, the precautions to be adopted when beta-alanine is used as a food supplement are due to its potential to induce paresthesia.

Given that high doses of beta-alanine (above 10 mg/kg b.w./day) may produce paresthesia, individuals with a predisposition to paresthesia should refrain from taking this food supplement.

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5.4 L-alanine

5.4.1 Proposal

The AESAN has proposed the inclusion of L-alanine in Royal Decree 1487/2009 without specifying a maximum daily amount. The proposal is based on the fact that Regulation (EC) No 953/2009 includes L-alanine among the substances that may be added for specific nutritional purposes in foods for particular nutritional uses (EU, 2009).

5.4.2 Characteristics and sources

L-alanine (α-aminopropionic acid) is a non-essential amino acid which is hydrophobic, nonpolar and aliphatic. The fact that its alpha carbon is linked to the methyl group makes it one of the most simple alpha amino acids. Its chemical formula is $CH_3CH(NH_2)COOH$.

L-alanine is one of the most abundant amino acids in proteins and is present in a large quantity of foods. These include meat, fish, shellfish, eggs and dairy products.

The EFSA considers that it is safe to use L-alanine as a flavouring (EFSA, 2008).

5.4.3 Nutrition and metabolism

L-alanine plays a central role in the metabolism of nitrogen and together with glutamate and aspartate it is a substrate of the main aminotransferases that directly connect the amino acids to the intermediary keto acids from glycolysis and the Krebs cycle. Therefore, L-alanine intervenes in the alanine-glucose cycle, which enables the liver to obtain glucose from muscular degradation. The glucose obtained is used for muscular contraction.

L-alanine is released to the blood by the cells of the intestinal mucosa and the skeletal muscle as a result of its synthesis from other amino acids and subsequently it may be captured by the liver for transformation into glucose.

Based on food intake data for the period 1988-1994 (NHANES III), the estimated mean intake of L-alanine from foods and food supplements is approximately 3.6 g/day. The highest intake mentioned, 8.5 g/day, is for the 99th percentile of men aged between 51 and 70.

In the United States, the database of food supplements of the National Library of Medicine (NLH) lists around 40 supplements that contain L-alanine in their composition (NLH, 2012). The maximum recommended daily amounts of L-alanine range from 10 mg to 3.6 g per day.

The clearance and bioavailability studies of L-alanine, conducted on healthy subjects and subjects with hepatic cirrhosis have demonstrated that both parameters are high (Schricker et al., 1995).

5.4.4 Safety

Studies in animals and humans of food intake, growth and haematological changes resulting from the oral intake of L-alanine have not provided sufficient data to propose a LOAEL (Lowest Observed Adverse Effect Level) or a NOAEL (No Observed Adverse Effect Level) (LSRO, 1992).

The intake of L-alanine is not associated with severe adverse effects (Garlick, 2004). In rats with a protein-poor diet, a slight decrease in growth and in the intake of food was observed (Harper et al., 1970).

In animals, L-alanine exhibits neural inhibitory action and hypothermogenicity (Glyn and Lipton, 1981). Insufficient data are available to define the dose-response ratio of L-alanine in animals.

In human beings, no patent secondary effects were mentioned when up to 4 g of L-alanine was administered to 48 boys in an oral rehydration solution for 2 days (Da Costa Ribeiro and Lifshitz, 1991), or when administering up to 132 g of L-alanine, also in an oral rehydration solution, for four days to 20 boys under the age of one year (Patra et al., 1989). Nor were adverse effects mentioned in adults following an intravenous infusion of up to 35 g of L-alanine for 5 minutes (Genuth and Castro, 1974). In various studies, oral doses of up to 50 g of L-alanine did not have adverse effects other than slight nausea and stomach cramps (Genuth, 1973) (Koeslag et al., 1985a) (Koeslag et al., 1985b).

The scarce available data on the adverse effects of the intake of L-alanine from food supplements (Genuth, 1973) (Genuth and Castro, 1974) are considered insufficient to calculate the dose-response ratio and the proposed deduction of an UL for L-alanine.

No mutagenicity tests were indicated when various strains of *E. coli* were incubated with L-valine, L-histidine or L-tyrosine in concentrations of up to 5,000 mg/plate without metabolic activation (Fluck et al., 1976) (Martinez et al., 2000).

5.4.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, L-alanine presents a low toxicity and that maximum daily quantities such as those of supplements marketed in other countries of up to 3.6 g are acceptable from the safety point of view for use as food supplements.

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5.5 L-arginine

5.5.1 Proposal

The AESAN has recommended a maximum daily amount of 3 g of L-arginine. This proposal has been referred to the industry.

In Italy, L-arginine is authorised in food supplements (legislative proposal) without the establishment of a maximum daily amount (Italy, 2012).

5.5.2 Characteristics and sources

L-arginine is a basic amino acid, with a positive charge and neutral polarity containing two amino groups and one carboxyl group.

The most important dietary sources of L-arginine are dairy products, meat, fish, nuts and seeds.

The EFSA has published a scientific opinion regarding the verification of health claims relating to L-arginine: i) growth and maintenance of muscular mass; ii) improvement of immune system; iii) improved formation of red blood cells; iv) regulation of blood pressure; v) improvement of endothelium-dependent vasodilatation; vi) improved physical condition; vii) improved erectile function and spermatogenesis; viii) improved gastrointestinal function; and ix) maintenance of ammonia clearance. Based on the information presented, the NDA Panel of the EFSA has not been able to establish a cause and effect relation in any case (EFSA, 2011).

5.5.3 Nutrition and metabolism

L-arginine is a conditioned essential amino acid. Therefore, in adults with an adequate intake of proteins, endogenous synthesis is sufficient to cover physiological needs. However, in some states with a high catabolic demand (major burns, multiple trauma, severe/serious infection and advanced cancer) or during periods of rapid growth, body requirements may be higher than the body's capacity to synthesise.

Endogenous synthesis of L-arginine takes place in various steps from the amino acids, aspartate and citrulline. This synthesis takes place through the L-arginine synthase enzyme and mainly occurs in the kidneys or the liver (Wu and Morris, 1998).

L-arginine has an essential metabolic role in the formation of factors with a major physiological function (nitric oxide, urea and creatine). Moreover, it is important in the synthesis of proteins and the release of hormones (Guest et al., 2004). Consequently, the most important functions of L-arginine include: i) increasing the secretion of hormones such as the growth hormone, insulin, glucagon and prolactin (Isidori et al., 1981) (McConell, 2007); ii) improving the endothelial function (Diguardi, 2011); iii) improving the immune function (Munder, 2009); and iv) it has ergogenic functions (Elam, 1988). These functions imply that the possible clinical uses of supplementation with L-arginine are numerous.

In accordance with the joint technical report issued by the FAO/WHO/UNU, the requirements for L-arginine in adults are 117 mg/kg b.w./day (WHO, 2007). The normal intake of an adult with a correct mixed protein diet is 5.4 g/100 g of mixed proteins (NRC, 2002). Due to the lack of adequate scientific information, the tolerable upper intake level has not yet been established.

5.5.4 Safety

Studies conducted on humans who were administered with L-arginine orally are varied with respect to the size and variety of the sample, type of clinical test or nutritional intervention, dose, duration, analysed effects and the presence of other active components. In general, in all these studies no clear adverse effects have been found that may be attributed to L-arginine. In addition, all the literature analysed reveals a fairly high level of safety for supplementation with L-arginine (Shao and Hathcock, 2008).

In this respect, the highest dose used in a clinical test, in patients with cystic fibrosis was 42 g/day for 6 weeks (Grasemann et al., 2005). Furthermore, the longest-lasting test was conducted on patients with a kidney transplant and lasted 3 years, during which 9 g/day was administered (Alexander et al., 2005).

Nevertheless, there have been some studies that describe gastrointestinal disturbances following oral supplementation with L-arginine (Grimble, 2007). However, these studies were not correctly controlled or included patients suffering from cancer or who were receiving chemotherapy.

In the absence of studies that reveal toxicity it is very difficult to establish a NOAEL or a LOAEL. This occurs for amino acids that are usually consumed in the diet. Consequently for these cases, some authors (Shao and Hathcock, 2008) have proposed the use of the OSL (Observed Safe Level) as an alternative. In this respect, technically the highest dose of L-arginine used in a clinical test of 42 g/day (Grasemann et al., 2005) may be considered to be the OSL. However, given the scarcity of other clinical tests in which this dose or similar were used, there is insufficient evidence in order to consider this dose as safe and a relatively high uncertainty level is established.

Another study that has used high intakes of L-arginine is the study conducted by Chin-Dusting et al. (1996). This is a double-blind placebo-controlled clinical test on healthy subjects, in which 20 g/day of L-arginine was administered for 4 weeks. No adverse effects were observed, or any changes in the analytic parameters. Subsequently, a further six clinical tests were published, all without adverse effects or alterations of any type, using doses of between 21-42 g/day (Shao and Hathcock, 2008). This means that, for the supplementation with L-arginine of healthy subjects, an OSL of 20 g/day can be considered appropriate with a sufficient confidence level.

5.5.5 Conclusion

In the revision of 38 clinical tests on humans, no adverse effects or clinical alterations were found, not therefore permitting the establishment of a value for the NOAEL or LOAEL for the oral administration of L-arginine. Therefore, based on clinical tests and given their quality, an OSL of 20 g/day for supplementation with L-arginine has been established.

The Scientific Committee concludes that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum daily amount of 3 g of L-arginine is acceptable from the safety point of view for use as a food supplement.

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5.6 L-carnitine

5.6.1 Proposal

The AESAN proposes a maximum daily amount of L-carnitine of 2 g using L-carnitine and L-carnitine hydrochloride as sources and of 3 g if using L-carnitine tartrate as a source. This proposal is based on the opinion of the EFSA (2003) and the authorisation in Denmark of L-carnitine and L-carnitine-L-tartrate in food supplements (Denmark, 2011).

5.6.2 Characteristics and sources

L-carnitine (β-hydroxy-γ-trimethylamine-butyrate) is a derivative of the amino acids L-lysine and L-methionine. It is widely distributed in all mammal tissue and is abundant in muscular tissue.

Homeostasis of L-carnitine is maintained thanks to a modest synthesis which takes place in the kidneys and the liver, through the diet and by means of an efficient renal reabsorption (Rebouche and Seim, 1998). Milk and dairy products, meat and fish are rich in L-carnitine.

The administration of L-carnitine as a food supplement may take place in three different ways: L-carnitine, propionyl L-carnitine and acetyl L-carnitine. However acetyl L-carnitine is normally used in its hydrochloride form or forming a salt with tartaric acid (L-carnitine tartrate).

The EFSA has published a scientific opinion regarding the verification of health claims relating to L-carnitine: i) faster recovery of muscular fatigue after exercise; ii) repair of skeletal muscle after exercise; iii) improvement of aerobic capacity; iv) regulation of LDL-cholesterol levels; v) it assists spermatogenesis; and vi) improvement of circulating levels of free fatty acids during pregnancy. Based on the information presented, the NDA Panel of the EFSA has not been able to establish a cause and effect relation in any case (EFSA, 2011).

5.6.3 Nutrition and metabolism

L-carnitine is synthesised from the essential amino acids L-lysine and L-methionine. In addition, for its biosynthesis it requires ascorbic acid, iron, niacin and vitamin B_6. In the liver, kidneys, heart and skeletal muscle N-trimethyl-L-lysine is synthesised from L-lysine and S-adenosyl L-methionine. Subsequently, after some intermediate steps, γ-butyrobetaine is synthesised and, lastly, L-carnitine is synthesised only in the liver and the kidneys (Broquist, 1982).

The mean intake of L-carnitine of a population with a varied diet is 100-300 mg/day (Feller and Rudman, 1988). There are a series of factors that may affect the synthesis of L-carnitine, such as the L-carnitine content in the diet and certain pathological states (kidney failure, diabetes, alcoholism and cancer). Free L-carnitine is absorbed to between 50-90 % in the small intestine (Rebouche and Seim, 1998).

L-carnitine plays an important role in the energy metabolism, as it is responsible for facilitating the entrance of long-chain fatty acids to the mitochondrial matrix, where they are oxidised. It also helps the exit of short-chain fatty acids from the mitochondria to the cytosol, reduces the production of lactate and improves the stability of the cell membranes.

As L-carnitine is an amino acid derivative widely distributed in all mammal tissues, no dietary reference intakes (DRI), or average requirements (AR) have been established. L-carnitine deficiency is very rare (approximately 100 cases have been diagnosed in the past 40 years). Nevertheless, premature babies and neonates have a low

capacity for L-carnitine synthesis, and in addition if they are fed with milk- or soy-based formulae, exempt of L-carnitine, there is a significant drop in L-carnitine plasma levels (Gil and Sánchez de Medina, 2010).

5.6.4 Safety

The bioequivalence of L-carnitine tartrate and of L-carnitine hydrochloride with L-carnitine is complete (Schmidbaur et al., 1998).

Acute toxicity studies conducted on rats show that doses of L-carnitine tartrate of up to 5.0 g/kg b.w. did not have toxic effects (IBR, 1991a). The mutagenicity studies carried out did not exhibit any mutagenic effects up to doses of 5.0 g/plate (IBR, 1991b) (Hendler and Rorvik, 2001). With respect to L-carnitine hydrochloride, the data from L-carnitine tartrate can be extrapolated (Bioresco, 2003).

As regards research on tolerance in humans, intakes of up to 15 g L-carnitine/day are usually well-tolerated, although in some people they cause gastrointestinal upsets and diarrhoea (Lurz and Fischer, 1998). In the case of L-carnitine tartrate, there is a randomised double-blind crossover study with a 1 week wash-out period, in which the administration of 3 g/day of L-carnitine tartrate for 3 weeks did not affect the biochemical and haematological parameters or the liver and kidney functions (Rubin et al., 2001). Nevertheless, the same study indicates that doses of 4-6 g/day may cause gastrointestinal upsets and diarrhoea.

Lastly, it must be remembered that acetyl-L-carnitine may interfere with the thyroid metabolism (Hendler and Rorvik, 2001) (Zdanowicz, 2001), therefore the intake of supplements of any form of acetyl-L-carnitine is not recommended for individuals receiving treatment for thyroid disease or with any thyroid–associated pathology.

5.6.5 Conclusion

In general, human tolerance to L-carnitine is high; nevertheless doses of 4-6 g/day of L-carnitine tartrate may cause gastrointestinal disorders. Therefore, the Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AESAN proposal of a maximum daily amount of 2 g of L-carnitine, using L-carnitine or L-carnitine hydrochloride as sources and of 3 g if L-carnitine tartrate is used is acceptable from the safety point of view for use as a food supplement.

Nevertheless, dietary intakes should not be exceeded in the case of individuals with thyroid pathologies or receiving treatment for thyroid diseases.

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5.7 L-cysteine

5.7.1 Proposal

The AESAN has recommended the inclusion of a maximum daily amount of L-cysteine of 300 mg.

The proposal is based on the fact that Regulation (EC) No 953/2009 (EU, 2009) includes L-cysteine among the substances that may be added for specific nutritional purposes in foods for particular nutritional uses.

In Italy, a maximum daily amount of 1,190 mg of L-cysteine + L-methionine is authorised in food supplements (legislative proposal) (Italy, 2012).

5.7.2 Characteristics and sources

L-cysteine $C_3H_7NO_2S$ (CH_2SH-$CHNH_2$-COOH) is a conditionally essential sulphur-containing amino acid, obtained from L-methionine and from serine. In normal physiological conditions the body is able to obtain L-cysteine in sufficient quantity. However premature babies cannot synthesise it and must obtain it through diet.

To satisfy the L-cysteine and L-methionine requirements a sufficient intake is required. Pulses are deficient in sulphur-containing amino acids; these are mainly found in cereal proteins and animal proteins. Although the presence of sulphur-containing amino acids in proteins is less abundant than that of other amino acids, it is important from a metabolic point of view, to the extent that the relative requirement for maintenance is probably higher than for growth.

Recommended dietary intakes for adults (over 19 years old) (IoM, 2005) are: average requirements (AR): L-methionine + L-cysteine: 15 mg/kg b.w./day; dietary reference intakes (DRI): L-methionine + L-cysteine: 19 mg/kg b.w./day.

The requirements (WHO, 2007) for adults are: cystine: 4 mg/kg b.w./day; L-methionine + L-cysteine: 15 mg/kg b.w./day.

5.7.3 Nutrition and metabolism

L-cystine is converted into L-cysteine with the action of cystine reductase that requires NADH as cofactor. L-cysteine and cystine are interconvertible and are usually considered together. In the human body, L-cysteine is the central compound in the metabolism of sulphur. In proteins, the formation of disulfide bonds among the thiol groups of L-cysteine plays an important role in the ternary structure and in enzyme activity; nevertheless L-cysteine is always incorporated as such in the polypeptide chain.

L-cysteine is an amino acid of great metabolic interest. Apart from being a precursor of taurine, it forms part of molecules as important as the coenzyme A or glutathione. It has functions like an antioxidant and is used in xenobiotic conjugation and in the formation of leukotrienes. An important characteristic of L-cysteine is its facility of oxidation to cystine (Sánchez de Medina, 2010).

L-cysteine is degraded to pyruvate in two stages, elimination of sulphur and transamination. L-cysteine can be metabolised to taurine and CO_2 through the L-cysteine sulfinate path, the initial step of which is oxidation, catalysed by L-cysteine dioxygenase, to cysteine sulfinate. Cysteine sulfinate can be decarboxylated to produce taurine or metabolised via beta-sulfinyl-pyruvate (intermediary case) to pyruvate and sulfite and then to CO_2 and sulfate (Stipanuk, 1986).

In the United States, based on the data from NHANES III (1988 - 1994), an average dietary intake of L-cysteine from food and food supplements of 1.0 g/day has been estimated. The highest intake is for men aged between 51 and 70 years old and is 2.2 g/day in the 99th percentile.

5.7.4 Safety

Low-protein diets with L-cysteine contents of 0.5 to 10 % reduce the increase of weight and the intake of food and therefore increase animal mortality (Harper et al., 1970). They also modify the cholesterol concentrations in plasma that increase in rats which receive cholesterol-poor diets and fall in those with cholesterol-rich diets (Rukaj and Sérougne, 1983) (Sérougne and Rukaj, 1983) (Sérougne et al., 1987). Moreover, histopathological changes have been systematically observed in the liver and kidneys (Harper et al., 1970).

Acute adverse effects in animals

L-cysteine is mutagenic in bacteria (Glatt, 1989), but not in mammal cells (Glatt, 1990).

A single oral dose of 3 g L-cysteine/kg b.w. in *Swiss Webster* albino mice, aged 10 to 12 days, produced hypothalamic neuron necrosis and retinal lesions, observed 5 hours after the intake (Olney and Ho, 1970). Furthermore, the subcutaneous injection of 1.2 mg of L-cysteine/kg b.w. of *Wistar* rats aged 9 to 10 days caused permanent dystrophy in the interior layers of the retina (Karlsen and Pedersen, 1982).

The intraperitoneal injection of 1.0 mmol L-cysteine/kg b.w. of male *Wistar* rats led to a maximum blood concentration of some 2 mM after 30 minutes (Calabrese et al., 1997). After one hour, exposure resulted in high concentrations of malondialdehyde in the substantia nigra of the brain. The subcutaneous injection of 0.5 g of L-cysteine/kg b.w. to 4-day-old *Sprague-Dawley* rats did not have an ulterior effect on the neurotransmitters or neuropeptide systems in the striatum at 35 days old (Sivam and Chermak, 1992).

The acute administration to rats of a dose of 1.9 g L-cysteine/kg has been indicated to cause ultrastructural alterations of testicular Sertoli cells and spermatids (Bernacchi et al., 1993).

L-cysteine was identified as a neuroexcitotoxin due to its interaction with N-methyl-D-aspartate (NDMA) receptors (Olney, 1994). The perinatal administration of L-cysteine in mice or rats with an immature blood-brain barrier produces neurotoxicity. In new born animals effects are observed in the brain (hypothalmus) and retina similar to those induced by L-glutamic acid (Olney, 1994).

Acute adverse effects in humans

In humans, oral doses of 5 and 10 g of L-cysteine provoked nausea and slight dizziness (Carlson et al., 1989). In addition, healthy people who were administered with increasing doses of up to 20 g of L-cysteine (with tranylcypromine) exhibited fatigue, dizziness, nausea and insomnia depending on the dose (Davies et al., 1972).

There is no information available regarding the chronic administration of L-cysteine in humans.

The information available on the adverse effects of the intake of L-cysteine and L-cystine from food supplements is not considered adequate for dose-response assessment nor for the proposal of an UL for L-cysteine (IoM, 2005).

5.7.5 Conclusion

The Scientific Committee concluded that, based on the information available to date and taking into account the considerations reflected in this report, the proposal of the AESAN of a maximum daily amount of 300 mg of L-cysteine is lower than the requirements of L-methionine + L-cysteine established by the WHO (World Health Organitation) and is very far from the doses of L-cysteine that cause dizziness and nausea, therefore it considers it acceptable from the safety point of view for use as a food supplement.

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5.8 L-glutamine

5.8.1 Proposal

The AESAN has recommended a maximum daily amount of 2,000 mg of L-glutamine. This proposal is based on the authorisation in Denmark of a total maximum amount that must not exceed 2,000 mg of L-glutamine per recommended daily dose (Denmark, 2011).

In Italy, L-glutamine is authorised in food supplements (legislative proposal) without the establishment of a maximum daily amount (Italy, 2012).

5.8.2 Characteristics and sources

L-glutamine is the amide form of L-glutamic acid. In normal physiological circumstances it is synthesised in sufficient quantity to satisfy the body's needs, and therefore it has traditionally been considered as a non-essential amino acid. Nevertheless, in recent years it has been confirmed that in conditions of metabolic stress, the demand for L-glutamine increases and the human being is unable to synthesise it in adequate quantity. Therefore, at present, it is thought that it should be included among the conditionally essential amino acids.

It is the most abundant amino acid in the blood. It is also found in the greatest quantity in cells. In addition, it constitutes 61 % of the amino acids of the skeletal muscle, equivalent therefore to half of the total body amino acids.

The most important sources of L-glutamine are beef, chicken, fish, eggs, dairy products, wheat, cabbage, beetroot, beans, spinach and parsley.

The EFSA has published two scientific opinions regarding the verification of health claims relating to L-glutamine: i) growth and maintenance of muscular mass; ii) faster recovery of muscle glucogen deposits after strenuous exercise; iii) increase in the repair of muscular tissue after exercise; iv) improvement of the neurological function and increase in attention span; v) improved memory; vi) increase in the synthesis of intestinal proteins; vii) improvement of the immune system; and viii) improvement of defence mechanisms against intestinal pathogens. Based on the information provided, the NDA Panel of the EFSA has not been able to establish a cause and effect relation in any case (EFSA, 2009, 2011).

5.8.3 Nutrition and metabolism

In the body, L-glutamine is synthesised from glutamate and from ammonia, in a reaction catalysed by L-glutamine synthetase, with the collaboration of the ATP. Hydrolysis of L-glutamine is catalysed by glutaminase, which regenerates the glutamate and ammonia.

The synthesis of L-glutamine and its release into the blood predominates in the muscle, adipose tissue, lungs and brain. In these tissues, the synthesis of L-glutamine is a means of detoxifying ammonia from the tissues. Hydrolysis of L-glutamine takes place in the intestinal mucosa, where it is used as a source of energy and for the synthesis of purines. The hydrolysis of L-glutamine in the renal cortex serves to regulate the acid-base equilibria. Finally, the liver is able to synthesise and hydrolyse L-glutamine.

In addition to the above functions, L-glutamine has other important physiological functions: i) regulation of insulin release; ii) reduction in the synthesis of glucocorticoids; iii) nitrogen reservoir; iv) regulation of protein turnover; v) regulation of the gene expression; vi) immune regulation; vii) metabolic fuel for fast-growing tissues

and tissues that require it; viii) inhibition of apoptosis; ix) synthesis of amino sugars and glycoproteins; and x) purine and pyrimidine synthesis (Brasse-Lagnel et al., 2009) (Stanley, 2009) (Wu, 2009).

In the last two decades, the importance of the use of L-glutamine in various catabolic states (sepsis, multiple trauma, cancer, bone marrow transplant, intensive chemotherapy and radiation), and in inflammatory intestinal diseases has been revealed (Ballestero et al., 2010) (Kuhn et al., 2010) (Xi et al., 2011). In these situations, L-glutamine is administered in parenteral solutions as a dipeptide. This improves the response to metabolic stress, cellular immunity, the integrity of the intestinal barrier, the synthesis of L-arginine and the nitrogen balance, preserving muscular L-glutamine (Cardona, 1998) (Wenerban, 2008). The efficiency of oral supplementation with L-glutamine is doubtful, but recent clinical evidence indicates how its use in enteral nutrition improves the immune response and reduces energy expenditure in critical patients (De Legge, 2008).

Given that in the absence of disease, L-glutamine is a non-essential amino acid, no dietary reference intakes (DRI) or average requirements (AR) have been established. In addition, scientific evidence of the beneficial effects of supplementation with L-glutamine takes place at far higher doses than those reached with diet. In this respect, the NDA Panel of the EFSA has published two reports in which they propose daily doses of L-glutamine of 50-900 mg/kg b.w./day in order to obtain beneficial effects (EFSA, 2009, 2011).

5.8.4 Safety

There are numerous studies on the use of L-glutamine as a food supplement, but only four have been designed with the objective of assessing its safety (Garlick, 2001). From these studies it is concluded that L-glutamine is safe in adults and neonates but that there is insufficient data to permit the identification of one or more adverse effects. Moreover, the studies carried out have not permitted the establishment of a NOAEL for L-glutamine (Garlick, 2001). The maximum doses used in the few studies carried out were 0.3 g/kg b.w. in a single oral dose or 0.57 g/kg b.w. administered intravenously, for 30 days. There is also a study which used between 20-40 g/day, but only for 24 hours.

Nevertheless, a series of considerations must be taken into account: i) there are no studies of any sort on the use of L-glutamine in healthy subjects over long periods of time; ii) the studies have always been conducted on patients under strict medical supervision; iii) individual susceptibility must be studied, as there is a study that demonstrates intolerance to L-glutamine in doses of 0.1-1.0 g/kg b.w./day, in cases where it is used to treat Crohn's disease (Akobeng et al., 2000); iv) there are no toxicity data for the elderly, in whom high doses of L-glutamine could obstruct the hepatic and renal processing of an increased nitrogen load; and v) L-glutamine is metabolised to glutamate and ammonia, compounds that have adverse neurological effects, and therefore studies on the possible psychological and behavioural effects are required.

Finally, the Scientific Committee of the Norwegian Food Safety Authority (VKM, 2011) established that L-glutamine presents a low risk. This is understood as when no changes take place in the biomarkers, nor are there any adverse effects to health.

5.8.5 Conclusion

The Scientific Committee considers that no adverse effects have been observed in either the safety studies conducted with L-glutamine or in its use at high doses in clinical nutrition. Therefore, and although the safety of L-glutamine has not been assessed in healthy subjects or in chronic administrations, this Scientific Committee

concludes that the AESAN proposal of a maximum daily amount of 2,000 mg of L-glutamine is acceptable from a safety point of view for use as a food supplement.

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5.9 L-histidine

5.9.1 Proposal

The AESAN has recommended the inclusion of L-histidine in Royal Decree 1487/2009 with a maximum daily amount of 750 mg.

The proposal is based on the fact that Regulation (EC) No 953/2009 (EU, 2009) includes L-histidine among the substances that may be added for specific nutritional purposes in foods for particular nutritional uses.

In Belgium, L-histidine is authorised in food supplements without the establishment of a maximum daily amount (Belgium, 1992).

In Italy, a maximum daily amount of 1,120 mg of L-histidine is authorised in food supplements (legislative proposal) (Italy, 2012).

5.9.2 Characteristics and sources

L-histidine (His) $C_6H_9N_3O_2$ is an essential amino acid for children.

The essential nature of L-histidine has been well established to date. It is known that this amino acid cannot be synthesised in the body but, on the other hand, it is difficult to demonstrate its deficiency, as it is an abundant amino acid in haemoglobin and in muscle proteins. There are carnosine and anserine dipeptides in the muscle that contain L-histidine (Sánchez de Medina, 2010).

L-histidine is an important component of haemoglobin (8 %). Diets without L-histidine reduce the rate of erythropoiesis and in adults they reduce haemoglobinemia, changes which revert when the intake of L-histidine is restored (Kopple and Swendseid, 1975). In addition, the dipeptide, carnosine found in skeletal muscle, is a large store of L-histidine (Christman, 1971). As the L-histidine pool in the body is large, a prolonged period of time (more than 60 days) is required to deplete L-histidine in an adult.

5.9.3 Nutrition and metabolism

L-histidine is an important component of haemoglobin and is the precursor of histamine which is formed by the decarboxylation of L-histidine through L-histidine decarboxylase.

L-histidine is necessary for the regulation and use in the body of trace elements such as zinc, copper, iron, manganese and molybdenum.

L-histidine is mainly degraded in the liver and in skin cells. The process starts with the deamination of the amino acid through the action of the histidase forming urocanic acid. This acid is hydrolysed and transformed in various enzymatic stages to glutamate in the liver.

Recommended dietary intakes for adults (over 19 years old) (IoM, 2005) are: histidine AR: 11 mg/kg b.w./day; histidine RDA: 14 mg/kg b.w./day (IoM, 2005).

The requirements (WHO, 2007) for adults of L-histidine are 10 mg/kg b.w./day.

Based on the distribution of the NHANES III data (1988-1994), the mean daily intake of L-histidine from food and food supplements for all age groups, at all stages of life, in men and women, is 2.2 g/day. The highest intakes correspond to the 99th percentile of men aged 51 to 70 years old and the value is 5.2 g/day (IoM, 2005).

5.9.4 Safety

Adverse effects in animals

The administration of L-histidine by intraperitoneal or intravenous injection has been observed to provoke changes in the amino acid content in the brain (Oishi et al., 1989) and the histamine content (Schwartz et al., 1972). Young rats, aged 4 to 5 weeks old, treated with a histidinase inhibitor exhibit a reduction in their locomotive activity after receiving an intraperitoneal injection of L-histidine (250 mg/kg b.w.) (Dutra-Filho et al., 1989). Pilc et al. (1982) indicate a "strange behaviour" in rats administered with intraperitoneal L-histidine in concentrations of 400 to 800 mg/kg b.w. These effects have not been indicated in rats administered with L-histidine orally.

Low protein diets enriched with L-histidine, administered to rats, for 3 to 4 weeks, led to significant weight loss after some days. Nevertheless, the effects diminished when increasing quantities of high-quality proteins were added to the diet (Benevenga and Steele, 1984).

In studies on rats fed for short periods of time (7 to 46 days), with between 2 and 4 g/kg b.w./day of L-histidine, delayed growth, hepatomegaly and hypercholesterolemia were observed (Solomon and Geison, 1978) (Harvey et al., 1981) (Ohmura et al., 1986) (Hitomi-Ohmura et al., 1992). Harvey et al. (1981) indicated significant reductions in the plasma concentrations of copper and zinc and a reduction in the copper content in rat livers after receiving, for 46 days, diets containing 8 % of L-histidine (~4 g/kg b.w.). Hypercholesterolemia is eliminated with a diet simultaneously enriched with L-histidine and copper, supporting the hypothesis that L-histidine-induced hypercholesterolemia is a consequence of changes in copper status. Oral administration to mice of 1.3 g L-histidine/kg b.w. for 21 days led to an increase in the absorption and use of zinc, and to a higher zinc content in the liver, muscular tissue, spleen and pancreas (Van Wouwe et al., 1989).

The long-term toxicity and the carcinogenicity of L-histidine monohydrochloride (HMHC) was studied in 50 male rats and 50 female rats (Ikezaki et al., 1996). The male rats were administered diets containing 0.47 and 0.96 g/kg b.w./day of HMHC for 104 weeks and the females 0.56 and 1.1 g/kg b.w./day for the same period of time. No significant increases were observed in the appearance of tumours related to the treatment when compared to paired controls. Nor were any neoplastic changes mentioned in either the control groups or the treatment groups. In the male rats that received 0.96 g of HMHC/kg b.w./day increases were observed in the red blood cell count, haemoglobin and hematocrit concentration. No sperm granuloma tests were observed in male rats that received either 1.6 g of HMHC/kg b.w./day for 13 weeks or 0.97 g/kg b.w./day for 104 weeks (Ikezaki et al., 1996).

Adverse effects in humans

Thirty rheumatoid patients and 20 controls received daily for 30 weeks, capsules of 4.5 g of L-histidine in a double-blind test, followed by open treatment in which 19 patients received the same dose for another 10 months. The authors of the study (Pinals et al., 1977) indicate the absence of adverse effects as a consequence of the L-histidine therapy, although it is not clear as to what the adverse effects examined were. Similarly, in a double-blind test 42 patients (16 chronic uremics and 26 subjected to maintenance dialysis) were treated with oral doses of 4 g of L-histidine/day for 17.5 weeks. No adverse effects were mentioned, although it is not clear from the report which effects were examined (Blumenkrantz et al., 1975).

Studies of the effects of L-histidine on taste and smell acuity produced contradictory results. Henkin et al. (1975) indicated a reduction in acuity in both senses in six patients who received 8 to 65 g of L-histidine/day, for 24 days. In view of the increase in the urinary excretion of zinc and the decrease in the serum level, the authors

postulate that the effects of the administration of L-histidine are due to a zinc deficiency. In another study in which eight healthy men were administered with 4 g of L-histidine/day, for 2 weeks, the effects on taste and smell acuity were not mentioned (Schechter and Prakash, 1979). This also occurred with the administration of oral doses of L-histidine, of between 24 and 64 g/day, for 4 weeks. Nevertheless, even at the lowest dose (4 g/day) adverse effects were observed. These included headaches, weakness, stupor and nausea, whereas at the highest doses (24 and 64 g/day) anorexia, feeling of pain in the eyes and changes in visual acuity in two women were reported (Geliebter et al., 1981).

In children who received total parenteral total nutrition an increase is mentioned of 70 % in the urinary excretion of zinc when the fluid contains 165 mg of L-histidine/kg b.w./day, compared to the 95 mg of L-histidine/kg b.w./day of the controls. Although this is parenteral administration, it provides further evidence that in humans an intake of L-histidine in excess may cause L-histidine/zinc interactions, the result of which is a zinc deficiency (Zlotkin, 1989).

Evaluation of the dose-response

The study by Ikezaki et al. (1996) is the only dose-response test on animals relating to the oral administration of L-histidine. Nevertheless, only two doses were used in this study, neither of which caused any adverse effects. In addition, no data are mentioned to indicate the possible effect of the doses on the zinc and copper metabolisms, which has been indicated in both humans and in animals used for research.

It should be noted that the studies in humans to assess the effects of L-histidine were designed to study their efficiency as a therapeutic agent in some diseases, the objective was not to obtain an UL in apparently healthy individuals, and therefore their use in this respect is limited. The chronic study of the effects of L-histidine administered orally to rodents is not considered appropriate for obtaining an UL.

Evidence is available that in human beings, doses of L-histidine of between 4 and 4.5 g/day above the dietary content do not have adverse effects. However this evidence must be considered tentative in view of the limited number of individuals studied and the lack of information relating to dose-response. In addition, there is evidence from studies in animals used for research and on humans of the effect of high intakes of L-histidine on the metabolism of copper and zinc. Nevertheless, the lack of dose-response data does not permit the identification of the L-histidine intakes required to provoke these responses. From this, it is concluded that the available scientific data are inadequate for deriving an UL for the chronic oral intake of L-histidine from food supplements.

5.9.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AESAN proposal of a maximum daily amount of 750 mg of L-histidine is of the order of the requirement established by the WHO (World Health Organisation) and therefore acceptable from the safety point of view for use as a food supplement.

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5.10 L-isoleucine

5.10.1 Proposal

The AESAN has recommended a maximum daily amount of 1,500 mg of L-Isoleucine. This proposal is based on the protein reference intake recommended by the WHO for the adult population (WHO, 2007).

In Italy, a maximum daily amount of 910 mg of L-isoleucine is authorised in food supplements (legislative proposal) (Italy, 2012) and in Belgium L-isoleucine is authorised in food supplements without establishing a maximum daily amount (Belgium, 1992).

5.10.2 Characteristics and sources

L-isoleucine (2-amino-3-methylpentanoic acid) is an aliphatic branched-chain alpha amino acid with the following chemical formula: $HO_2CCH(NH_2)CH(CH_3)CH_2CH_3$. It is considered as a hydrophobic amino acid and together with L-threonine is the only amino acid to have a chiral-type side chain. There are four possible stereoisomers of L-isoleucine, but in nature it only exists in its enantiomeric form ((2S,3S)-2-amino-3-methylpentanoic acid).

Although this amino acid is not synthesised in animals, relatively high levels are found in the animals. The most important food sources of L-isoleucine are fish (tuna, sea bream, grouper and mackerel), meat (chicken, beef, lamb and turkey), eggs and soybeans.

5.10.3 Nutrition and metabolism

L-isoleucine is an essential amino acid and therefore cannot be supplied by the body.

The degradation of L-isoleucine shares the first two steps with the other branched-chain amino acids. Therefore, its catabolism occurs in the muscular tissue, where it undergoes a reversible transamination process resulting in alpha-ketoisocaproate. Subsequently, it undergoes irreversible oxidative decarboxylation resulting in alpha-methyl-butyryl-CoA and acyl-CoA derivatives. Lastly, after several catabolic steps succinyl-CoA and acetyl-CoA are produced, which may be used as energy sources through oxidative metabolism, through the Krebs cycle in the muscle. In the final phases of the catabolism, the L-isoleucine produces acetyl-CoA and propionyl-CoA, therefore it is considered as a glucogenic and ketogenic amino acid (IoM, 2005) (Lehninger, 2005). The presence of biotin is necessary for the catabolism of L-isoleucine.

L-isoleucine serves as a structural base for protein synthesis, but it also takes part in protein synthesis, it is a source of energy for the muscle in the absence of glycogen and regulates the metabolism of the glucose (Yoshizawa, 2012). In this respect, it has been seen that L-isoleucine stimulates the muscle's glucose uptake, increases the total body oxidation of the glucose and reduces hepatic gluconeogenesis. All these effects give it a hypoglycemiant nature (Yoshizawa, 2012). In addition, it intervenes in the synthesis of haemoglobin and in platelet aggregation (Honig, 1967).

At present there are no experimental data which can be used to calculate the requirements of L-isoleucine. Therefore, given that L-isoleucine is a branched amino acid like L-leucine, it shares catabolic paths of oxidation with it, and that the daily requirements mainly reflect the basal levels of its catabolism, the joint technical report of the FAO/WHO/UNU has estimated the requirements of L-leucine assuming a proportionality with L-leucine and considering the proportion of L-isoleucine in the body proteins. According to these calculations, the requirements of L-isoleucine are 20 mg/kg b.w./day (WHO, 2007).

The usual daily intake of L-leucine in the Western population is 3-4 g/person per day, although male adults between 50-70 years old may take up to 8 g/day (IoM, 2005).

5.10.4 Safety

There are very few studies, either on animals used for research, or on humans, which assess the toxicity of L-isoleucine in isolation and not as part of the other branched-chain amino acids or forming part of a tripeptide (L-isoleucine-proline-proline).

An acute toxicity study on rats (2.0 g/kg b.w./day, 14 days orally) did not find any adverse effects (EFSA, 2010). In experiments carried out on piglets given doses of L-isoleucine 10 times higher than the usual for 3 weeks, no adverse effects were found (EFSA, 2010). There is one subchronic toxicity study on rats in which they were administered, for 13 weeks, oral doses of up to 3.2 g/kg b.w./day. In doses of up to 2.0 g/kg b.w./day no adverse effects were observed. In doses of 3.2 g/kg b.w./day only a slight increase in the size of the kidneys and the volume of urine were observed (Kawabe et al., 1996). Mutagenicity and genotoxicity studies on four strains of *Salmonella* and *E. coli* did not exhibit any alteration (EFSA, 2010). In addition, the capacity of the isoleucine to produce mutagenesis was tested on a line of mouse lymphoma and on the CHO line. In neither of the cases, with doses of up to 1.31 mg/ml were chromosomic alterations observed (EFSA, 2010). Chronic toxicity studies on rats used doses of up to 5.0 g/kg b.w./day for 104 weeks. In the highest doses, minor analytic modifications were observed, together with an insignificant increase in the size of the kidneys and a slight reduction in testicle size in male rats (EFSA, 2010). In addition, studies on the toxicity of L-isoleucine in the reproductive function and embryonic development have demonstrated that doses of up to 4.2 g/kg b.w./day, for the 14 days prior to mating did not affect fertility or embryonic development (EFSA, 2010). This was also the case when L-isoleucine was administered in doses of up to 5.0 g/kg b.w./day, from fertilisation to birth (EFSA, 2010).

Nevertheless, it should be noted that there are discrepancies as regards a possible effect of L-isoleucine on the development of bladder cancer. Nishio et al. (1986) in a test on rats given L-isoleucine for 40-60 weeks, after the induction of bladder tumours with nitrosamine, observed a significant increase in the development of bladder cancer. On the other hand, Kawabe et al. (2006) in a similar study, using doses of up to 3.2 g/kg b.w./day, did not observe any carcinogenic effects.

All these studies have led the EFSA to establish a NOAEL of 0.92 g/kg b.w./day (EFSA, 2010).

5.10.5 Conclusion

The Scientific Committee concludes that, although there are no toxicity studies in humans, toxicity studies on different animal models establish that there is a high tolerance level to L-isoleucine. In addition, the mean intake data for L-isoleucine in humans range between 3 and 8 g/person/day. Therefore, it considers that on the basis of the available information to date, the AESAN proposal of a maximum daily amount of 1,500 mg of L-isoleucine is acceptable from the point of view of its safety for use as a food supplement.

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5.11 L-leucine

5.11.1 Proposal

The AESAN has recommended a maximum daily amount of 2,950 mg of L-leucine. This proposal is based on the protein reference intake recommended by the WHO for the adult population (WHO, 2007).

In Italy, a maximum daily amount of 1,330 mg of L-leucine is authorised in food supplements (legislative proposal) (Italy, 2012) and in Belgium L-leucine is authorised in food supplements without establishing a maximum daily amount (Belgium, 1992).

5.11.2 Characteristics and sources

L-leucine is a branched-chain alpha amino acid with the following chemical formula: $HO_2CCH(NH_2)CH_2CH(CH_3)_2$. It is considered a hydrophobic amino acid.

It is the most abundant amino acid in food proteins and tissues. The most important food sources of L-leucine are soybean, beef, peanuts, fish, wheat germ, almonds, chicken, eggs and oats.

5.11.3 Nutrition and metabolism

L-leucine is an essential amino acid and therefore cannot be supplied by the body. L-leucine is absorbed in the small intestine in its free form or forming part of peptides. Subsequently it is transported to the liver, where a part is used for protein synthesis and the rest is distributed to the different body tissues (PDNRS, 2008).

Most of the L-leucine metabolism occurs in the muscular tissue, where it undergoes a reversible transamination process resulting in alpha-ketoisocaproate. Subsequently, it undergoes irreversible oxidative decarboxylation resulting in isovaleryl-CoA and acyl-CoA derivatives. Lastly, after several catabolic steps acetoacetate and acetyl-CoA are produced, which may be used as energy sources through oxidative metabolism (IoM, 2005). The urea resulting from the catabolism of L-leucine is eliminated through the kidneys. In general, mammals have a high capacity for metabolising L-leucine, and the maximum oxidation level is 8.9 g/kg b.w./day (Sakai et al., 2004).

L-leucine serves as a structural base for protein synthesis, but is also a powerful activator of the mammalian target of rapamycin (mTOR), a serine/threonine kinase involved in numerous cellular processes. Consequently, L-leucine exercises a number of metabolic functions, which depend on its intracellular concentration. These functions include: i) stimulating protein synthesis; ii) controlling satiety by acting directly on the hypothalamus and stimulating the release of leptin; iii) controlling body weight by reducing fatty mass; iv) controlling energy expenditure; and v) improving the metabolism of glucose and sensitivity to the same (Li et al., 2011). Lastly, L-leucine may act as a source of energy for the muscle in the absence of glycogen (MacDonald et al., 2005).

In accordance with the joint technical report issued by the FAO/WHO/UNU, the requirements for L-leucine in adults are 39 mg/kg b.w./day (WHO, 2007). For lactating infants the requirements are between 90 and 165 mg/kg b.w./day (WHO, 2007). For children and adolescents the values range between 39 and 73 mg/kg b.w./day (WHO, 2007). The usual daily intake of L-leucine in the Western population is between 6 and 9 g/person per day, although male adults between 50-70 years old may take up to 14 g/day (IoM, 2005).

5.11.4 Safety

The toxicological studies conducted on animals used for experiment, and the clinical trials and nutritional intervention tests on humans indicate that the degree of tolerance to L-leucine is high and that its toxicity is low.

Tsubuku et al. (2004), in a study conduced on rats for 13 weeks, established a NOAEL of 3.33 (female rats) and 3.83 (male rats) g/kg b.w./day for the oral intake of L-leucine. In the case of the reproductive function and embryonic development, the NOAEL is 1.0 g/kg b.w./day (Sakai et al., 2004).

Mutagenicity tests show that in concentrations of up to 2 mM, L-leucine does not produce any alteration to the different strains of *E. coli* (Sargentini and Smith, 1986). In addition there is scientific evidence that L-leucine does not enhance the growth of neoplastic cells but that even at high doses it diminishes its proliferation (Wakshlag et al., 2006).

Work conducted on humans corroborates the existing data on the safety of L-leucine in animals used in experiments. In healthy subjects, the intake of 45 mg/kg b.w./day for 3 months did not have produce adverse effects (Verhoeven et al., 2009). There are studies in which it is administered orally in doses of up to 24 g/day without finding adverse effects (Scarna et al., 2003).

5.11.5 Conclusion

Although it has not been possible to establish a NOAEL or LOAEL for the oral intake of L-leucine, from the studies reviewed, it can be concluded that: i) the usual intake of L-leucine, in the population, is between 6 and 9 g/person/day; ii) the capacity to metabolise L-leucine is high (8.9 g/kg b.w./day); and iii) healthy subjects are able to tolerate intakes of 45 mg/kg b.w./day for long periods of time.

Therefore, the Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, a maximum daily amount of 3,000 mg of L-leucine is acceptable from the safety point of view for use as a food supplement.

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5.12 L-lysine

5.12.1 Proposal

The AESAN has recommended a maximum daily amount of 2,250 mg of L-lysine. This proposal is based on the protein reference intake recommended by the WHO for the adult population (WHO, 2007).

In Italy, a maximum daily amount of 1,120 mg of L-lysine is authorised in food supplements (legislative proposal) (Italy, 2012) and in Belgium L-lysine is authorised in food supplements without establishing a maximum daily amount (Italy, 2012) (Belgium, 1992).

In Denmark L-lysine hydrochloride is authorised in food supplements with a total maximum amount that must not exceed 100 mg per daily recommended dose (Denmark, 2011).

5.12.2 Characteristics and sources

L-lysine is an alpha amino acid with the chemical formula $HO_2CCH(NH_2)(CH_2)_4NH_2$. It is a basic amino acid.

The most important food sources of L-lysine are protein foods such as eggs, meat, cheese and some fish (especially sardines and cod). Soybean is also rich in L-lysine.

L-lysine is also used in its monohydrochloride form as a food ingredient in levels of up to 1.55 % (1.2 % of L-lysine) (FDA, 2011). Its function is to reduce the formation of acrylamides in frying processes.

The EFSA has published a scientific opinion regarding the verification of health claims relating to L-lysine: i) improvement of the immune response to the herpes virus; ii) regulation of LDL-cholesterol levels; iii) increase of appetite; iv) increase of protein synthesis; v) maintenance of bone mass; and vi) increase of bone mass. Based on the information presented, the NDA Panel of the EFSA has not found it possible to establish a cause and effect relation in any of the cases (EFSA, 2011).

5.12.3 Nutrition and metabolism

As it is an essential amino acid it cannot be synthesised by the body. Its catabolisation takes place preferably in the liver. The two nitrogen groups are transferred to the alpha-ketoglutarate. Outside the liver, one of the nitrogen groups is separated as ammonia by the L-lysine oxidase. Lastly, the carbon skeleton produces acetoacetyl-CoA. Therefore it is a ketogenic amino acid.

Its functions include its incorporation to collagen, giving it consistency and it is a precursor of L-carnitine. Moreover, it intervenes in protein synthesis and in the intestinal absorption of calcium.

According to the joint technical report issued by the FAO/WHO/UNU L-lysine requirements in adults range between 12 and 30 mg/kg b.w./day, according to the type of study used (isotopic tracers, oxidation of the amino acid or nitrogen balance) (WHO, 2007). For lactating infants the requirements are 103 mg/kg b.w./day (WHO, 2007). For children and adolescents the values range between 30 and 64 mg/kg b.w./day (WHO, 2003). For a healthy adult, the mean intake of L-lysine ranges between 7 and 16 mg/kg b.w./day (FDA, 2011). The studies of Flodin (1997) and the Food and Drug Administration (FDA, 2011) indicate that in individuals who eat large quantities of snacks, the daily intake of L-lysine may range between 3.5 and 4 g/day.

5.12.4 Safety

Studies on the teratogenic effects of L-lysine, and on reproduction in animals used for experiment show that doses of up to 2.25 g/kg b.w./day are not harmful (Funk et al., 1991). In addition, in a study conducted on women with familiar hyperlysinemia (plasma levels of L-lysine of up to 10 times higher than normal), it was observed that one woman gave birth to a normal baby (Dancis et al., 1983).

The oral LD_{50} for L-lysine is 10 g/kg b.w. (Breglia et al., 1973). In a subchronic toxicity study conducted on rats for 13 weeks (Tsubuku et al., 2004) no alterations were observed. This work established a NOAEL for L-lysine of 3.6 g/kg b.w./day. In chronic toxicity studies on rats, doses of 740 mg/kg b.w./day were used for 2 years, and no adverse effects were found (Flodin, 1997). This serves to confirm that the toxicity of L-lysine is low.

Studies conducted on humans confirm the data obtained from the animals. In this respect, supplementation of between 1.0 and 3.0 g/day for 6 months did not have any adverse effects on healthy individuals (Flodin, 1997). The same study confirmed that L-lysine was also well-tolerated in babies and children. The use of daily doses of between 0.4 and 1.5 g/day of L-lysine for up to 3 years to treat the herpes simplex virus did not have secondary effects (Griffith et al., 1978).

5.12.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AESAN proposal of a maximum daily amount of 2,250 mg of L-lysine is acceptable from the safety point of view for use as a food supplement.

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5.13 L-methionine + L-cysteine

5.13.1 Proposal

The AESAN has proposed a maximum daily amount for the sum of L-methionine and L-cysteine of 1,100 mg and maximum daily amounts of 800 mg of L-methionine and 300 mg of L-cysteine. This proposal is based on the protein reference intake recommended by the WHO for the adult population (WHO, 2007).

The proposal is based on the fact that Regulation (EC) No 953/2009 (EU, 2009) includes L-cysteine among the substances that may be added for specific nutritional purposes in foods for particular nutritional uses.

In Italy, a maximum daily amount of 1,190 mg of the sum of L-methionine and L-cysteine is authorised in food supplements (legislative proposal) (Italy, 2012) and in Belgium L-methionine is authorised in food supplements without establishing a maximum daily amount (Belgium, 1992).

5.13.2 Characteristics and sources

L-methionine is an essential alpha amino acid, nonpolar, very hydrophobic, with the chemical formula $HO_2CCH(NH_2)CH_2CH_2SCH_3$. L-cysteine is a neutral conditionally essential alpha amino acid, with the chemical formula $C_3H_7NO_2S$ (CH_2SH-$CHNH_2$-COOH). Both are sulphur amino acids.

The principal food sources of L-methionine are eggs, chicken, fish and meat, cheese, soybean, sesame seeds and Brazil nuts. Sources of L-cysteine are all animal proteins and red peppers, garlic, onions, Brussels sprouts, broccoli and oats (USDA, 2009).

The EFSA has published a scientific opinion relating to the verification of health claims regarding L-methionine and the maintenance of blood cholesterol levels. Based on the information presented, the NDA Panel of the EFSA has not been able to establish a cause and effect relation (EFSA, 2010).

5.13.3 Nutrition and metabolism

Both L-methionine and L-cysteine are proteinogenic amino acids. L-methionine is the principal donor of methyl groups. Its derivative S-adenosylmethionine serves as a donor of methyl groups that can be transferred to other compounds. L-methionine is an intermediary of homocysteine, L-cysteine and taurine. L-cysteine is a precursor of taurine and forms part of the coenzyme A and of the glutathione. In this respect it has antioxidising functions. In addition, L-cysteine intervenes in xenobiotic conjugation and in the formation of leukotrienes. Finally, L-cysteine plays an essential role in the maintenance of the quaternary structure of proteins, thanks to its capacity to form intracatenary disulfide bridges (Brosnan and Brosnan, 2006).

In normal physiological conditions the body is able to obtain L-cysteine in sufficient quantity. However premature babies cannot synthesise it and must obtain it through diet.

The metabolism of L-methionine starts with its conversion to S-adenosylmethionine in a reaction with the ATP and in which the enzyme L-methionine adenosyltransferase intervenes. Subsequently, the S-adenosylmethionine transfers its methyl group to another acceptor and S-adenosylhomocysteine is formed, a compound that is hydrolysed creating homocysteine (Brosnan and Brosnan, 2006). L-cysteine as a conditionally essential sulphur amino acid, is obtained from L-methionine and from serine. Specifically, the enzyme cystathionine beta-synthase may give cystathionine from homocysteine and serine. Subsequently, the cystathionine may be broken into L-cysteine and alpha-ketobutyrate (Lehninger et al., 2000). An important

aspect of L-cysteine is that it can oxidise to cystine. In addition, L-cysteine is degraded to pyruvate in two stages, elimination of sulphur and transamination. L-cysteine can be metabolised to taurine and CO_2 through the L-cysteine sulfinate path, the initial step of which is oxidation, catalysed by L-cysteine dioxygenase, to cysteine sulfinate. L-cysteine sulfinate can be decarboxylated to produce taurine or metabolised via beta-sulfinyl-pyruvate (intermediary case) to pyruvate and sulfite and then to CO_2 and sulfate (Stipanuk, 1986).

Recommended dietary intakes for adults (over 19 years old) (IoM, 2005) are: i) AR for L-methionine + L-cysteine 15 mg/kg b.w./day and ii) RDA for L-methionine + L-cysteine 19 mg/kg b.w./day. According to the WHO (2007), adult requirements are 4 mg/kg b.w./day for L-cysteine and 15 mg/kg b.w./day for L-cysteine + L-methionine. With respect to the daily consumption of L-methionine, the study by Ward et al. (2000) based on consumption frequency questionnaires, estimates that the mean intake of L-methionine of a healthy adult is 2.3 ± 0.9 g/day, with an interval of 0.5-5 g/day (7-70 mg/kg b.w./day). In children, mean intakes range between 62 and 97 mg/kg b.w./day (Garlick, 2006). In the United States, based on the data from NHANES III (1988-1994), an average dietary intake of L-cysteine from food and food supplements of 1.0 g/day has been estimated. The highest intake is for men aged between 51 and 70 years old and is 2.2 g/day in the 99th percentile (CDC, 1997).

5.13.4 Safety

Safety of L-cysteine

Low-protein diets with L-cysteine contents of 0.5 to 10 % reduce the increase of weight and the intake of food and therefore increase animal mortality (Harper et al., 1970). They also modify the cholesterol concentrations in plasma that increase in rats which receive cholesterol-poor diets and fall in those with cholesterol-rich diets (Rukaj and Sérougne, 1983) (Sérougne and Rukaj, 1983) (Sérougne et al., 1987). Moreover, histopathological changes have been systematically observed in the liver and kidneys (Harper et al., 1970).

Acute adverse effects in animals

L-cysteine is mutagenic in bacteria (Glatt, 1989), but not in mammal cells (Glatt, 1990).

A single oral dose of 3 g L-cysteine/kg b.w. in *Swiss Webster* albino mice, aged 10 to 12 days, produced hypothalamic neuron necrosis and retinal lesions, observed 5 hours after the intake (Olney and Ho, 1970). Furthermore, the subcutaneous injection of 1.2 mg of L-cysteine/kg b.w. of *Wistar* rats aged 9 to 10 days caused permanent dystrophy in the interior layers of the retina (Karlsen and Pedersen, 1982).

The intraperitoneal injection of 1.0 mmol L-cysteine/kg b.w. of male *Wistar* rats led to a maximum blood concentration of some 2 mM after 30 minutes (Calabrese et al., 1997). After 1 hour, exposure resulted in high concentrations of malondialdehyde in the *substantia nigra* of the brain. The subcutaneous injection of 0.5 g of L-cysteine/kg b.w. to 4-day-old *Sprague-Dawley* rats did not have an ulterior effect on the neurotransmitters or neuropeptide systems in the striatum at 35 days old (Sivam and Chermak, 1992).

The acute administration to rats of a dose of 1.9 g L-cysteine/kg has been indicated to cause ultrastructural alterations of testicular Sertoli cells and spermatides (Bernacchi et al., 1993).

L-cysteine was identified as a neuroexcitotoxin due to its interaction with N-methyl-D-aspartate (NDMA) receptors (Olney, 1994). The perinatal administration of L-cysteine in mice or rats with an immature blood-brain barrier produces neurotoxicity. In new born animals effects are observed in the brain (hypothalmus) and retina similar to those induced by L-glutamic acid (Olney, 1994).

Acute adverse effects in humans

Single oral doses of 5 and 10 g of L-cysteine caused nausea and slight dizziness in healthy human beings (Carlson et al., 1989). In addition, healthy people who were administered with increasing doses of up to 20 g of L-cysteine (with tranylcypromine) exhibited fatigue, dizziness, nausea and insomnia depending on the dose (Davies et al., 1972).

There is no information available regarding the chronic administration of L-cysteine in humans.

The information available on the adverse effects of the intake of L-cysteine and L-cystine from food supplements is not considered adequate for dose-response assessment nor for the proposal of an UL for L-cysteine (IoM, 2005).

Safety of L-methionine

In the last 25 years, there have been numerous studies on the toxicity of L-methionine in humans. These studies have tried to establish a relation between the intake of L-methionine, the elevation of homocysteine plasma levels and endothelial dysfunction. The most frequently used test is known as the L-methionine load test. In this load test a single dose of 100 mg of L-methionine/kg b.w. (approximately seven times the recommended intake of sulphur amino acids (L-methionine + L-cysteine)) was usually administered (Garlick, 2006). After the load test, it was confirmed that there is an increase in the circulating levels of homocysteine that are apparent after 2 hours and reach their maximum after 4 hours. This increase in homocysteine produces an endothelial dysfunction (Chambers et al., 1999). Subsequent research clarified that the endothelial dysfunction was not directly caused by L-methionine, but that it was due to the increase of homocysteinemia (Hanratty et al., 2001). Nevertheless, the load test studies concluded that the effects observed were transitory and that they disappeared four hours after loading with L-methionine (Garlick, 2006). In addition, it has been confirmed that intakes of 100 mg/kg b.w. are not safe in patients with schizophrenia and with innate errors in the metabolism of sulphur amino acids (Garlick, 2006). Lastly, a meta-analysis study that reviewed various studies in which the L-methionine load test took place (some 6,000 adult subjects in total) concluded that apart from the above-mentioned transitory increase of homocysteinemia, the administration of a single dose of L-methionine only produces minor secondary effects (dizziness, somnolence, nausea, polyuria and slight changes in blood pressure). It should be noted, however, that there is one study in which, due to an error, one person was given a dose of 1 g of L-methionine/kg b.w. (approximately 70 times the recommended intake of sulphur amino acids) resulting in the death of the individual after 30 days (Cottington et al., 2002).

As regards the chronic administration of L-methionine it has been confirmed that the daily intake, for one week, of 100 mg of L-methionine kg b.w./day induces a prolonged increase in the plasma levels of homocysteine. The authors of the study conclude that this type of intake is not safe. The same study demonstrated that the intake of 250 mg per day of L-methionine, for 30 days, did not produce any significant increase in the plasma levels of homocysteine, and that it is therefore a safe dose (McAuley et al., 1999). In children, there is only one study on the toxic effects of intakes of high doses of L-methionine. In this study a group of 10 children were given, due to an error, formula milk with a high content of L-methionine. The children's intakes of L-methionine were between 1.5 and 6.5 times higher than the usual intakes, detecting values 250 times higher than the normal values of homocysteinemia. Nevertheless, in spite of this, no long-term adverse effects were observed (Mudd et al., 2003).

5.13.5 Conclusion

The Scientific Committee concluded that, based on the information available to date and taking into account the considerations reflected in this report, the proposal of the AESAN of a maximum daily amount of 300 mg of L-cysteine is lower than the requirements of L-methionine + L-cysteine established by the WHO and is very far from the doses of L-cysteine that cause dizziness and nausea, therefore it considers it acceptable from the safety point of view for use as a food supplement. Nevertheless, it recommends reducing the maximum daily amount of L-methionine to 250 mg. With this modification, the sum of the maximum daily intake of sulphur amino acids is 550 mg.

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5.14 L-ornithine-alpha-ketoglutarate

5.14.1 Proposal

The AESAN proposes a maximum daily amount of L-ornithine-alpha-ketoglutarate of 2,000 mg. This proposal is based on the existing authorisation in Italy (legislative proposal) of a maximum daily amount of 2 g/day in food supplements, with the warning that it should not be consumed by pregnant women, children or for prolonged periods of time without medical supervision (Italy, 2012).

5.14.2 Characteristics and sources

L-ornithine-alpha-ketoglutarate (OKG) is an ionic salt that contains two molecules of L-ornithine and one of alpha-ketoglutarate. L-ornithine is an amino acid required for the normal functioning of the urea cycle. Alpha-ketoglutarate is the carbon skeleton of glutamate. It is an intermediary in the Krebs cycle and plays an important role in a large variety of transamination reactions.

OKG is a precursor of the amino acids L-glutamine, L-arginine and proline and increases the secretion of anabolic hormones, such as insulin and the growth hormone.

5.14.3 Nutrition and metabolism

The OKG stimulates the release of insulin and the growth hormone, at the same time that it increases levels of amino acids and their metabolites, presumably making them available for protein synthesis. It also reduces the catabolic markers of protein degradation. Although the action mechanism is not well-known, it is believed that the metabolic activities of insulin and the growth hormone contribute to the influence of the OKG on the protein metabolism (Salvucci et al., 1987).

L-ornithine and alpha-ketoglutarate may follow different metabolic paths to produce a common product, L-glutamic acid. This may explain the metabolic interaction between alpha-ketoglutarate and L-ornithine during the administration of the OKG. The L-ornithine from the OKG may increase the tissue content of polyamine which stimulates the synthesis of proteins, RNA and DNA (Jeevanandam et al., 1988).

In human beings, the administration of a 2:1 molar combination of L-ornithine and alpha-ketoglutarate modifies the metabolism of the amino acids and the hormonal patterns differently when both are administered separately, indicating that the simultaneous administration of L-ornithine and alpha-ketoglutarate is necessary to obtain the expected anabolic effects (Vaubourdolle et al., 1988) (Cynober et al., 1990).

5.14.4 Safety

No studies have been found that assess the safety/toxicity of the OKG. However it has been used successfully, administered enterally and parenterally, in patients with burns or surgical trauma, or those suffering from chronic malnutrition (Cynober et al., 1984a) (Leander et al., 1985).

The kinetics and metabolic effects of the OKG administered orally have also been studied. In a study, 10 healthy individuals (five men and five women), subject to a standardised regimen, were given 10 g of OKG orally. The rapid fall in the L-ornithine blood concentrations to the basal values, the absence of an increase in the alpha-ketoglutarate content and the elimination of urea through the urine following the administration of the OKG

show the intense metabolism and use of both compounds. This would indicate that the hyperornithinemia observed, after 4 hours fasting, in patients with traumatisms who receive OKG, is more a reflection of a metabolic alteration in the use of said amino acid than a hypothetical slow use. In addition, the OKG induces an increase in insulinemia which causes hypoglycemia and probably a reduction in the plasma content of different amino acids (Cynober et al., 1984b).

Another study analysed the metabolism and kinetics of L-ornithine and the OKG metabolites after its administration to patients suffering from burns (35 men and 7 women). The patients wee distributed randomly and given oral doses of 10 g of L-ornithine (n = 13), doses of 10, 20 or 30 g/day by continuous gastric infusion (for 21 hours, n = 13) or an isonitrogenated control (n = 16). The OKG had a mean life of 89 minutes and was fully metabolised to produce L-glutamine, proline and L-arginine. The production of proline was dose-dependent and quantitatively similar in the different forms of OKG administration. The production of L-glutamine and L-arginine was not dose-dependent and was greater in the group that received the oral dose than in those groups that received it by infusion (Le Bricon et al., 1997).

5.14.5 Conclusion

The Scientific Committee considers that: 1) studies to assess the safety/toxicity of L-ornithine-alpha-ketoglutarate in humans have not been found; 2) the OKG is metabolised to L-glutamine, proline and L-ornithine; 3) no adverse effects have been mentioned on administering daily doses of 10 g of OKG to healthy individuals or to burn patients. Therefore, the Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, a maximum daily amount of 2,000 mg of L-ornithine-alpha-ketoglutarate is acceptable from the safety point of view for use as a food supplement.

Its use by pregnant women and children is not recommended. Nor should it be taken for prolonged periods of time without medical supervision.

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5.15 L-taurine

5.15.1 Proposal

Taurine is listed in the European Commission's Report on substances present in food supplements in the European Union (DG SANCO, 2008).

The AESAN has recommended a maximum daily amount of 1,000 mg of taurine. This proposal is based on the authorisation of this substance in Denmark with a maximum total amount that must not exceed 1,000 mg per recommended daily dose (Denmark, 2011), although in Italy the amount of taurine authorised in food supplements (legislative proposal) is 500 mg/day (Italy, 2012).

Regulation (EC) No 953/2009 (EU, 2009) includes taurine among the substances that may be added for specific nutritional purposes in foods for particular nutritional uses. Specifically, it may be added to dietary foods for particular nutritional purposes, including foods intended for special medical purposes, and excluding infant formulae, follow-on formulae, processed cereal-based foods and baby food for infants and young children.

Directive 2006/141/EC (EU, 2006) on infant formulae and follow-on formulae and its transposition in Spain to Royal Decree 867/2008 (BOE, 2008) permit, on establishing the basic composition of infant formulae when reconstituted according to the instructions of the manufacturer, the use of taurine in a concentration that must not exceed 2.9 mg/100 kJ (12 mg/100 kcal).

5.15.2 Characteristics and sources

Taurine (2-aminoethanesulfonic acid) is a free amino acid, one of the most abundant in the body, it is not incorporated in the proteins but nevertheless plays a decisive role in a number of important physiological functions such as bile acid conjugation, neurological and retina development, osmoregulation, modulation of cellular calcium levels and the immune function (Huxtable, 1992, 1996). Taurine is present in relatively high quantities in the retina, skeletal muscle and cardiac tissue. Taurine is synthesised endogenously in the liver from L-cysteine, through various enzymatic stages, and is therefore considered a non-essential or conditionally essential amino acid as in some cases endogenous production is insufficient and it is necessary to provide taurine in the diet. Breast milk and the proteins derived from meat and fish are sources of taurine in the diet (Rana and Sanders, 1986).

5.15.3 Nutrition and metabolism

In the last decade, a large number of therapeutic benefits have been proposed linked to the supplementation of taurine in diet, including diabetes treatment (Franconi et al., 2006), high blood pressure (Militante and Lombardini, 2002), heart failure (Sole and Jeejeebhoy, 2000), retina degeneration (Militante and Lombardini, 2004) and skeletal muscle disorders (Trip et al., 2006). Sports drinks contain taurine mainly due to its high concentration in muscular tissue and the role played by taurine in osmoregulation and the modulation of cellular calcium levels (Seidl et al., 2000).

Taurine is involved in a wide range of biological processes. The principal effects that have been investigated include: 1) its antioxidant activity, especially relevant at mitochondrial level; 2) anti-inflammatory effects; 3) effects on glucose homeostasis; and 4) its osmoregulatory action (Hansen, 2001).

It has been demonstrated that diabetes is linked to oxidative stress and to a fall in the levels of endogenous antioxidants, particularly taurine (Shaffer et al., 2009). Taurine acts as a powerful antioxidant, prevents the overload of intracellular calcium and restores the levels of antioxidant enzymes making the cells more resistant to toxic attacks (Nonaka et al., 2001) (Wu et al., 2005). Taurine acts as an antioxidant, not as a scavenger of reactive oxygen species (ROS) but by inhibiting the generation of these or interfering in their oxidant actions. Nevertheless there are exceptions; taurine is able to act as a scavenger of hypochlorous acid, an oxidant that activates the L-tyrosine kinase that leads to the formation of inflammatory mediators. Hypochlorous acid is produced by polymorphonuclear leukocytes and eosinophils and acts as a bactericide, but when produced in excess it causes oxidative stress. Park et al. (2003) revealed that the taurine-chloramine complex exercises anti-inflammatory activity, inhibiting the formation of nitric oxide and the tumour necrosis factor (TNF-α). In this context, it is important to note that type 1 diabetes is an inflammatory disease triggered by the destruction of pancreatic cells mediated by neutrophils.

Several studies have revealed that taurine is involved in glucose homeostasis through two mechanisms: a) due to its effect on the secretion of insulin, and b) by interfering with the signalling of insulin and other postreceptor steps (Franconi et al., 2004). Taurine has also been accorded nephroprotective properties probably due to a reduction in the activity of the NADPH oxidase renal enzyme. There is evidence that taurine reduces albuminuria and glomerulopathy, both present in diabetes; taurine may suppress the progression of diabetic nephropathy thanks to its antioxidant effects (Winiarska et al., 2009).

Experimental data and *in vitro* studies suggest that taurine as a food supplement might play a relevant role as a protector against oxidative stress and the development of atherosclerosis (Xu et al., 2008). In humans, taurine forms bile acid conjugates, mainly with cholic acid, resulting in taurocholate bile salt, the principal bile salt which extracts cholesterol from the plasma. Dietary supplementation of taurine leads to an improved lipid profile. Zhang et al. (2004a) show that in humans a dose of 3 g taurine/day, for 7 weeks, results in a significant reduction in triglyceride levels; Mizushima et al. (1996) demonstrated the reduction of LDL and LDL-cholesterol plasma levels in humans, treated for 3 weeks with 6 g/day of taurine.

It is known that hyperglycaemia is the principal factor in the development of endothelial dysfunction in diabetic patients. Endothelial dysfunction is a precursor to atherosclerosis. Taurine has a protective effect on endothelial dysfunction (Ulrich-Merzenich et al., 2007).

Taurine reduces blood pressure by interfering with the angiotensin II, which is the cause of vasoconstriction and an increase in blood pressure. Another mechanism that may also explain the hypotensive effect of taurine is due to inhibition of nitric oxide and prostaglandin E_2. Fuyita et al. (1987) demonstrated in a controlled study of hypertensive patients with a diet supplemented with 6 g taurine/day a significant decrease in the systolic and diastolic blood pressure.

5.15.4 Safety

It is necessary to highlight that the beneficial properties described above of taurine on health have mainly been observed in studies carried out on animal models and *in vitro*. Clinical studies in humans are limited, and therefore further investigation is necessary to define the efficiency and safety of the use of taurine as a food supplement. Nevertheless, taurine appears to be of particular use in the therapy of diabetic complications such as retinopathy, nephropathy and particularly in cardiovascular disorders.

There are more than 30 clinical trials on humans published, involving the oral administration of taurine. Of these, only 11 represent random studies (placebo-controlled) of safety in adult individuals, after oral intake for at

least one week. However, these studies exclude research into the acute effects, bioavailability and parenteral administration. In addition, the size of the sample, the dose and the duration, and the measurements of the potential effect vary among the studies. These clinical tests also involve healthy individuals and individuals with a wide range of diseases or health conditions. The highest oral dose used was 10 g/day for 6 months (Durelli et al., 1983). The longest-lasting study was for 12 months, with a dose range of 500-1,500 mg/day, in patients with cystic fibrosis (Colombo et al., 1996). Taurine levels in the body appear to be partly regulated by the kidneys (Chesney et al., 1985). Therefore, excess taurine in the diet is excreted in the urine. With the exception of the gastrointestinal disorders described in only one study (Jeejeebhoy et al., 2002), no adverse effects have been observed in any of the clinical studies revised. Although all the clinical studies were principally designed to assess the beneficial effects of taurine as a food supplement, the studies are short-term studies and the longest, as mentioned above, lasted 12 months.

The estimated consumption of taurine in food by adults in the United States is up to 400 mg/day (Rana and Sanders, 1986) (Laidlaw et al., 1990) (Hayes and Trautwein, 1994), suggesting that the doses used in the clinical tests published (up to 20 times greater than that typically consumed in a normal diet) are suitable for assessing the safety of the food supplement. The absence of adverse effects observed in relation to the oral administration of taurine supports the safety idea of this amino acid with confidence.

None of the clinical tests on humans revised showed adverse effects related to the oral administration of taurine. Therefore, by definition, there is no basis for identifying a NOAEL or LOAEL. In the absence of these two values, it is not generally possible to derive an UL (IoM, 1998). Consequently, for each clinical test the OSL method is applied, defined as the highest nutrient level observed with scientific evidence of safety for which toxicity has not been identified at any level (WHO, 2006). The value of the OSL identified in tests does not require correction for dietary intakes or endogenous synthesis (that is UF = 1.0) and may be identified as the SUL (Safe Upper Level for Supplements). The results are described below.

The most relevant published clinical tests on humans administered oral doses of taurine in the range of 500 mg/day to 10 g/day (Azuma et al., 1983, 1985) (Durelli et al., 1983) (Fuyita et al., 1987) (Colombo et al., 1996) (Mizushima et al., 1996) (Jeejeebhoy et al., 2002) (Sirdah et al., 2002) (Chauncey et al., 2003) (Brons et al., 2004) (Zhang et al., 2004a, 2004b) (Spohr et al., 2005). All the studies were double blind, random and controlled studies on healthy adults and on patients with different diseases, assessing the relevant clinical measurements or parameters.

The highest dose of taurine tested was 10 g/day (Durelli et al., 1983). In this study, patients with myotonic dystrophy were treated in a crossover study with 100-150 mg/kg/day (equivalent to 7-10 g/day in adults of 70 kg b.w.); the first dose was administered parenterally, followed by oral doses for 6 months. In this study, duplicated levels of taurine were observed and an increase of taurine in the urine. No adverse effects were described. Although this test was over a long period of time, given that the sample size is small (n = 18) and due to the lack of relevant clinical measurements, the authors did not consider the use of this study appropriate for the identification of an OSL.

In a series of two clinical studies conducted by Azuma et al. (1985, 1983) in 14 and in 58 patients with cardiac failure at doses of 6 g of taurine/day for 4 weeks, no adverse effects were observed; these studies were principally designed to assess the blood pressure and heart rate. Although no adverse effects were observed, the size of the sample and the short duration of the test again did not permit the use of this study in the identification of an OSL (Azuma et al., 1985, 1983).

Another series of clinical studies were performed with a lower dose, 3 g taurine/day for a period of between 12 days and up to 4 months. When the studies described by Colombo et al. (1996), Sirdah et al. (2002), Brons et

al. (2004), Zhang et al. (2004a, 2004b) and Spohr et al. (2005) are analysed collectively, the results (no changes were observed in the biochemical blood measurements or adverse effects) were considered adequate for selecting an OSL of 3 g taurine/day (Shao and Hathcock, 2008).

With respect to the toxicity data for animals, there are no acute, subchronic and chronic toxicity tests for taurine after oral administration, therefore only the published clinical tests on humans have been analysed.

To conclude, an OSL of 3 g taurine/day has been identified from the data on individuals who consume a large variety of diets, apart from the endogenous synthesis of taurine itself. The additional source of taurine supplied in the diet does not need to be extracted from the OSL to identify an ULS. Therefore, an ULS based on toxicity data from clinical trials on humans is also 3 g taurine/day, with a UF of 1.0 (Shao and Hathcock, 2008).

Summary of the results of the risk analysis for the amino acid, taurine

- NOAEL or LOAEL in humans: > 10 g/day.
- OSL: 3 g/day.
- Estimated intake through dietary sources: 40-400 mg/day.
- ULS: 3 g/day.

5.15.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AESAN proposal of a maximum daily amount of 1,000 mg of taurine is acceptable from the safety point of view for use as a food supplement.

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5.16 L-tyrosine + L-phenylalanine

5.16.1 Proposal

The AESAN has proposed a maximum daily amount for the sum of L-tyrosine and L-phenylalanine of 1,900 mg. This proposal is based on the protein reference intake recommended by the WHO for the adult population (WHO, 2007).

In Italy, a maximum daily amount of 1,330 mg of L-tyrosine + L-phenylalanine is authorised in food supplements (legislative proposal) (Italy, 2012).

5.16.2 Characteristics and sources

L-phenylalanine is an aromatic alpha amino acid with a benzene ring. The chemical formula is $C_9H_{11}NO_2$. It is also known as 2-amino-3-phenyl-propanoic acid. It is a nonpolar, hydrophobic amino acid with a neutral electric charge.

L-tyrosine is also known as 4-hydroxyphenylalanine or L-2-Amino-3-(4-hydroxyphenyl) propanoic acid. The chemical formula is $C_9H_{11}NO_3$. It is also an aromatic amino acid but with a polar group.

Food sources of L-phenylalanine and L-tyrosine are usually protein foods (meat, eggs, fish, dairy products and pulses). L-phenylalanine is also found in the sweetener, aspartame.

The EFSA has published a scientific opinion regarding the verification of health claims relating to L-phenylalanine: i) improves alertness; ii) improves mood; iii) improves memory; iv) analgesic; v) assists spermatogenesis; and vi) improves circulating levels of free fatty acids during pregnancy. Based on the information presented, the EFSA Panel has not found it possible to establish a cause and effect relation in any of the cases (EFSA, 2010). In addition, the EFSA has published a scientific opinion relating to the verification of health claims relating to the claim that L-tyrosine contributes to the normal synthesis of dopamine. Based on the information presented, the NDA Panel of the EFSA concludes that L-tyrosine does contribute to the synthesis of dopamine (EFSA, 2011a). In addition, there is another scientific opinion regarding the verification of health claims relating to L-tyrosine: i) the normal synthesis of catecholamines; ii) improved attention; and iii) contribution to muscular contraction. Based on the information presented, the NDA Panel of the EFSA has only found it possible to establish a cause and effect relation for the first of the claims (EFSA, 2011b).

5.16.3 Nutrition and metabolism

L-phenylalanine is an essential amino acid that is catabolised to L-tyrosine through the enzyme L-phenylalanine hydroxylase and with the participation, as cofactor, of tetrahydrobiopterin. The catabolism of L-tyrosine leads to fumarate and ketoacetate through transamination reactions. Therefore, both amino acids are considered as glucogenic and ketogenic.

Furthermore, L-tyrosine is an intermediary in the biosynthesis of dopamine, epinephrine and norepinephrine. The first step of the biosynthetic route of the catecholamines from L-tyrosine is catalysed by tyrosine hydroxylase. In addition, L-tyrosine intervenes in the synthesis of melanin. Lastly, both amino acids are proteinogenic.

Studies of nitrogen balance and oxidation with aromatic amino acid markers indicate that the daily requirements of both are 25 mg/kg b.w. and for children they range between 25 and 90 mg/kg b.w. (WHO, 2007).

At present it is not possible to establish requirements for each of the amino acids separately. No data are available for the estimated intakes of aromatic amino acids in the adult or infant population.

5.16.4 Safety

A significant number of studies carried out prior to the eighties concluded that increases in the intake of L-phenylalanine (diets enriched with 3-7 % L-phenylalanine) imply an increase in the circulating levels of L-tyrosine. Therefore, the toxic effects of L-phenylalanine are linked to those of L-tyrosine (Harper et al*.,* 1970) (Benevenga and Steele, 1984).

The administration of L-phenylalanine, in doses of between 0.3-4 g/kg b.w./day, for 2-4 weeks, to recently weaned rats induces a: i) reduction in body weight; ii) a reduction in the weight of the brain; iii) cerebral demyelination, partially affecting the cerebellum; and iv) a modification in the lipid profile of the myelin (Prensky et al., 1974) (Shah and Johnson, 1978).

In recently weaned rats, supplementation with L-tyrosine (3-5 %) for 2 weeks produced a reduction in growth of up to 30 %, the appearance of cataracts and skin injuries (Boctor and Harper, 1968) (Goldsmith, 1975). In addition, studies on the acute administration of L-tyrosine in rodents (up to 5 g/kg b.w.) and chronic administration (28 days, doses of up to 50 mg/kg b.w./day) in rodents, dogs and pigs, did not give any signs of toxicity (Baldrick et al., 2002). It should be noted that in these studies, L-tyrosine was administered by intramuscular or subcutaneous routes. In addition, genotoxicity studies (up to 1 g/cell culture plate of *Salmonella typhimurium*) have concluded the absence of genotoxic effects of L-tyrosine (Baldrick et al., 2002).

Studies in humans have demonstrated that L-tyrosine is safe in doses up to 150 mg/kg b.w./day (Van Spronsen et al., 2001), producing in only some people minor secondary effects (nausea, diarrhoeas, headaches or insomnia). In addition, it has been observed that in patients with hyperthyroidism, the intake of L-tyrosine may increase the plasma levels of the thyroid hormone (Van Spronsen et al., 2001). No studies are available on the intake of L-tyrosine for pregnant women.

5.16.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AESAN proposal of a maximum daily amount of 1,900 mg for the sum of L-tyrosine and L-phenylalanine is acceptable from the safety point of view for use as a food supplement.

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5.17 L-threonine

5.17.1 Proposal

The AESAN has recommended the inclusion of L-threonine in Royal Decree 1487/2009 with a maximum daily amount of 1,150 mg. This proposal is based on the protein reference intake recommended by the WHO for the adult population (WHO, 2007).

The proposal is based on the fact that Regulation (EC) No 953/2009 (EU, 2009) includes L-threonine among the substances that may be added for specific nutritional purposes in foods for particular nutritional uses.

In Belgium, L-threonine is authorised in food supplements without the establishment of a maximum daily amount (Belgium, 1992).

In Italy, a maximum daily amount of 630 mg of L-threonine is authorised in food supplements (legislative proposal) (Italy, 2012).

5.17.2 Characteristics and sources

L-threonine (Thr) (alpha-amino-beta-hydroxybutyric acid) is a polar amino acid with a lateral chain that contains a hydroxyl group. The nutritional requirement of L-threonine is especially important, as it has been insinuated that, after the sulphur amino acids, it is the second limiting amino acid of the maintenance requirements, probably because it is considered as the most important individual component in ileal loss in the large intestine. It is present in small quantities in cereal proteins (WHO, 2007).

L-threonine is a food additive, the direct addition of which is authorised by the United States FDA, provided that: 1) the additional quantity does not exceed that which is reasonably required to obtain the physical, nutritional or other technological effects in foods; and 2) it is food grade and is treated as a food ingredient.

5.17.3 Nutrition and metabolism

L-threonine is an essential amino acid that the body uses in tissue protein synthesis in the production of mucin by the intestinal tract enterocytes. In mammals it makes a significant contribution to the synthesis of collagen and elastin and to the formation of tooth enamel. It is a precursor to glycine.

L-threonine, as with L-lysine, does not take part in transamination reactions. Glycine, serine and L-threonine are metabolically inter-related, such that the metabolism of L-threonine is closely linked to glycine and serine.

In mammals, L-threonine has two catabolic routes, it can be catabolised in the cytosol as a result of the action of L-threonine dehydratase (TDH) to NH_4^+ and 2-ketobutyrate which is quickly and irreversibly transformed to CO_2 or it can be metabolised in the mitochondria by L-threonine dehydrogenase (TDG) to form 2-amino-3-ketobutyrate that is decomposed by the action of the 2-amino-ketobutyrate CoA ligase to produce glycine and acetyl-CoA (Dale, 1978) (Bird and Nunn, 1983).

Recommended dietary intakes for adults (over 19 years old) of L-threonine (IoM, 2005) are: AR: 16 mg/kg b.w./day; RDA: 20 mg/kg b.w./day (IoM, 2005).

The requirements (WHO, 2007) for adults are: 15 mg/kg b.w./day.

Based on the distribution of the NHANES III data (1988-1994), the mean daily intake of L-threonine from food and food supplements for all age groups, at all stages of life, in men and women, is 3.0 g/day. The highest intakes correspond to the 99th percentile of men (aged 51 to 70 years old) and the value is 7.1 g/day (IoM, 2005).

5.17.4 Safety

Adverse effects in animals

In rats fed with a diet that contains 19 % of casein and 5 % of additional L-threonine, a reduction was observed in the increase in weight, with respect to the controls who received the same diet without the addition of L-threonine, but no changes were observed in the weight of the liver or in the hepatic DNA, RNA or protein content (Muramatsu et al., 1971). The available data indicate that L-threonine in excess is transformed into carbohydrates, lipids in the liver and CO_2 (Yamashita and Ashida, 1971).

In weaned piglets, the addition of 0.5, 1, 2 or 4 % of L-threonine to a diet containing 20 % of gross protein does not have an effect, if compared with the controls, on the weight increase, the intake of food or the proportional weight increase: animal feed (Edmonds and Baker, 1987) (Edmonds et al., 1987).

Adverse effects in humans

No information has been found relating to apparently healthy human beings who consume food supplements with L-threonine. However L-threonine has been used clinically to increase the glycine concentrations in the cerebrospinal fluid of patients with spasticity, without observing adverse clinical effects when doses of 4.5 to 6.0 g/day are administered for 14 days (Growdon et al., 1991).

Individuals who received up to 22.5 g of L-threonine intravenously complained of headaches and backaches (Floyd et al., 1966).

In premature children, when the intake of formula milk is increased, the serum concentrations of L-threonine are also increased, particularly in those children who were given whey-based formula (which is especially rich in L-threonine), without observing any adverse effects (Jarvenpaa et al., 1982).

The L-threonine in low birth weight infants has also been studied and the serum concentrations were found to be directly related to the L-threonine content of the formula milk (Rigo and Senterre, 1980). The authors indicated that, in premature children, L-threonine intakes should not exceed 140 mg/kg b.w./day.

Evaluation of the dose-response

There is no available data relating to the adverse effects of the intake of L-threonine from food supplements for a dose-response assessment and the extrapolation of an UL in apparently healthy humans.

5.17.5 Conclusion

Based on the information available to date, and considering that the AESAN proposal of a maximum daily amount of 1,150 mg is in line with the L-threonine requirement established by the WHO, the Scientific Committee concludes that the proposed amount is acceptable from the safety viewpoint for use as a food supplement.

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5.18 L-tryptophan (obtained by protein hydrolysis)

5.18.1 Proposal

The AESAN has proposed a maximum daily amount of L-tryptophan obtained by protein hydrolysis of 300 mg, with the warning that it is not recommended in individuals receiving treatment with antidepressants. This proposal is based on the protein reference intake recommended by the WHO for the adult population (WHO, 2007).

In France, following the conclusions of the Committee on Toxicity of the Food Standards Agency (FSA) of the United Kingdom, a quantity of L-tryptophan of 220 mg/day has been favourably assessed in food supplements, but its intake by individuals receiving treatment with antidepressants is not recommended (AFSSA, 2008).

In Italy, a maximum daily amount of 350 mg is authorised in food supplements (legislative proposal) (Italy, 2012).

5.18.2 Characteristics and sources

Tryptophan is also known as 2-amino-3-(1H-indol-3-yl) propionic acid. The chemical formula is $C_{11}H_{12}N_2O_2$. It is an amino acid which contains an indole functional group.

Tryptophan is usually found in protein food. These include dairy products, meat, eggs, fish, poultry, soybean and some nuts and seeds.

The EFSA has published a scientific opinion regarding the verification of health claims relating to L-tryptophan: i) improves sleep; ii) improves mood; iii) increases the cognitive function; and iv) contributes to maintaining a suitable body weight. Based on the information presented, the NDA Panel of the EFSA has not found it possible to establish a cause and effect relation in any of the cases (EFSA, 2011).

5.18.3 Nutrition and metabolism

Tryptophan is an essential amino acid that intervenes in protein synthesis and is the biochemical precursor of serotonin, melatonin, niacin and the coenzymes, NAD and NADP.

Tryptophan is metabolised through the kynurenine-niacin pathway. This catabolic process starts in the liver.

In a healthy adult that follows the dietary recommendations for the intake of proteins, the intake of L-tryptophan ranges between 600 and 1,200 mg per day (Brown, 1994). At present, the daily recommendations of L-tryptophan are 4 mg/kg b.w. (WHO, 2007). These recommendations are based on the studies of oxidation of L-tryptophan in well-nourished people (Lazaris-Brunner et al., 1998) and on the analysis of the plasma response curve to the amino acid (Young et al., 1971).

5.18.4 Safety

Studies were carried out on the mutagenicity of L-tryptophan on five different strains of *Salmonella typhimurium* and on cell lines of mice, hamsters and humans. No mutagenic effects were observed in any of these. Moreover, the compounds derived from the metabolism of L-tryptophan did not produce any effects. This leads us to reject the possible mutagenic effect of the metabolites formed by intestinal bacteria (Herbst, 1994).

Acute toxicity studies established, in mice, rats and rabbits, a LD_{50} of between 2-16 g/kg b.w., when L-tryptophan was administered orally, intraperitoneally or intravenously (Herbst, 1994). These studies allow us to conclude that the acute toxicity of L-tryptophan is low.

As regards subchronic toxicity studies, intraperitoneal administration to rats of L-tryptophan in doses of 0.5, 1.0 and 2.0 g/kg b.w./day for 30 days caused, in the highest doses, hepatic damage, loss of appetite, weight loss, reduction in motor activity and eventually the death of the animals (Herbst, 1994).

The chronic toxicity studies are contradictory in the sense that some studies highlight the capacity of L-tryptophan for favouring the appearance of hepatic tumours (Herbst, 1994). Nevertheless, these studies used extremely high doses of L-tryptophan and tumorigenesis induction agents. In addition, there are numerous studies that indicate that L-tryptophan in high oral doses and for long periods of time does not have any carcinogenic potential (Herbst, 1994). These studies include a test conducted by the National Cancer Institute in the United States (NCI, 1978), on mice (n = 35) and rats (n = 35). In this study, the animals were given L-tryptophan at 2.5 and 5 % in drinking water (equivalent to 2.5-5 mg/kg b.w.), five times a week for 78 weeks. A tumorogenic effect or enhancer of tumour induction was not observed in any case.

Research on rats, given L-tryptophan at 1 % in drinking water, for seven generations, did not have any effect on reproduction or embryonic development (Herbst, 1994). However, in the golden hamster, a species of rodent that does not have the hepatic enzyme, apo-tryptophan oxidase, equal doses of L-tryptophan in water did produce lower weight and size babies with a higher perinatal mortality rate (Pevet et al., 1981).

With respect to humans, it should be noted that there are almost no studies on toxicity as such, and therefore conclusions can only be extracted from clinical trials which use L-tryptophan. Doses of up to 10-15 g/day were used in these clinical trials for long periods of time. Nevertheless, the authors indicate that doses of 1-5 g/day (up to 70 mg/kg b.w./day), for log periods of time, are well-tolerated and do not involve any risk (Kimura et al., 2012). Lastly, it was confirmed that one of the enzymes responsible for the hepatic metabolism of L-tryptophan (tryptophan-2,3-dioxygenase) did not work correctly in pregnant women, neonates, lactating infants and patients with adrenal insufficiency (Herbst, 1994).

5.18.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AESAN proposal of a maximum daily amount of 300 mg of L-tryptophan is acceptable from the safety point of view for use as a food supplement.

It must not be consumed by pregnant women, or those individuals receiving treatment with antidepressants or who suffer from kidney failure.

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5.19 L-valine

5.19.1 Proposal

The AESAN has recommended a maximum daily amount of 1,950 mg of L-valine. This proposal is based on the protein reference intake recommended by the WHO for the adult population (WHO, 2007).

In Italy, a maximum daily amount of 910 mg of L-valine is authorised in food supplements (legislative proposal) (Italy, 2012) and in Belgium L-valine is authorised in food supplements without establishing a maximum daily amount (Belgium, 1992).

5.19.2 Characteristics and sources

L-valine is an alpha-amino acid that contains branched aliphatic moiety. Therefore it is a branched-chain amino acid. It is a proteinogenic amino acid. The chemical formula is $HO_2CCH(NH_2)CH(CH_3)_2$. It is classified in the nonpolar amino acids and is hydrophobic.

The most important food sources are cheese, fish, poultry, peanuts, sesame seeds and lentils.

5.19.3 Nutrition and metabolism

As it is an essential amino acid it cannot be synthesised by the body. The catabolisation takes place preferably in the muscle and the first two stages of its degradation are the same as for other branched-chain amino acids. Firstly, there is a transamination followed by oxidative decarboxylation of the resultant ketoacids. The degradation of the acyl-CoA results in succinyl-CoA.

This is an amino acid that intervenes in protein synthesis and glucose synthesis. It has also been observed that it can take part in the binding and recognition of hydrophobic ligands such as the lipids (Betts and Russell, 2003).

In accordance with the joint technical report issued by the FAO/WHO/UNU, the requirements for L-valine in adults are 26 mg/kg b.w./day. For lactating infants, the value is 49 mg/kg b.w./day and for children and adolescents the value is 29 mg/kg b.w./day (WHO, 2007). There is no available data on the usual intake of L-valine in the western population.

5.19.4 Safety

Few studies are available on the toxicity of L-valine. In fact, there are few studies on the acute effects of the intake of high doses of L-valine. In the majority of studies, L-valine is administered together with other branched-chain amino acids (L-leucine and L-isoleucine). As the joint administration of these amino acids has antagonistic effects, it is very difficult to extrapolate the results of these studies for the case of L-valine. In a study carried out on rats, L-valine was administered in drinking water in concentrations of 1.25-5 % for 13 weeks. This study did not find any harmful effects on health. The authors indicate that the NOAEL for L-valine is 2-3 g/kg b.w./day (Tsubuku et al., 2004).

In the case of humans, there are no studies of the administration of L-valine separately and it is not clear whether the study conducted by Tsubuku et al. (2004) on growing rats can be extrapolated to adult humans. As it has been observed that the excessive intake of branched-chain amino acids may reduce cerebral levels of L-

tryptophan, L-phenylalanine, L-tyrosine, noradrenaline, dopamine and serotonin (Baker, 2005), an excess of L-valine may have a psychological effect and an effect on behaviour. Nevertheless, the establishment of specific studies in humans is required.

5.19.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AESAN proposal of a maximum amount of 1,950 mg/day of L-valine is acceptable from the safety point of view for use as a food supplement.

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6. Dipeptides and peptides

6.1 Glutathione

6.1.1 Proposal

The AESAN has recommended a maximum daily amount of 50 mg of glutathione. This proposal is based on the favourable assessment of this substance in France (AFSSA, 2008) and on the authorisation in Italy in food supplements (legislative proposal) of a maximum daily amount, in both cases, of 50 mg (Italy, 2012).

6.1.2 Characteristics and sources

Glutathione is a tripeptide (γ-glutamyl-cysteinyl-glycine) ubiquitously present in all human tissues and those of other animals.

Glutathione is naturally found in several food of animal and plant origin in variable contents, ranging from 10 to 100 µg/g in fruit and vegetables, and up to 500 µg/g in meat products. In addition, glutathione, in its purified form, is marketed in some countries as a food supplement, normally in capsule, powder or tablet form, in doses ranging from between 50 to 600 mg per day (PDRHealth, 2006).

In spite of the presence of glutathione in numerous foods, there is no reliable data on its normal consumption. Estimations of dietary intake may vary considerably due to the differences in the glutathione content in foods and to the variability in the frequency of intake. Wierzbicka et al. (1989) indicated that the estimated food intake of glutathione in the United States ranged between 2.9 and 131 mg/day, whereas Flagg et al. (1994) estimated daily intakes for the same population between 13 and 110 mg/day. Nor is there any reliable data on the intake of glutathione in Europe.

6.1.3 Nutrition and metabolism

Glutathione is synthesised from its precursor amino acids, glutamate, L-cysteine and glycine, in two stages. Although all the tissues have the necessary enzymes for this synthesis, the liver is the main organ in which it is produced. In healthy tissues and cells, more than 90 % of the total glutathione is in reduced form (GSH) and the rest in the disulfide form (GSSG). An increase in the GSSG/GSH ratio is considered a sign of oxidative stress.

In the body, glutathione is principally found in its reduced form (GSH) and plays a major antioxidant and cellular protection function. GSH is oxidised nonenzymatically to GSSG (oxidised form) and may react with electrophilic substances, including the reactive species of oxygen and nitrogen and the free radicals. In normal conditions, cellular levels of GSH remain constant due to the regulation of the enzyme GSH reductase. However, in conditions of oxidative stress there may be a build-up of GSSG, which is secreted to the exterior of the cell, where it is degraded, resulting in the loss of intracellular GSH. It has been indicated that the exogenous intake of GSH, orally, may be useful for increasing the plasma and tissue concentrations of this compound (Favilli et al., 1997).

In human beings, a reduced concentration of glutathione and/or a disproportionate GSH/GSSG ratio have been linked to diseases such as cancer, hepatitis, type 2 diabetes, Parkinson's and cystic fibrosis. Although there is currently no clear scientific evidence that supplements of glutathione may help to prevent these diseases, some observational studies suggest that a higher dietary intake of glutathione is associated with a lower risk of cancer. The beneficial effects of glutathione against these diseases would be based on its antioxidant capacity.

The glutathione present in food is generally found in its reduced form (GSH), which is the form in which it can be absorbed in the small intestine (Hagen and Jones, 1989), although it may also be found in its oxidised state (GSSG) with a very low bioavailability at intestinal level (Wierzbicka et al., 1989) (Hagen et al., 1990a) (Jones et al., 1992). Nevertheless, the small intestine seems to have a reduction mechanism able to reduce GSSG to GSH (Hagen et al., 1990a), thus potentially increasing the absorption of GSSG from food sources.

The question of the intestinal absorption of glutathione is, however, controversial. Studies that involve the oral administration of GSH to laboratory animals indicate that this is absorbed in the gastrointestinal tract intact, observing an increase in plasma and tissue content (Hagen et al., 1990b). Specifically, the inclusion of GSH in the diet of rats has been observed to increase their plasma levels (the intake of 9 mg doubles the plasma concentration) and tissue levels (jejunum, lung, heart, liver and brain). In rats, GSH is mainly absorbed in the upper jejunum through a sodium-dependent uptake system (Hunjan and Evered, 1985) (Hagen et al., 1990a). The circulating GSH is metabolised and mainly eliminated through the kidneys (Hahn et al., 1978).

In human beings the absorption of glutathione in the diet has not been definitely demonstrated; there are few studies in this respect and the results are contradictory. Some studies indicate that glutathione absorption is anecdotic and that its plasma and tissue levels are regulated by the liver and red blood cells. Thus, Witschi et al. (1992) did not observe variations in the plasma content of glutathione after the administration of a single oral dose of 3 g to healthy volunteers. To the contrary, Hagen and Jones (1989) indicated an increase in the plasma content of GSH in four of the five volunteers who orally received 15 mg of GSH/kg b.w. In this case, the plasma content of GSH increased 300 % with respect to the basal level one hour after its administration, and after 3 hours it fell to approximately 200 % of its basal value. Similarly they indicated that the administration to human beings of GSH constituent amino acids did not produce the same increase in plasma levels of GSH, demonstrating that this peptide is absorbed intact.

6.1.4 Safety

The safety assessment for glutathione is based on toxicological and clinical studies, the results of which did not indicate adverse effects linked to its intake, and also on its long history of safe consumption and due to its endogenous presence in the biological systems and its important role in cellular detoxification.

Glutathione toxicity studies, principally acute and chronic, conducted on animals (Nozaki et al., 1972) (Suzuki et al., 1972) (Brown et al., 1996) (Sugimura and Yamamoto, 1998), and clinical studies in humans (KOHJIN, 2008) conclude the safety of this substance. In the chronic toxicity study of GSH in dogs conducted by Suzuki et al. (1972) a NOAEL was established at 300 mg/kg b.w./day.

Clinical studies on the efficiency of treatment with glutathione do not describe any adverse effects attributable to the treatment, thus supporting the safety of this peptide (Murao et al., 1974) (Dalhoff, 1992) (Allen and Bradley, 2011).

Since 2008 in the United States, glutathione has been considered a Generally Recognised As Safe (GRAS) substance for use as a food ingredient (KOHJIN, 2008).

Given the ubiquitous nature of the enzymes involved in both the synthesis and the metabolism and transport of the glutathione, it would seem important to also consider the safety of the amino acids in this peptide to assess the safety of glutathione in oral doses. For glutamate and L-cysteine, please see the section corresponding to their safety in this report.

Glycine

The intravenous administration of a dose of 3 g/kg of glycine to mice led to the death of 70 % of the animals. However, with an oral dose, after a supplementation with glycine of 1 and 5 g/kg to mice for 3 months a significant reduction was only observed in the expression levels of the N-type calcium channels of the parietal cortex (Shoham et al., 2001). This effect reflects a functional adaptation to the increase in the circulating glycine concentration and is not considered as evidence of neurotoxicity for glycine administered orally.

With respect to the clinical studies in humans, Gannon et al. (2002) showed that the acute oral administration of 1 mmol of glycine/kg b.w. (approximately 4.5 g) has a favourable effect on the glycemic control, without causing any type of secondary effect. Similar results are also available from various long-term studies in patients with schizophrenia (Rosse et al., 1989) (Javitt et al., 1994) (Heresco-Levy et al., 1999) (Potkin et al., 1999) (Javitt et al., 2001) (Heresco-Levy et al., 2004). Nevertheless, in the study conducted by Heresco-Levy et al. (2004), two of the seven patients in the study who received doses of 0.8 g of glycine/kg b.w. (from 40 to 60 g per day according to the patient) abandoned the treatment due to the appearance of nausea and digestive disturbances.

The dietary intake of glycine in the population of Western countries is approximately 3.2 g per day. The AFSSA considers that a total daily intake of 4.5 g per day is absolutely safe (AFSSA 2008b).

With the data available to date, the AFSSA considers that there is insufficient reason for glutathione supplementation in healthy humans with a varied and balanced diet and a calorie intake adequate for their requirements. However, it also indicates that there is no scientific basis for opposing glutathione supplementation at a dose of 50 mg/day (AFSSA, 2008a).

6.1.5 Conclusion

The Scientific Committee considers that the toxicological assessments made of glutathione have not revealed any risks for the health of consumers. Similarly, clinical studies carried out to assess the efficiency of glutathione in different pathological situations have not revealed any secondary adverse effects which may be attributed to this peptide.

In view of the above, the Scientific Committee concludes that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum daily amount of 50 mg of glutathione is acceptable from the safety point of view for use as a food supplement.

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6.2 Lactoferrin

6.2.1 Proposal

The AESAN proposes a maximum daily amount of 200 mg for lactoferrin. This proposal is based on the Italian legislative proposal which authorises a maximum daily amount of 200 mg in food supplements (Italy, 2012) and on the assessment made by the French Agency (AFSSA, 2008).

6.2.2 Characteristics and sources

Bovine lactoferrin (bLF) is a component of the protein fraction of cow's milk. It is a glycoprotein of some 77 kDa. It has a single polypeptide chain of 689 amino acids, without free sulfhydryl groups but with intramolecular disulfide bonds. It is glycosylated in two different places by N-glycans of the N-acetyllactosamine type. These glycans are characterised for containing traces of galactose alpha-1.3- bonded to the non-reducing terminal position. Unlike human lactoferrin, bLF also contains oligomannosidic glycans. The tertiary structure of lactoferrin has two bonding points that allow it to attach two Fe (III) irons per protein molecule. The sequences of human and bovine lactoferrin exhibit a homology of around 70 % (EFSA, 2012).

bLF has a minimum protein content of 93 %, of which more than 95 % corresponds to lactoferrin. It may contain small quantities of other milk proteins, principally casein, alpha-lactoglobulin and beta-lactoglobulin. It usually contains 120 mg of iron/kg, at maximum 4.5 % water and 1 % ashes. The total content of heavy metals (cadmium, lead, arsenic, mercury, copper) does not exceed 1 mg/kg (EFSA, 2012). The AFSSA agrees with the Dutch Committee that the information relating to the specification of bovine lactoferrin permits its characterisation (AFSSA, 2008).

bLF is stable at ambient temperature as indicated by the results obtained in stability trials lasting up to 6 years, maintaining its chemical characteristics (protein content, dry and mineral material, pH and iron binding capacity) and microbiological characteristics (bacterial count). However, heat treatment of bLF may modify its native state (molecular structure in space) and the related properties. A number of parameters affect the degree of denaturation (heat treatment intensity, pH and iron-saturation degree). Data is available for the stability of bLF added to milk derivatives, infant and follow-on formulae, etc.

The Dutch Committee and the AFSSA indicate that the relative claim that heat treatments do not denature bLF is not substantiated. Therefore, a study shows that pasteurization of milk may induce a modification of the tertiary structure of the bLF and consequently the related properties (Schwarcz et al., 2008). However, the AFSSA questions the nutritional equivalence between native bLF and more or less denatured lactoferrin, whereas the Dutch Committee considers that the denatured product is the same as the native product, and therefore in its assessment they consider that all lactoferrin is in native form.

6.2.3 Nutrition and metabolism

Humans have consumed bovine lactoferrin for centuries in the form of cow's milk, which contains between 20 and 200 mg/l, with an average of 100 mg lactoferrin/l of milk (King et al., 2007). In the Netherlands the intake (P90) of lactoferrin through dairy products is estimated at 73, 75 and 50 mg/day in children, young people and adults, respectively. Based on the consumption data for milk and milk derivatives, it was concluded that the Dutch (over the age of one year) consume 40 mg of lactoferrin per day. In Scandinavia this figure is higher, but

there is little information for the Southern European countries. In the United States, the consumption of bLF in food supplements ranges between 10 and 1,200 mg/day (EFSA, 2012).

According to the AFSSA, the intake data for adults in France indicates a mean intake of native bLF of between 20 and 50 mg/day, depending on the type of milk derivatives consumed. In addition, it indicates that approximately 5.3 mg of this daily intake comes from raw milk and cheese made with this milk (AFSSA, 2008).

According to the NDA Panel of the EFSA the relevance of data for the intake of bLF in food (milk and its derivatives) is limited, as the majority of these have been subjected to heat treatment and only a small fraction remain as native proteins, whereas bLF is used in native form as a food ingredient or supplement.

At present, in the United States and in the European Union, bLF is authorised and marketed as a food supplement and as an ingredient for food for sports people at a concentration of 100 mg per portion of the product. bLF received the GRAS status in 2001, indicating that on the labelling it must be declared as lactoferrin from milk (FDA, 2001). In 2008, the AFSSA issued a ruling on the assessment of the initial report of the Dutch authorities relating to the introduction in the market of bLF based on the substantial equivalence with lactoferrin from cow's milk (AFSSA, 2008).

In human beings, and due to the progressive development of the intestinal function, digestion of bLF depends on age (AFSSA, 2010). In newborns, proteolysis of bLF is relatively slow and incomplete, therefore it can be found in infant stools. The non-degraded lactoferrin from breast milk can be absorbed and enters the systemic circulation, but it has not been demonstrated that this is the case with bLF, although it has been seen to occur in adult mice with a complete intestinal function (Fischer, 2007).

There is no reason to suppose that the digestion of lactoferrin is different to that of other proteins in the diet, although it appears that bLF is relatively resistant to the proteolytic enzymes of the intestinal tract. In any case, there is not enough information regarding the intestinal absorption of bLF in humans and in particular in adults. It has been indicated that the resistance of bLF to proteolysis may favour the absorption of the more or less intact molecule and even its action in the digestive tract (AFSSA, 2008).

6.2.4 Safety

In *in vitro* genotoxicity tests with bacteria (Ames test) conducted on four strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium* and one of *Escherichia coli* (WP2uvrA), with and without metabolic activation, no mutagenic activity was detected at the highest tested dose of 5 mg bLF/plate (Yamauchi et al., 2000a). The results of these tests therefore permit the exclusion of any mutagenicity due to bLF and consequently there do not appear to be any problems of safety as regards the genotoxicity.

With respect to the allergenicity, the EFSA Panel considers that the risk of allergic reactions is no different to that of other milk derivatives coming from cattle sources (EFSA, 2012).

For studies in animals, the NDA Panel of the EFSA considers that rats are a suitable species for assessing the safety of bLF in humans as its absorption at intestinal level has been demonstrated (Kitagawa et al., 2003). bLF administered orally was detected by ELISA in the liver, kidneys, gall bladder, spleen and brain of adult mice (Fischer et al., 2007).

Acute toxicity: in one study (4 weeks) an oral dose of 0, 200, 600 or 2,000 mg bLF/kg b.w./day was administered using a stomach tube to adult *Sprague-Dawley* rats. No adverse effects were observed to the highest dose administered, and therefore according to this test, the NOAEL is 2,000 mg/kg b.w. (Nishimura, 1998).

Subchronic toxicity: in a study lasting 13 weeks, *Sprague-Dawley* adult rats received an oral dose of 0, 200, 600 or 2,000 mg bLF/kg b.w./day using a stomach tube once a day in groups of 12 male rats and 12 female rats (n= 12/gender/dose) (Nishimura, 1998) (Yamauchi et al., 2000b). No relevant adverse effects were observed in any of the groups. In the intake, weight, blood, biochemical and urine tests no statistically relevant differences were observed between the groups and the control group, nor were there any differences in the ophthalmological examinations. The determination of the weight of the selected organs and microscopic examinations during the necropsy did not reveal any toxic effects either. Exceptionally, in male rats, fibrosis was observed in the islet cells of the pancreas, with a slightly higher incident rate and degree of severity in the treated groups than in the control groups. Fibrosis of the pancreas islet cells is an injury that occurs with a relatively high frequency as a phenomenon that accompanies the ageing of this type of rat. Nevertheless in the histopathological examinations, the non-existence of morphological differences between the control group and the treated group with respect to fibrosis of the islet cells, allows the authors to conclude that this alteration cannot directly be attributed to the administration of bLF and therefore concludes that the highest dose administered (2,000 mg/kg b.w./day) may be considered as the NOAEL.

The available toxicity data is taken from a dossier relating to the application for GRAS status without details of the test protocols used. Moreover, it does not specify whether Good Laboratory Practices were applied in the studies or not. Nevertheless, the AFSSA considers the quality level of the 13 week study on rats to be acceptable (AFSSA, 2010).

Taking into account the available data, the AFSSA considers that it is not conclusive as regards the existence or non-existence of a causal relation between the intake of bLF and the appearance of fibrosis in the pancreatic islet cells of the rat.

The scientific opinion of the EFSA (2012) indicates that 11 studies on children do not mention adverse effects related to bLF, although in 10 of these, body weight and size are the only relevant final points linked to safety that are not affected by bLF. Given the limitations of the studies, its importance is considered relative.

There have been at least 15 studies on adult patients to assess the efficiency of bLF on diverse pathologies. In one of these on post-operation adult patients, the effect of a dose of 20 mg bLF/day for 5 days was assessed with respect to the immune system (Zimecki et al., 2001); in other studies the efficiency of intakes of bLF of between 400 and 7,200 mg/day for 12 months was evaluated in patients with chronic hepatitis C (Tanaka et al., 1999) (Okada et al., 2002) (Ishii et al., 2003) (Ueno et al., 2006) (Kaito et al., 2007); in other cases it was assessed in patients infected with *Helicobacter pylori* (Di Mario et al., 2003, 2006) (Okuda et al., 2005) (Zullo et al., 2005, 2007) (De Bortoli et al., 2007) (Tursi et al., 2007), and in patients with Sjorgen syndrome (Dogru et al., 2007) or with *tineapedis* (Yamauchi et al., 2000b). The authors of five of these studies indicate the absence of statistically significant differences between the treated group and control group (De Bortoli et al., 2007) (Kaito et al., 2007) and that none of the adverse effects observed was related to the intake of bLF (Yamauchi et al., 2000b) (Di Mario et al., 2003) (Ueno et al., 2006).

In various studies on healthy individuals, carried out to demonstrate the efficiency of bLF on different immunological and haemostatic functions (Yamauchi et al., 1998) (Paesano et al., 2006) (Koikawa et al., 2008) (Mulder et al., 2008), treated with different doses (in an interval of 100 mg to 2,000 mg/day) and of variable duration (from 2 to 4 weeks, according to the study), no adverse effects attributable to the lactoferrin were observed.

The NDA Panel of the EFSA concluded that in 19 studies on adults, no adverse effects relating to bLF were mentioned, although the studies were not designed to study the safety of lactoferrin and in general the size of the sample was reduced (EFSA, 2012).

As it has been indicated that: a) there is a high prevalence of antibodies to lactoferrin in patients with diabetes mellitus type 1 (Taniguchi et al., 2003); b) the bLF administered orally was found as a native protein in mice brains (Fischer et al., 2007); c) increased concentrations of lactoferrin and/or its receptors were found in the characteristic lesions of patients with neurodegenerative diseases; and d) that the dietary intake of bLF may interfere with the production and function of endogenous lactoferrin, the history of the use of bLF and the available tests do not appear sufficient to doubt the safety of bovine lactoferrin.

In the assessment of bLF, the NDA Panel of the EFSA considers that this is essentially a cow's milk protein, mainly found in its non-denatured form. It also observed that lactoferrin is a normal component in human milk, and the expected consumption of bLF is found in the contents interval for human lactoferrin (native form) in the breast milk consumed by infants.

The NDA Panel of the EFSA observed that the average estimated intake of "lactoferrin", of some 210 mg/kg b.w./day for children of up to one year old, would be ten times lower than the highest dose (2,000 mg/kg b.w./day) tested in a subchronic study for 13 weeks on rats, that did not exhibit any adverse effects relating to bLF. In the case of adults over 19 years old, the intake would be 100 times lower. The available data for human beings indicates the absence of adverse effects of bLF at the levels of intake proposed, although the studies were not designed to assess safety. In view of the above, the NDA Panel of the EFSA concluded that the new food ingredient, bLF is safe for the proposed uses and levels.

The AFSSA in turn, and agreeing with the Dutch Committee, estimated that bLF is sufficiently characterised and that its manufacturing process is not of any concern. It indicates that the clinical supplementation tests did not exhibit any undesirable effects linked to bLF in adults, children and infants, although the objective of said studies was to assess the efficiency and not the safety of bLF. In addition, it considers that the toxicological data presented in a 13-week study is not sufficient to draw a conclusion as to the existence of a causal relation between the intake of bLF and the appearance of fibrosis in the pancreatic islet cells in rats. Therefore, it concludes that it is not possible to establish a NOAEL from this data and that long-term toxicity study would be required, i.e., a study that assessed the safety/harmlessness of bLF, with an adequate sample size.

If a NOAEL of 2 g/kg b.w./day is considered, the AFSSA indicates that a safety factor of 100 should be applied, therefore the levels of enrichment should be adjusted to ensure an intake, through diet, of less than 20 mg/kg b.w./day, including food supplements.

6.2.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum amount of 200 mg/day is acceptable from the safety point of view for use as a food supplement.

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7. Coenzymes

7.1 Coenzyme Q-10 or ubiquinone

7.1.1 Proposal

The AESAN has recommended a maximum daily amount of 200 mg of coenzyme Q-10 or ubiquinone. This proposal is based on the authorisation existing in Belgium for food supplements with a minimum limit of 4 mg/day and a maximum of 200 mg/day (Belgium, 2009). In Italy, a maximum daily amount of 200 mg of the coenzyme Q-10 is authorised in food supplements (Italy, 2012).

In France there is a favourable assessment of 30 mg/day for this substance, but it is not recommended for individuals receiving treatment with vitamin K antagonist anti-coagulants (AFSSA, 2008).

In Denmark the coenzyme Q-10 is authorised in food supplements with a total maximum amount that must not exceed 180 mg per daily recommended dose (Denmark, 2011).

It is also listed in the European Commission Report on substances present in food supplements in the European Union (DG SANCO, 2008).

7.1.2 Characteristics and sources

The coenzyme Q-10 or ubiquinone (CoQ10) is a molecule whose functional group is benzoquinone which is synthesised by the human body. Its complete chemical name is: 2,3-dimethoxy, 5-methyl, 6-poly-isoprene parabenzoquinone. CoQ10 is a compound from the family of benzoquinones that is mainly found in the human body. It has a poly-isoprene chain with 10 units, hence its name. CoQ10 may be in three oxidation states: totally reduced as ubiquinol (CoQ10H2), radical semiquinone, the intermediate form (CoQ10H) and the totally oxidised form, ubiquinone (CoQ10) (Crane, 2001).

This purified coenzyme is a highly lipophilic crystalline powder with a high molecular weight making it insoluble in water. These characteristics make its addition to foods for fortification difficult, especially those with a low fat content.

In spite of the difficulties in the quantitative determination of CoQ10 in food, due to the problem in extracting it from the different food matrices and the diversity of methods used in its determination, data is available on the content of this coenzyme in the foods. Pravst et al. (2010) have classified the food in five categories (A, B, C, D and E) according to their richness in CoQ10. Category A includes foods containing more than 50 mg/kg and category E contains those with less than 1 mg/kg. Meat and fish are the food sources that are the richest in CoQ10. However, in the same species, the content in the different tissues is different depending on its function (heart, liver, muscle, etc.). The highest concentrations of CoQ10 were found in reindeer meat (158 mg/kg), in the hearts of different animals (beef, pork, chicken) and in chicken livers (all class A). Oily fish are also major sources of CoQ10, and the highest concentrations were found in herring and mackerel, in the viscera and muscle, especially red muscle (five times greater than in white muscle). The richest vegetable sources are the oils. Concentrations were found ranging from 100 to 280 mg/kg in soybean, maize and olive oil. In general the oils with the highest content in CoQ10 are those from the Brassicaceae and Fabaceae families. Nuts and cereals also contain CoQ10 but in lower quantities (Pravst et al., 2010).

In 2010 the EFSA settled the application for health claims relating to the intake of CoQ10. These claims were related to their contribution to a normal energetic metabolism through the mitochondrial synthesis of ATP, the maintenance of normal blood pressure levels, protection of DNA, lipids and proteins from oxidative damage, its

contribution to the maintenance of a normal cognitive function, to the maintenance of adequate blood-cholesterol levels and to the increase in resistance capacity and/or performance. These claims were all rejected as it was not possible to establish, in any of the cases, a cause and effect relation between CoQ10 and the health claim (EFSA, 2010).

7.1.3 Nutrition and metabolism

In the body, CoQ10 is distributed in all the cell membranes bonded to enzymatic systems of the membrane, as occurs in the mitochondria, or floating in the lipid bilayer of different cell membranes (Crane, 2001). The CoQ10 found in the internal membrane of the mitochondria takes part in the cellular respiration process, forming part of the enzymatic systems of the respiratory chain. Through this process, the cell obtains the chemical energy (ATP) necessary for its growth and structural and functional maintenance.

This compound has other functions in addition to those described above. It acts in the body as a non-enzymatic endogenous antioxidant protecting the cells, in particular the cell membranes, from the damage that free radicals may cause to important components such as DNA, lipids and proteins. In fact, plasma concentration of CoQ10 was used as a marker of oxidative stress. It is known, as mentioned above, that the quinone group can be oxidised (quinone) or reduced (quinol). In addition to its direct antioxidant action, CoQ10 also recovers tocopheryl radicals reducing them to tocopherol. The percentage of the reduced form present in the membranes and in the serum ranges between 30 and 90 % depending on the metabolic state of the cell.

Other actions of this compound have also been proposed in different cell signalling pathways and in gene expression through the production of peroxides and the modulation of the *redox* state of the thiol groups (Crane, 2001).

As it is a liposoluble substance, it is absorbed with the lipids in the diet with a mechanism similar to that of vitamin E. It has been demonstrated that in the same enterocyte, the absorbed ubiquinone is transformed to its reduced form, ubiquinol which is the main circulating form (95 % of the total CoQ10). After its intestinal absorption, it is released to the bloodstream with the chylomicrons that, as remnants, are taken up by the liver and thus the CoQ10 is incorporated in this organ. The liver again exports the CoQ10 to the different tissues through the lipoproteins VLDL and LDL (Crane, 2001).

In general, the tissues with the greatest energy requirements or with a high metabolic activity such as the heart, kidney, liver and muscle contain higher relative levels of CoQ10. In addition, due to its chemical nature, it is linked to the lipid content of the different tissues and organs. The higher proportion of tissue content of the coenzyme is found in the reduced form except in the lungs and brain, which appears to reflect the greater oxidative stress of these organs (Crane, 2001).

Its bioavailability is very low due to its hydrophobicity and molecular size. Therefore, with respect to nutritional supplements, the quantity of substance absorbed will depend in the nature of the formula used, where the soluble formulas are the most bioavailable.

Plasma concentrations of CoQ10 in healthy individuals range between 0.20 and 1.91 µmol/l (Bhagavan and Chopra, 2006).

Its metabolism is not well-known although the maximum plasma concentration is described as being reached, in the majority of pharmacokinetic studies, 6.5 hours after its oral intake, with a second increase 24 hours later. Excretion is mainly through the bile and stools although a small fraction is excreted in urine (principally the phosphorylated metabolites). There is a certain enterohepatic circulation which may explain the second peak 24

hours after its oral intake, together with its redistribution that takes place from the liver to the circulation. The mean life is 33.19 hours.

The total quantity of CoQ10 in the human body is approximately 2 g, requiring the daily replacement, by endogenous synthesis and through diet, of 0.5 g (Bliznakov and Wilkins, 1998), therefore the mean turnover is approximately 4 days (Ernster and Dallner, 1995). The importance of the exogenous sources is even greater when there are problems in the endogenous synthesis (Pravst et al., 2010).

The CoQ10 content in the diet in developed countries ranges between 3 and 6 mg/day and comes, predominantly, from the consumption of meat (including poultry) while plant foods contribute in a lesser extend (Weber et al., 1997) (Mattila and Kumpulainen, 2001) (Kubo et al., 2008) (Pravst et al., 2010). 50 % corresponds to ubiquinol. The dietary intake of CoQ10 does not significantly affect the plasma levels of the coenzyme (Kaikkonen et al., 1999).

In normal circumstances, and due to endogenous biosynthesis, *de novo,* the tissues are not dependent on an exogenous intake of this coenzyme and therefore their exogenous intake, in addition to dietary, is not necessary (Bhagavan and Chopra, 2006). However, some authors indicate that in some conditions, such as stress and ageing, the endogenous production is not able to meet the demands for this coenzyme (Bhagavan and Chopra, 2006), and therefore it must be provided through exogenous sources.

At present, the intake of CoQ10 food supplements has become widespread due to the functions it performs in the body linked to the cellular generation of energy in the mitochondria, its role in the antioxidising defence against stress caused by the formation of reactive species of oxygen and as a cell signalling molecule in certain functional routes and in gene expression. These supplements are, in some cases, taken by healthy individuals for their anti-ageing and health properties due to their role in the prevention of certain chronic diseases that, in their origin, seem to be due to an imbalance between the production of reactive species of oxygen and antioxidant defences (cardiovascular disease, diabetes, etc.) (MedlinePlus, 2012).

The CoQ10 content available in food supplements for healthy individuals ranges between 15 and 100 mg (Bhagavan and Chopra, 2006). In patients with cardiovascular disease, the doses range between 100 and 200 mg/day (Langsjoen and Langsjoen, 1998). Higher doses, to the order of 15 mg/kg/day, are being used in cases of mitochondrial cytopathies (Gold and Cohen, 2001). Doses of 600 to 1,200 mg/day have been used in patients with neurological alterations (Huntington and Parkinson) (Kieburtz, 2001) (Shults et al., 2002). The intake CoQ10, suggested by some authors, coming from exogenous sources has been established, for healthy individuals, at between 30 and 100 mg and from 600 to 1,200 mg when used as a complementary therapy in different pathological conditions (Bonakdar and Guarneri, 2005) (Challem, 2005) (Jones et al., 2002).

The intake of CoQ10 supplements causes increases in the plasma levels of the coenzyme. These increases of the coenzyme in plasma are determined by: 1) the dose taken; 2) the formula; and 3) the time for which it is taken.

With respect to the formula, the products which are currently available in the market include tablets, chewable tablets, hard powder-filled capsules, soft capsules with oily suspension and in recent years, CoQ10 formulas which are soluble in soft gels and liquids.

With respect to the dose, food supplements aimed at the general population contain a wide dose interval; between 30 and 300 mg are considered as low or medium doses, whereas CoQ10 preparations for therapeutic purposes contain amounts ranging from 600 to 3,000 mg. These are considered as high doses, although on occasions they are used, for these purposes, to supplement lower doses (for example, 300 mg) (Bhagavan and Chopra, 2007).

Studies with low or medium doses show that there is a dose response relation in the plasma concentrations of CoQ10. However, if it is expressed per 100 mg of CoQ10 taken, the plasma levels fall as the dose increases. This shows that the bioavailability of the coenzyme decreases as the dose increases. When we use preparations with high doses, the behaviour is similar although the plasma levels reached are greater. If we consider the whole dose range (30 mg and 3,000 mg) we observe a plateau for supplements containing 2,400 mg, where the plasma concentrations do not increase with doses of 3,000 mg. For high doses, a large reduction is observed in the net increase of the plasma level of CoQ10 when it is expressed per 100 mg consumed. This behaviour is to be expected as CoQ10 is a liposoluble compound which, when taken in high doses, reduces the efficiency of absorption (pharmacologic doses). It is also worth noting that, at high doses, CoQ10 preparations contain significant quantities of vitamin E (800-1500 UI) and it is known that this vitamin interferes with the absorption of the coenzyme, therefore the effects observed cannot only be attributed to the high CoQ10 content (Bhagavan and Chopra, 2007).

With respect to the type of pharmaceutical preparation, it is agreed that the bioavailability, both in acute and chronic administration, of CoQ10 supplements increases in those that contain the principal asset in soluble form compared to those that are in oily suspension (soft capsules) and, with less bioavailability, those that are powder-based (normal or chewable tablets and hard powder-filled capsules) (Bhagavan and Chopra, 2007).

As mentioned above, ubiquinone (non-reduced form of CoQ10) is transformed to ubiquinol in the enterocyte, before being released into the lymphatic circulation. In addition, around 95 % of the circulating CoQ10 is in the form of ubiquinol. Therefore, in recent years a large quantity of dietary supplements of reduced CoQ10 (ubiquinol) have been marketed. It has been confirmed that this reduced compound is absorbed better than ubiquinone and after its intake, the plasma levels obtained are higher than those obtained following the intake of ubiquinone in similar doses, in any of its pharmaceutical forms and for low, medium and high doses. In the case of high doses, efficiency is increased if it is administered in split doses in two intakes (Bhagavan and Chopra, 2007).

The highest blood concentration of CoQ10 found in humans after the intake of a supplement is 10.7 µmol/l, although it has not been established whether this would be the plasma upper limit. Nor is it clear whether these levels provide the maximum therapeutic benefits. This maximum level was obtained with a soluble ubiquinol supplement (Bhagavan and Chopra, 2006).

Regardless of whether the supplement is in the form of ubiquinone or ubiquinol, soluble forms are better absorbed and result in higher rises in the CoQ10 plasma levels.

In addition to the above pharmaceutical types and dosses, other factors may affect CoQ plasma concentrations, including the amount of fat in the diet, with a direct relation, vitamin E, cholesterol, triglyceride content, in the supplement or in the diet, gender and age (Bhagavan and Chopra, 2007).

One of the aspects relating to the healthy function of CoQ10 in the body is, after absorption and the increase in plasma levels, its uptake by the tissues and in particular by cell membranes and more specifically the mitochondrial membrane. For its uptake by tissues and so that it can cross the blood-brain barrier, the plasma levels must be higher than the above-mentioned normal levels. The threshold of plasma levels for tissue uptake varies, depending on the tissue in question. Therefore, in patients with congestive heart failure, the uptake of CoQ10 is observed after plasma levels of 2.4 µg/ml (2,780 µmol/l). Another study in the same type of patients establishes the threshold at 35 µg/ml (4,054 µmol/l). In the case of patients with neurodegenerative disease (Huntington and Parkinson) the brain uptake thresholds are higher (Bhagavan and Chopra, 2007).

There is firm evidence that exogenous CoQ10 does not affect, down regulating, its endogenous synthesis as the plasma levels of the coenzyme return to their base levels when the supplementation is no longer taken,

regardless of the dose administered and whether the substance administered is ubiquinone or ubiquinol (Ikematsu et al., 2006) (Bhagavan and Chopra, 2007) (Hosoe et al., 2007).

One of the aspects that has emerged in the use of food supplements is the possibility of the existence of interactions between drugs and supplements. In this respect, in the case of CoQ10, its interactions with the anthracyclines and statins have been described with significant support from scientific literature. The anthracyclines, such as doxorubicin and daunorubicin are anti-cancer drugs of proven efficiency but they are also cardiotoxics, causing irreversible damage to the myocardial mitochondria. This adverse effect may be counteracted with the administration of CoQ10 without affecting its antitumoral effect. Moreover, the statins, hypocholesteremiant drugs due to their inhibition of hydroxy-methy-glutaryl-CoA reductase, also block the synthesis of CoQ10 leading to its tissue depletion. This depletion has been described as leading to myopathies in some cases, and in other extremes to rhabdomyolysis. These effects may be reverted with the administration of CoQ10. In addition, the beta-blockers may affect the status of CoQ10 due to the inhibition of the enzymes dependent on this coenzyme. This also occurs with some oral hypoglucemiants such as glyburide, phenphormin and tolazamide. It has also been described that CoQ10 improves glycemic control in diabetics and it has been suggested that patients who take CoQ10 supplements should adjust the dose of the hypoglycaemic drug. Lastly, and due to its structural similarity with vitamin K, the possible procoagulation action of CoQ10 has been suggested. Therefore, those patients receiving anticoagulant therapy may need to control their INR (Internationalised normalised ratio of prothrombin time) and adjust the anticoagulating dose accordingly (Bhagavan and Chopra, 2006).

7.1.4 Safety

The safety of CoQ10 supplements has been studied by different authors, both that of ubiquinone and of ubiquinol, considering that it is a molecule found in is natural form in our body.

The studies conducted with CoQ10, in any of its forms are based on the methods of the Council for Responsible Nutrition (CRN) (Hathcock, 2004) that include the characteristics of the determination of a UL value from the US Food and Nutrition Board (FNB) and the modification of the observed safe level (OSL) adopted as the highest observed intake (HOI) by the FAO/WHO. As the data available for the different clinical tests on humans, random and placebo controlled and of adequate size and duration, did not establish adverse effects, the OSL of the CRN and the HOI of the FAO/WHO are used instead of the NOAEL or the LOAEL to establish the UL.

No systematic pattern of adverse effects with relatively high doses has been observed in patients suffering from Parkinson's (2,400 mg, 1,200 mg and 600 mg). In other studies, effects have not been observed with dose ranges of 390-100 mg/day in healthy individuals with different pathologies (Hathcock and Shao, 2006).

Some studies observed the appearance of nausea, heartburn, gastric upset or related effects at doses of 600 mg in cardiac patients and 1,200 mg in Huntington and 120-180 mg in angina pectoris, in HFD and heart attack patients and 60 mg in subjects with oligospermia. Nevertheless, many of these disturbances also appeared in the placebo group and no significant differences exist. Together the studies provide strong evidence that there is no solid pattern for the incidence of nausea, related gastrointestinal effects or other adverse effects in periods of a few months (Hathcock and Shao, 2006).

Doses of 3,000 mg/day were administered in three studies of small groups of patients without adverse effects (although there was no control group). The largest study of the three offers substantial but not conclusive evidence of safety at this dose (Hathcock and Shao, 2006).

The most important study in terms of size, 80 subjects, and duration, 16 months, on patients with Parkinson's, at doses of 300-1,200 mg/day shows that there were no adverse effects such as nausea or other related symptoms (Shults et al., 2002).

None of the studies have a dose-response study for the appearance of nausea or other adverse effects. This indicates that there may be causality with a component of the supplement. Nausea was described at doses of 60 mg/day and 1,200 mg/day, with an equal incidence rate and severity in the placebo group in the majority of studies. As possible causes, the supplement vehicle (oil) or the capsule material have been mentioned. It may be that in patients with pathologies involving pain (angina, heart attack, etc.), this precipitates the nausea (Hathcock and Shao, 2006).

On the whole, the risk assessment obtained from the analysis of the different clinical tests at doses ranging from 3,000 mg/day to 600 mg/day has permitted the establishment of an OSL for healthy subjects of 1,200 mg/day/person, as at this dose there were no adverse effects attributable to CoQ10, and according to the authors (Hathcock and Shao, 2006) (Hidaka et al., 2008) there is no known mechanism that suggests that patients of Huntington and Parkinson's disease are less susceptible to the adverse effects of CoQ10 than healthy adults.

The toxicity studies in animals have permitted the establishment of an ADI of 12 mg/kg/day. Nor have any genotoxic effects been observed (Hidaka et al., 2008).

From all the available data it is concluded that CoQ10 does not present acute, subacute, chronic or reproduction or development effects at the doses proposed (Hosoe et al., 2007) (Hidaka et al., 2008).

There is not enough information regarding the safety of the use of CoQ10 during pregnancy and breastfeeding.

7.1.5 Conclusion

The toxicological studies performed did not reveal any adverse effects from the coenzyme Q10. Therefore, considering that an OSL of 1,200 mg/day has been established, the Scientific Committee concludes that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum amount of 200 mg/day of the coenzyme Q10 is acceptable from the safety point of view for use as a food supplement.

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8. Flavonoids and carotenoids

8.1 Astaxanthin from shellfish and fish

8.1.1 Proposal

The AESAN has proposed a maximum daily amount of 4 mg of astaxanthin from shellfish and fish. This proposal is based on the existence of an authorisation in the United Kingdom of 4 mg/day of astaxanthin in food supplements established through a substance equivalence (article 5 of Regulation (EC) 258/1997 (EU, 1997)) with astaxanthin from alga *Haematococcus pluvialis* with respect to capsules containing dried *H. pluvialis* previously marketed in the European Union (ACNFP, 2004). In addition, there is a favourable assessment in France, considering 6 mg/day (AFSSA, 2008) and in Italy astaxanthin is authorised in food supplements without having established a maximum daily amount (Italy, 2012).

8.1.2 Characteristics and sources

Astaxanthin (3S,3'S-dihydroxy-β, β-carotene-4-4'dione) is a carotenoid with a molecular weight of 596.85 daltons and CAS number 472-61-7, characterised by the presence of hydroxyl and ketone groups on the terminal cycles.

It is found naturally in yeasts (*Xanthophyllomices dendrorhus*) and in certain microscopic algae present in phytoplankton (*Haematococcus pluvialis*). The higher plants and animals do not have the required enzyme systems for its synthesis *de novo.* Nevertheless, fish such as salmon and trout and shellfish such as prawns or lobsters, as they feed on phytoplankton, may be considered as a source of astaxanthin.

In nature, astaxanthin is found conjugated with proteins or esterified with one or two fatty acids (oleic, linoleic, palmitic, linolenic acids) as astaxanthin acyl monoester or diester (Kidd, 2011). The isomer of natural origin is the 3S3'S whereas the synthetic form used in the fish farms for the breeding of salmon and trout is the isomer 3R3'S (Guerin et al., 2003).

In the European Union astaxanthin is authorised as a colorant in animal food (BOE, 1994) (EU, 2008).

8.1.3 Nutrition and metabolism

The bioavailability of astaxanthin is comparable to that of beta-carotene. After intake, the esterified forms are hydrolysed by the digestive enzymes. Free astaxanthin undergoes passive absorption, favoured by the presence of lipids in the diet and is distributed by bonding to high and low density lipoproteins (Guerin et al., 2003) (Kidd, 2011). The maximum plasma concentration is reached 6 hours after administration (1.2 mg/l) and may persist up to 76 hours, depending on the dose administered (Kidd, 2011). It has been confirmed that very small daily doses (1 mg), administered for four weeks, may significantly increase the base plasma levels of astaxanthin in adults (Miyazawa et al., 2011).

Unlike other carotenoids, the hepatic mechanism does not result in the production of active metabolites, although *in vitro* studies on hepatic cells have shown that high concentrations of astaxanthin increase the activity of the CYP3A4 and CYP2B6 enzymes (Kistler et al., 2002).

Although scientific literature attributes a large number of beneficial effects to astaxanthin, applications to the EFSA under Regulation (EC) No 1924/2006 (EU, 2006) on regarding its healthy properties have not been approved (EFSA, 2009, 2011).

Astaxanthin is widely used as an additive in animal feed in order to give a pink colour to salmon flesh and other aquaculture products. Wild salmon may contain up to 40 mg of astaxanthin/kg of flesh. The astaxanthin content in aquaculture salmon and trout is estimated at 1-9 mg/kg and 0-25 mg/kg, respectively. The EFSA, in relation to an additive used in animal feed that contains 11 % astaxanthin dimethyl succinate, considered that enrichment in fish food at concentrations of 100 mg/kg does not pose an additional risk to the consumer (EFSA, 2007).

There is no data available for the intake of astaxanthin but the EFSA, based on different sources, estimated that in the European adult population the highest intake (97.5 percentile) of astaxanthin is due to the consumption of fish (103 g/person/day) or of fish and shellfish (165 g/person/day). With this data and considering that all the fish is salmon or trout from fish farms fed with the highest contents of astaxanthin, the intake would be between 1.6 (fish) and 4.1 mg/person/day (fish and shellfish). If the astaxanthin content in red salmon is considered (*Oncorhynchus nerka*) the intake would be between 3.0 and 6.3 mg/person/day (EFSA, 2005).

In Spain fish consumption is higher than in other European countries. Whereas in Europe the same EFSA study indicated an average intake of 13 g of trout and salmon/person/day and 80 g of trout, salmon and shellfish/person/day, in Spain the mean intake in the consumer population of trout, salmon and shellfish in general is, according to the data collected in the National Survey of Spanish Dietary Intake, 77.48, 51.89 and 28.51 g/person/day, respectively and in the 97.5th percentile the intake reaches 266.67, 100 and 110 g/person/day (data not published). Based on this data, the maximum intake of astaxanthin in the Spanish population of consumers of these foods may be estimated at 1.95 mg/day.

8.1.4 Safety

Studies *in vivo* and *in vitro*

The study of the acute and subchronic toxicity of astaxanthin in *Wistar* rats determined a LD_{50} for oral doses above 12 g/kg b.w. In the subchronic test in which the rats were fed for 90 days with 1, 5 and 20 % of biomass of *H. pluvialis* rich in astaxanthin (weight/weight) no changes were observed in the weight of the rats or alterations in the haematological parameters with respect to the control group. At higher doses a slight increase was observed of alkaline phosphatase and an increase in the weight of the kidneys in both genders. The hystopathological analysis did not reveal any adverse effects, only an increase was observed in the pigmentation of the proximal tubule of the kidney in half of the rats. The value of the NOAEL estimated in this test was 465 mg and 557 mg astaxanthin/kg b.w./day for males and females, respectively (Stewart et al., 2008). The assessment of the distribution of the astaxanthin in different rat tissues showed a build-up of the same in the kidneys, spleen and adrenal glands and to a lesser extent in the liver, heart and eyes (Petri and Lundebye, 2007).

The effect of astaxanthin on the metabolic activation of promutagen benzo(a)pyrene induced by the enzyme CYP1A was also studied on *Wistar* rats. In the livers of the rats treated for 3 days with an oral dose of 100 mg of astaxanthin/kg b.w./day, a significant increase was observed in the mRNA CYP1A1, of the protein and its activity (5.5, 8.5 and 2.5 times, respectively). Consequently, the mutagenicity of the benzo(a)pyrene in the Ames test was greater in the rats treated with astaxanthin in comparison to the control group (Ohno et al., 2011).

However, in contrast to that observed in rats, in a study of human hepatocytes carried out in 2002 the astaxanthin performed as an enzyme inducer for CYP3A4 and CYP2B6, but not for CYP1A (Kistler et al., 2002).

In addition, the EFSA in its safety assessment of the use of an additive containing approximately 11 % of astaxanthin dimethyldisuccinate include the results of the acute and subchronic toxicity studies for 90 days of rats (with negative results); of *in vivo* and *in vitro* genotoxicity (with negative results), of chronic toxicity for 52 weeks of rats and two carcinogenicity studies on rats and mice.

In the chronic toxicity study on rats, changes were observed in the lipid parameters and inflammatory nodules in the liver at doses of more than 125 mg/kg b.w/day.

From the results of the carcinogenicity tests, the EFSA established for the synthetic preparations of astaxanthin a NOAEL value of less than 40 mg/kg b.w./day in rats. In the evaluation of the carcinogenicity of astaxanthin in mice, after 6 months of treatment, anomalies were observed in the lipid metabolism and a NOAEL of 14 mg/kg b.w./day and a LOAEL of 300 mg/kg b.w./day were established (EFSA, 2007).

Studies in humans

In the case of humans, the majority of the studies carried out have been directed at determining the possible beneficial effects but very few preclinical studies offer information about the safety of astaxanthin. Several studies confirm that supplements of astaxanthin of 4, 6 and 21.6 mg/day for 3, 8 and 2 weeks respectively, do not have any secondary effects (AFSSA 2008) (Spiller and Dewell, 2003).

A study carried out with 73, 38 and 16 adult volunteers who were given a daily dose of 4, 8 and 20 mg of astaxanthin for 4 weeks did not show any significant alterations in the biochemical or haematological constants studied (Satoh et al., 2008).

The administration of 5 to 12 mg of astaxanthin for 4 weeks or 6 mg for 8 weeks to healthy volunteers was well-tolerated and did not produce any clinical signs of reference (EFSA, 2005).

Notwithstanding the above, according to the AFSSA (2008) the evaluation of signs of carcinogenicity must be conducted on models that consider the environmental exposure that would favour the pro-oxidant and carcinogenic effect of the carotenoids in general, which is predictably higher in the case of astaxanthin.

The teratogenic effects of this substance are unknown.

With an estimated NOAEL in rats of 14 mg of astaxanthin/kg b.w./day and considering a safety factor of 100, the safety limit for man would be 140 µg/kg b.w./day, that is, 9.8 mg/day for an adult weighing 70 kg.

8.1.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and considering that Spain is a country in which the consumption of seafood is higher, the maximum daily amount of 4 mg of astaxanthin in food supplements proposed by the AESAN may be considered within the safety limits for medium-term intake as, taking a maximum intake value of astaxanthin through seafood estimated at 1.95 mg/day, the upper exposure level, including the food supplements, would be less than 6 mg/day.

Due to the chemical characteristics and to assess the potential risk of its long-term intake, carcinogenicity studies must be conducted using models that include exposure to environmental contaminants.

Although studies in humans have not shown any adverse effects, the threshold dose from which it may interfere with the metabolism of certain medicines is not known. Due to the absence of studies on teratogenic effects, food supplements of astaxanthin are not recommended for pregnant women.

In addition, due to the lack of scientific information, the intake is not recommended for children and nursing mothers.

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8.2 Lycopene

8.2.1 Proposal

The AESAN has recommended a maximum daily amount of 15 mg of lycopene. This proposal is based on the decisions for the authorisation of the use of lycopene as a new food (Regulation EC 258/1997 (EU, 1997) with a maximum daily amount of lycopene of 15 mg (EU, 2009a, 2009b).

This concentration is in line with the authorisations in other countries in the European Union. In Denmark lycopene is authorised in food supplements with a total maximum amount that must not exceed 15 mg per recommended daily dose (Denmark, 2011). In Italy, a maximum daily amount of 15 mg is authorised in food supplements (Italy, 2012).

8.2.2 Characteristics and sources

Lycopene is a natural colorant belonging to the carotenoid group. It has an acyclic structure with an aliphatic chain that includes three double bonds, of which eleven are conjugated. Its chemical formula is $C_{40}H_{56}$ and molecular weight is 536.85 daltons.

The chemical names of lycopene are Ψ, Ψ-carotene; lycopene all *trans*; lycopene (all E) and (all E)-2, 6, 10, 14, 19, 23, 27, 31-octamethyl-2, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 30-dotriacontatridecaene (BOE, 2011).

As with the other carotenoids, lycopene is found in various geometric configurations. In the natural matrices, the majority form is the *trans*, which is the most thermodynamically stable conformation, although the forms 5-*cis*-, 9-*cis*- and 13-*cis*- are also found in lower proportions. The preparations intended of use in food are presented in the form of suspensions in edible oils or water-dispersible or soluble powders (BOE, 2011).

Lycopene is mainly found in the tomato (*Lycopersicon esculentum* Mill. =*Solanum lycopersicum* L.), but also in other fruits and vegetables (water melon, pink grapefruit, pawpaw, etc.). It can also be obtained synthetically or from the fungus *Blakeslea trispora*.

In ripe and fresh tomatoes approximately 83 % of the total number of carotenoids are lycopene, mainly in its all *trans* form. However, when exposed to sunlight or heat it may partially change to the *cis* configuration. Therefore, due to the heat process to which processed tomato products are subjected, the proportion of *cis* isomers may increase significantly (Olempska-Beer, 2006) (Vitale et al., 2010).

Lycopene is authorised in the European Union as a colorant for use in foods (E-160d) by Directive 2008/128/EC (EU, 2008) whether it is obtained by chemical synthesis or extracted from natural sources such as the red tomato or the fungus *Blakeslea trispora*.

In Spain, Order SPI/1957/2011, published by the Ministry of Health, Social Policy and Equality, (BOE, 2011) amending the annexe to Royal Decree 1465/2009 in order to adapt it to the provisions of he European Union, establishes the standards for the identity and purity of the colorants used in food products, highlighting with respect to synthetic lycopene (E-160d) and the lycopene from *Blakeslea trispora* that:

- The synthetic lycopene must contain no less than 96 % of total lycopenes of which 70 % are in the all *trans* form.
- The lycopene from the fungus *Blakeslea trispora* must contain no less than 95 % of total lycopenes of which no less than 90 % are in the all *trans* form.

8.2.3 Nutrition and metabolism

Its antioxidant property has been confirmed, relating it to the prevention of various pathological processes involving cellular oxidation although the scientific opinion of the EFSA does not consider claims concerning the protection against the oxidative damage of DNA, proteins and lipids, on skin (damage induced by UV), maintenance of the cardiac function and normal vision to be sufficiently established (EFSA, 2011).

Lycopene is an antioxidant molecule that reacts with the singlet oxygen $^1O_2$, generated by photonic exposure of the skin. Unlike beta-carotene it does not have a pro-vitamin A activity, and therefore cannot be considered a precursor of the retinoic acid involved in many processes of cell proliferation and differentiation (AFSSA, 2008).

It may be considered a regular ingredient in the human diet as it is consumed as part of the tomato and its processed products. This is why the carotenoid is found in greater concentrations in human plasma.

Factors affecting the bioavailability of lycopene in the body include the method of processing the food and the functional state of the intestine (Vitale et al., 2010) (WHO, 2010).

The lycopene present in the processed products subjected to heat has greater bioavailability as high temperatures easily release the lycopene present in the cell matrices. Given its lipophilic nature, the absorption of lycopene is greater when it is consumed with diets rich in fat (WHO, 2010).

Numerous studies have been conducted on animals (rats, mice, dogs, monkeys, calves) and in humans to determine its pharmacokinetic characteristics (absorption, distribution and excretion).

In humans, lycopene, as with all the carotenoids, due to its lipophilic nature, follows the same digestive and intestinal absorption process as the fats. Once released from its matrix in the food and dissolved in an oily medium, it bonds to the lipid mycela in the small intestine. It is carried in the plasma by the low density proteins and it builds up in the tissues rich in low density protein receptors. The target organ is the liver but it also builds up in the lungs, adrenal glands, adipose tissue, prostate, ovaries and uterus. Blood plasma may also contain detectable levels of lycopene (WHO, 2010).

In the plasma the most abundant isomers are the all *trans* form and the 5-*cis* lycopene that may represent more than 50 % of the total. It is unknown whether the greater presence of the *cis* form in the plasma is due to greater bioavailability or to a higher level of catabolism of the *trans* form. It has been suggested that the *cis* form is probably more bioavailable than the all *trans* form due to its structural configuration, its greater solubility in the mycelas and its lower aggregation capacity (WHO, 2010). It has a mean life in the plasma of between 6 and 4 hours.

The most important metabolites of lycopene are the 1,2 and 5,6-epoxide. Excretion is mainly through the stools and to a lower extent through urine and the lungs.

According to the data collected in the different intake surveys conducted in Europe, the mean intake of lycopene from natural sources is between 0.5 and 5 mg/day. Consumers of large quantities of fruit and vegetable and in particular products derived from tomatoes, may consume on extraordinary occasions quantities higher than 20 mg lycopene/day (EFSA, 2008a). However, considering all the sources, including that used as an additive in food, the daily intake of lycopene, according to the EFSA, may be as much as 43 mg/day (EFSA, 2008b) and from 420 to 500 µg/kg b.w./day in children, at the maximum level of exposure, exceeding the calculated ADI (Acceptable Daily Intake) by 44-55 % (EFSA, 2010). Furthermore the WHO, based on data from eight countries, estimates that daily exposure to lycopene from tomato or its derivatives is 10 mg/day and between 10 and 50 mg/day from all sources (WHO, 2010).

8.2.4 Safety

The toxicity of lycopene, from the extract of tomato, has been widely studied in animals and in humans.

Studies in animals

Studies of acute, chronic and subchronic toxicity with oral administration have been made of different animal species; toxicity studies on reproduction using multigenerational and development tests; and carcinogenicity and mutagenicity tests.

Lycopene did not present acute toxicity and the subchronic toxicity studies with oral administration in tests at 98 and 90 days have not shown toxic effects at the highest tested concentrations (500 and 586 mg/kg b.w./day, respectively). As a secondary effect, a yellow-orange coloration of the liver was observed (AFSSA, 2008).

In a test with rats, lasting 52 weeks, at the highest doses tested, on completion of the treatment in the females, a pigmentation was observed in the liver associated with histopathological alterations of basophilic foci. However, no structural alterations or signs of hepatic dysfunction were observed and therefore the significance of these alterations is not known, although it is thought that they are probably due to an intracellular accumulation of lycopene (WHO, 2010).

The carcinogenicity studies did not suggest the presence of negative effects with the administration of lycopene.

The different mutagenicity tests performed (chromosomal aberrations in mammal cells, mammalian erythrocyte micronucleus test, gene mutation and unprogrammed DNA synthesis) produced negative results (WHO, 2010).

The most relevant NOAEL values obtained from the different toxicological tests are (WHO, 2010):

- 586 mg/kg b.w./day in a toxicity study for 90 days on rats. Highest tested dose.
- 250 mg/kg b.w./day in a toxicity study for 1 year on rats.
- 50 mg/kg b.w./day in a toxicity and genotoxicity study for 2 years on rats.
- 500 mg/kg b.w./day in a multigenerational toxicity study on rats. Highest tested dose.
- 500 mg/kg b.w./day in a toxicity study on development in rats. Highest tested dose.
- 400 mg/kg b.w./day in a toxicity study on development in rabbits. Highest tested dose.

Studies in humans

From the tests on humans, although these were focussed on investigating the antioxidant capacity of the lycopene and not its safety, it can be concluded that in general, the administration of lycopene in diet is well tolerated. The adverse effects after several weeks of treatment with lycopene were limited to minor gastrointestinal disturbances and problems in the colouring of the skin (WHO, 2010).

Some cases of lycopenodermia have also been reported in scientific literature. The case of a woman has been described who consumed two litres a day of tomato juice for several years (equivalent to 160 mg or 2.3 mg/kg b.w./day of lycopene). The woman suffered abdominal pain associated with nausea, vomiting and diarrhoeas

and had a yellow-orange colouring on the skin of her hands and the sole of her feet. The clinical research revealed a high concentration of lycopene in the serum and liver (Reich et al., 1960). The same effects were detected in a woman who consumed tomato sauce for 3 years. The symptoms remitted when the levels of tomato in her diet were restricted.

Based on the available data, the EFSA, through the Panel of food additives, flavourings, processing aids and food contact materials, established an ADI of 0.5 mg/kg b.w./day for lycopene from all its sources, including the tomato (EFSA, 2008b). The WHO nevertheless considers that the lycopene has low toxicity and therefore it is not necessary to establish an ADI.

8.2.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum amount of 15 mg/day of lycopene is acceptable from the safety point of view for use as a food supplement.

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8.3 All *trans* lutein/zeaxanthin

8.3.1 Proposal

The AESAN proposes a maximum daily amount of *trans* lutein alone or associated with zeaxanthin of 20 mg as a food supplement.

This proposal is based on the decisions for authorisation as a supplement in other countries of the European Union. Thus, in France, the same amount is admitted, establishing a maximum limit of 20 mg/day (AFSSA, 2008), in Denmark the total maximum amount that can be used as a food supplement must not exceed 20 mg/day (Denmark, 2011) and in Italy lutein/zeaxanthin are authorised in food supplements without having established a maximum daily amount (Italy, 2012).

8.3.2 Characteristics and sources

Lutein (3R,3'R,6'R)-β,ε-carotene-3,3'-diol) and its zeaxanthin stereoisomer (all *trans*-(3R,3'R)-β-carotene-3,3'-diol) are carotenoids without pro-vitamin A activity, included in the group of vegetable pigments of the xanthophyll type, responsible for the yellow-orange colour of flowers and fruits. It is found in a number of vegetables including maize, alfalfa, green leaf vegetables (spinach, cabbage, broccoli, Brussel sprouts) and fruits including kiwi and melon. In green leaf vegetables and fruits it is found in its non- esterified forms, whereas in the other plants it is more frequent in the mono- or diester forms of fatty acids (palmitates and myristates). Its chemical formula is $C_{40}H_{56}O_2$ and molecular weight 568.88 g/mol.

The presence in its structure of three chiral centres implies the possibility of eight stereoisomers. In nature the majority isomers are all *trans* and *cis-trans*, in the form of di-palmitates. During the processing, there may be isomeric transformations (Khoo et al., 2011).

One of the main industrial sources are the flower petals of the French marigold, an ornamental plant belonging to the botanical species *Tagetes erecta* L. (= *T. patula* L.;= *T. remotiflora Junze*) from the *Asteraceae* family.

Lutein, a combination of lutein and zeaxanthin from *T. erecta*, is authorised in the European Union as a colorant for use in food (E-161b) (EU, 2009) and has been reassessed on two occasions by the group of experts in food additives and nutrient sources added to foods (EFSA , 2010, 2012).

In Regulation (EU) No 231/2012, lutein (E-161b) is listed as a "dark yellowy-brown liquid", "mixture of carotenoids, xanthophylls", obtained "by extraction with solvents" ("methanol, ethanol, propan-2-ol, hexane, acetone, methyl ethyl ketone, and carbon dioxide") "from strains of edible fruits and plants, grass, lucerne (alfalfa) and *Tagetes erecta*. The main colouring principle consists of carotenoids of which lutein and its fatty acid esters account for the major part. Variable amounts of carotenes will also be present. Lutein may contain fats, oils and waxes naturally occurring in the plant material". The Regulation indicates that the total content of colorants may not be less than 4 % expressed as lutein. However, the maximum quantities of lutein-zeaxanthin that may be contained in the preparations identified as E-161b are not specified (EU, 2012).

In *Tagetes erecta* 70-90 % of the colorants are lutein and 10-25 % zeaxanthin. In plant species other than *T. erecta*, the ratio between lutein and zeaxanthin is inverted, as is the case of maize in which the majority of the colorants are zeaxanthin.

At present different preparations are marketed in the European Union for use in foods, prepared from saponified extracts of *T. erecta* which differ according to the concentration of total carotenoids and the different ratios of lutein and zeaxanthin. Only three of these have been assessed by the EFSA (2011).

- Lutein with ≥80 % of carotenoids consisting of lutein (79 %) and zeaxanthin (5 %).
- Lutein with carotenoid content of ~5-12 %.
- Lutein from *T. erecta* with high concentrations (>60 %) of total carotenoids in the form of esters (>93 % lutein esters and the rest zeaxanthin esters).

Following a request from the European Commission, the Technical Commission on Dietary Products, Nutrition and Allergies issued a scientific ruling on the safety, bioavailability and suitability of a purified preparation of lutein obtained by saponification of the oleoresin obtained from an extract in hexane of *Tagetes erecta* flowers for the particular nutritional use by infants and small children. The preparation (*FloraGLO*®) contains at least 20 % lutein and 0.8 % zeaxanthin, suspended in sunflower oil. The ruling was favourable as regards bioavailability and safety (250 µg/l of lutein added to preparations with a low natural content of lutein of approximately 20 µg/l or less) but not as regards its nutritional or dietary value (EFSA, 2008).

The zeaxanthin is obtained by chemical synthesis, predominantly the all *trans* isomer (not less than 96 %). It is marketed in the form of "pearls" of dried powder, with gelatine or starch, that contain 5 % of zeaxanthin and alpha-tocopherol and ascorbyl palmitate as antioxidants; and in the form of suspension in maize oil with a richness of 20 % in zeaxanthin and alpha-tocopherol as antioxidant (AFSSA, 2010).

8.3.3 Nutrition and metabolism

Free or esterified lutein is absorbed in the small intestine, the zeaxanthin predominantly in the ileum. However, as occurs with the rest of the carotenoids, its absorption may be modified by nutritional or physiological factors (Evans et al., 2012). Although the degree of esterification does not appear to have a direct influence on bioavailability (EFSA, 2011), it may alter the absorption to the extent that it affects its release from the food matrix and to it micellar uptake. Therefore, the absorption of lutein will depend on its formula (Evans et al., 2012) and on the quantity of fat, fibre or other carotenoids, present in the diet.

In the enterocytes, the esters are hydrolysed by gastrointestinal enzymes. Free lutein, included in chilomicrons, is transported along the lymphatic pathway to the liver, distributing itself to the tissues (particularly the retina) through the blood circulation to the tissues bound to lipoproteins, preferably to high density proteins (Kijlstra et al., 2012).

The metabolisation is characterised by oxidation of the hydroxyl groups followed by reduction and epimerization (3'-epilutein). In humans an interconversion is observed between both xanthophylls, where the intermediate compound is 3'-epilutein, which can also be identified in human plasma (EFSA, 2009).

Of the quantity consumed orally, approximately 45 % is eliminated in the stools and 10 % in urine.

The physiological functions include the contribution to the density of the macular pigment and it probably takes part in the protection of the retina against damage from UV light. In animal food, lutein is used to intensify the yellow colour of egg yolks (EFSA, 2009).

Intake estimations for lutein and zeaxanthin do not differentiate between the two due to questions of an analytic order.

The mean dietary intake of lutein from natural sources is estimated at 2.5 mg/day both for adults and for children, equivalent to 0.04 and 0.1 mg/kg b.w./day respectively. In the 95th percentile the estimation reaches 7 mg/day for both groups equivalent to 0.12 mg/kg b.w./day for adults and 0.28 mg/kg b.w./day for children (EFSA, 2012).

The intake of lutein as a food colorant (E-161b) was estimated by the ANS Panel (Panel on Food Additives and Nutrient Sources Added to Food) of the EFSA in 2011 and reconsidered in 2012 based on the information provided by the food industry, considerably reducing the maximum intake levels, principally those relating to non-alcoholic drinks, sauces and desserts, including dairy products with flavours. Intake data from the United Kingdom, as the highest consumers of soft drinks in the European Union, was used for the estimation.

Table 2. Estimated intake of *trans* lutein including zeaxanthin as a colorant (E-161b) in mg/kg b.w./day

	Adults (UK)[1]	Children (UK)	Children (EXPOCHI)[2]
Maximum permitted levels			
Mean exposure	0.8	3.0	0.5-3.4
Percentile 95-97.5	3.2	7.2	0.2-2.2
Maximum intake levels			
Mean exposure	0.1	0.4	0.1-0.4
Percentile 95-97.5	0.3	1.0	0.1-1.0

[1]UK: UNESDA, NATCOL (Natural Food Colours Association) [2]EXPOCHI (Individual food consumption data and exposure assessment studies for children). **Source:** (EFSA, 2012).

In 2006, the JECFA (Joint FAO/WHO Expert Committee on Food Additives) estimated an ADI for lutein extracted from *Tagetes erecta* and for synthetic zeaxanthin of 0-2 mg/kg b.w./day (JECFA, 2006). However, in 2008, the EFSA estimated that the available toxicological studies did not permit the establishment of an ADI for synthetic zeaxanthin alone. In 2010, taking new toxicity studies as the base, the EFSA reconsidered the position of the JECFA and estimated an ADI of 1 mg/kg b.w./day for lutein extracted from *Tagetes erecta* with a minimum carotenoid content of 80 %, distributed between lutein and zeaxanthin.

Therefore, taking the sources of exposure to natural lutein and lutein as a food colorant together, in no case are exceeded in adults the mean values of the ADI of 1 mg/kg b.w./day proposed by the EFSA for this substance, although they are exceeded for children. However the mean exposure values do not reach this quantity in the majority of countries in the European Union, and in the percentiles 95-97 % they may be exceeded in certain cases, as occurs in the Netherlands and the United Kingdom (EFSA, 2010, 2011).

8.3.4 Safety

The toxicity of lutein, from the extract of *Tagetes erecta*, has been widely studied in animals and in humans. In 2006, the JECFA assessed the safety of the preparations of *Tagetes erecta* L. with high contents of lutein (>80 %) and of synthetic zeaxanthin, for use as food colorants and nutritional supplements. Taking into account the structural and physiological similarities and the absence of widely documented toxic effects for lutein from *T. erecta*, the JECFA (2006) established an ADI of 0-2 mg/kg b.w./day for both substances.

Subsequently, the EFSA (2011) reassessed this figure, considering the results of a study over 90 days performed on rats, taking into account a NOAEL value of 200 mg/kg b.w./day (highest tested dose), absence of toxicity for development at doses higher than 1,000 mg/kg b.w./day (highest tested dose), the non-observation of

effects on the reproductive organs, the absence of genotoxic effects (EFSA, 2006) and the fact that it is a substance which is found naturally in the human diet. Applying an uncertainty factor of 200 as there were no multigenerational studies on reproduction or chronic toxicity/carcinogenicity studies, an ADI of 1 mg/kg b.w./day was established for lutein from *T. erecta* with a carotenoid content >80 % (79 % lutein, 5 % zeaxanthin).

The toxicity studies conducted on another preparation of lutein, lutein with ≥60 % of carotenoid esters (>93 % of lutein and the rest zeaxanthin) permitted the establishment of a NOAEL of 1,000 mg/kg b.w./day, the highest dose and equivalent to 538 mg/kg b.w./day of lutein equivalents, far higher than the NOAEL of 200 mg/kg b.w./day that permitted the establishment of an ADI of 1 mg/kg b.w./day. This is why the EFSA (2011) considers this value of ADI acceptable for this preparation. To the contrary, it concludes that its application to other preparations of a lower purity or from other sources is not acceptable.

In recent years, some cases of toxidermia have been reported, attributable to the use of lutein-zeaxanthin preparations from *T. erecta* as food supplements. ANSES (2011) related these cases with the possible presence in preparations of thiophenes or sesquiterpenic lactones from other plants belonging to the Asteraceas family. Therefore it is important that the preparations intended for use as dietary supplements meet the specifications established in the safety assessments conducted by the EFSA.

8.3.5 Conclusion

Although there are other sources for obtaining lutein, to date safety studies have been carried out with *trans* lutein associated to *trans* zeaxanthin from *Tagetes erecta*, with the appearance of safety problems when other sources are used.

The Scientific Committee concludes that, based on the information available to date and taking into account the general considerations reflected in this report, the proposal of a maximum amount of 20 mg/day of *trans* lutein, associated with *trans* zeaxanthin, is acceptable from the safety point of view for use as a food supplement. However it should be noted that this estimate only refers to its intake as a supplement in adults.

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8.4 Quercetin

8.4.1 Proposal

The AESAN has proposed a maximum daily amount of quercetin of 75 mg with the warning "not recommended for pregnant women". This proposal is based on the existence of a favourable assessment in France considering 75 mg/day (AFSSA, 2008).

In Italy, a maximum daily amount of 300 mg of the quercetin is authorised in food supplements (Italy, 2012).

8.4.2 Characteristics and sources

Quercetin is a flavonoid from the group of flavonols (2-(3,4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4H-cromon-4-ona) naturally found in both its free form and combined in the glycoside form (O-β glycosides). It is widely distributed, mainly in the aerial parts of many plant species. It is an intrinsic component of several foods including green beans, broad beans, apples, apricots, cherries, grapes, blueberries, onions, tea leaves, cocoa fruit and red wine.

It is obtained by the hydrolysis of glycosides from plant sources and subsequent purification.

8.4.3 Nutrition and metabolism

Following the oral intake of the flavonoids, the glycosylated forms are hydrolysed by the microflora of the colon although some of them can also be hydrolysed in the small intestine, and the genins penetrate the epithelial cells through passive diffusion. Alternatively, the glycosylated forms can also be absorbed through a carbohydrate transporter (SGLT-1), be hydrolysed by the enzyme cytosolic beta-glucosidase and undergo subsequent conjugation with glucuronic acid.

Quercetin, once absorbed, may undergo a process of O-methylation, principally 3'-O-methylquercetin (isorhamnetin) and to a lesser degree 4'-O-methylquercetin or be conjugated with glucuronic acid or sulfate groups. These derivatives of quercetin and even the unaltered quercetin, in very low proportions, are metabolised in the liver and are eliminated through the kidneys and to a lesser degree through the bile. Alternatively they may be degraded by the colon microbiota to phenolic acid and CO_2, and are eliminated with the stools together with the unabsorbed quercetin.

Approximately half (52 %) of the quercetin consumed is though to be absorbed (Hollman et al., 1999). However, both the absorption and the maximum plasma levels and the elimination kinetics may vary depending on the food matrix in which it is included.

The mean intake of quercetin from diet is estimated to be in the range of 5 to 40 mg/day, however, for consumers of large quantities of fruit and vegetables, especially those who eat fruit and other vegetables rich in quercetin, including the skin, such as tomatoes, apples and onions, the estimate of daily intake may reach 200 to 500 mg/day (Harwood et al., 2007).

In populations who drink large quantities of tea, for example in the Netherlands, the daily intake is estimated to be 16 mg of quercetin. In Scotland the mean daily intake is estimated at 18 mg, in the United States from 12 to 14 mg and in Germany at 10 mg (AFSSA, 2008).

In the United States its use is authorised as a food supplement in a recommended dose of 200 to 1,200 mg/day (PDRNS, 2001). Since 2004, the quercetins of high purity (≥98,5 %) marketed as QU 985, QU 995, QU

998 and QU 1,000, have been considered GRAS for use as ingredients in different foods, with a level of use of 0.008-0.5 % or of 10-125 mg/portion.

Globally and taking into account its presence in very different types of foods a mean intake of 4.7 mg of quercetin/kg b.w./day (226 mg/day) is estimated in the 90th percentile.

8.4.4 Safety

In 1977, the JECFA were not able to reach a decision regarding the safety of this compound due to the absence of adequate toxicological studies (JECFA, 1977). However, subsequently the International Agency for Research on Cancer (IARC, 1999) classified this flavonoid in the group of substances without carcinogenic effect for humans (Group 3) although they did relate it with some cases of carcinogenicity in animals, toxic effects that were observed when high quantities of this flavonoid were used (Kylesova, 2011).

The monograph dedicated to quercetin mentions that the administration of this compound increased the incidence of intestinal and urinary bladder tumours in a study on rats, results that could not be confirmed in later studies. Similarly, it indicates that, in male rats, quercetin may produce an increase, slight but significant, in the incidence of adenomas on renal tumours (JECFA, 1977).

In the *in vitro* tests a mutagenic effect was observed in the Ames test and on human lymphocytes. However, there have been a number of *in vivo* tests, with different animal species, on which this effect was not observed.

As occurs with other compounds with powerful antioxidant activity it is necessary to consider that, in certain conditions of use, quercetin may induce a pro-oxidant effect, widely referenced in scientific literature. This activity, only demonstrated *in vitro*, is attributed to its capacity to auto-oxidate or to undergo an enzymatic transformation, resulting in quinonic derivatives (ortho-semiquinone and ortho-quinon/o-quinone methylurea) with a high affinity to the thiol groups of proteins (Moalin et al., 2012).

It is probable that part of the mutagenic activity detected *in vitro* is the consequence of this pro-oxidant activity which will be counteracted *in vivo* by the action of natural systems of antioxidant defence, by the metabolic transformation of quercetin to derivatives that do not present these activities (methylation), due to the low bioavailability of the free genin or the high affinity of quercetin and its derivatives to the serum proteins (albumin), which is considered a detoxifying mechanism.

Quercetin did not exhibit negative effects on the development and reproduction of the animals treated. However, the International Agency for Research on Cancer (IARC, 1999) describes the possibility of a delay in the fetal growth of rats treated orally with quercetin.

Although the principal objective of the majority of clinical tests performed to date has been the demonstration of its efficiency in the prevention and treatment of different diseases in humans, the results indicate very good tolerability and the absence of significant adverse effects in humans (Okamoto, 2005) (Harwood et al., 2007).

Supplementation of the diet with quantities of between 360 and 1,000 mg/day, for 28 days, did not have any harmful effects, nor were any adverse effects observed following the administration of doses of 760 mg/day, for 3 months (Kiesewetter et al., 2000). Using this concentration and after dividing by a safety factor of 10, the AFSSA estimated a maximum daily dose in the form of food supplements of 75 mg quercetin (AFSSA, 2008).

Recently, in the randomised double-blind, crossover and placebo-controlled clinical test, in which the different aspects relating to cardiovascular health in overweight individuals were assessed (BMI: 25-35 kg/m^2), it was observed that the administration of 150 mg/day of quercetin for 6 weeks, did not affect the biomarkers of the liver and kidney functions, nor the haematological and serum parameters. Nor were any adverse effects observed (Egert et al., 2009).

There are no scientific studies that guarantee the safety of its intake in the form of supplements for pregnant or nursing women or for children.

8.4.5 Conclusion

The Scientific Committee concluded that, based on the information available to date and taking into account the general considerations reflected in this report, the proposal of a maximum daily amount of 75 mg of quercetin submitted by the AESAN is acceptable from a safety point of view for use as a food supplement, provided that it is not administered concomitantly with other supplements that may provide the same compound by hydrolysis, as occurs with rutin, which would imply an increase in the recommended daily dose for this flavonoid.

According to the proposal made by the AFSSA, and as there are no scientific studies to guarantee its safety, it is not recommended for use by pregnant or nursing women or children.

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8.5 Rutin

8.5.1 Proposal

The AESAN has proposed a maximum daily amount of rutin of 150 mg with the warning "not recommended for pregnant women". This proposal is based on the existence of a favourable assessment in France considering that 150 mg/day of rutin provides 75 mg/day of quercetin (AFSSA, 2008).

8.5.2 Chemical nature and sources

Rutin, also known as rutosid is a derivative of quercetin which has a disaccharide (6-O-rhamnosyl-D-glucose= rutinose) on the hydroxyl located on the carbon 3 of the flavonoidic structure (quercetin-3-O-α-L-rhamnopyranosil-(1-6)-ß-D-glucopyranoside). Its molecular weight is 610.52 daltons. By acid hydrolysis one gramme of rutin produces approximately half a gramme of quercetin.

It is found in low concentrations in numerous vegetables, mainly in fruits from the *Citrus* family, cherries and apples. Some species are used as a source for obtaining rutin such as *Sophora japonica*, the floral button of which contain a proportion of 20 % of rutin to dry material, rue (*Ruta graveolens*) or buckwheat (*Fagopyrum esculentum* Moench) with leaves containing 8 % of rutin with respect to dry material and which constitute the main source of rutin in the human diet.

8.5.3 Bioavailability and metabolism

The rutosid as such is not absorbed in the digestive tube. After intake it is hydrolysed in the intestine, partially releasing more simple heterosides (isoquercitrin) or free genin, quercetin, which once absorbed, undergoes a methylation, conjugation and elimination process already described in section 8.4.3 of this document. The quercetin glycosides resulting from the hydrolysis of rutin are similarly hydrolysed in the small intestine using the enzyme systems of the microvilli or alternately they may be included in the enterocytes using a sodium-dependent glucose transporter (SGLT1). In the cell interior they are also hydrolysed by cytosolic beta-glucosidase, similarly changing into quercetin (Murota et al., 2010).

The rutin not absorbed in the small intestine may be transformed by the microflora of the large intestine into quercetin and its metabolites. In healthy volunteers, it has been confirmed that approximately 50 % of the rutin consumed (75 mg) is transformed by the microflora into metabolites of the quercetin from phenylacetic acid types (Manach et al., 2005).

In any case, the bioavailability studies performed on humans indicate that only a small part of the rutin consumed may enter the bloodstream in the form of quercetin conjugates. Therefore, the plasma concentrations of quercetin are lower when they are administered in the form of rutin (expressed in quercetin equivalents) than when they are administered in the form of simple glycosides or pure quercetin. The bioavailability of rutin is approximately 20 % of that corresponding to quercetin glycosides or pure quercetin (Manach et al., 2005).

There is very little intake data for rutin, only one study carried out in Japan estimates the intake of rutin from food at 1.5 mg/day (Kimira et al., 1998). The rutin content in foods may range between 200 and 300 mg/kg.

In the pharmaceutical area, rutosid is used in the preparation of medicines intended to improve vascular function, at present only applied topically.

In the food area, the scientific studies available to date do not sufficiently justify the assumption that the administration of rutin in the form of food supplements to healthy humans may suppose a health benefit or support an approval of the health claim (EFSA, 2010).

8.5.4 Safety

As occurs in the case of quercetin and precisely because it is a source of the same, some studies indicate that the administration of rutin may induce a mutagenic effect (Yu et al., 1986). However, in later studies carried out *in vitro*, on different cell lines (mouse bone marrow –RAE264,7-; human breast cancer; HTC hepatics), a cytotoxic effect is only observed at very high doses (800 µM) and after a long period of exposure (72 and 96 hours) which may be linked to the manifestation of a pro-oxidant activity due to the metabolic transformation of quercetin (Marcarini et al., 2011), although some authors indicate cytotoxic effects at lower concentrations on other cell types which may indicate differences with respect to the metabolic specifics of the different cell lines tested. The assessment of carcinogenicity in hamsters, following the administration of rutin at a concentration of 10 % of the diet, for 735 days was negative (Morino et al., 1982).

There are very few tests that validate the absence of genotoxicity of the rutin (Hardigree and Epler, 1978). It has been confirmed that very high concentrations of rutin (2 x 1,250 mg/kg b.w.), administered intraperitoneally, may induce slight damage to the DNA in the bone marrow cells of *Swiss-Webster* mice, however it is unlikely that a moderate consumption of this flavonoid in the form of an oral complement, may have clastogenic effects on humans, if in addition its low bioavailability is considered (Da Silva et al., 2002).

As regards chronic toxicity, in rats, the administration of 1 % of rutin in diet for 400 days did not induce negative effects on the physiological functions, or affect the organs of the animals treated (Wilson et al., 1947). According to the AFSSA, the toxicity studies carried out on animals *in vivo* did not exhibit negative effects, establishing a LD_{50} given orally of between 9.11 and 17.0 g/kg b.w. (AFSSA, 2008).

Although not directly referred to rutin, but to a derivative of the same, isoquercitrin, a product of its partial enzymatic hydrolysis through the loss of terminal rhamnose, it has been confirmed that supplementation of the diet of *Wistar* rats with concentrations of 0.2; 1 and 5 % of isoquercitrin, for 13 weeks, resulted in certain alterations in the male animals treated with the highest concentration (5 %), not displaying any effects in females. A slight inhibition was observed in weight gain, probably linked to a decrease in triglyceridemia and a fall in red blood cells, the concentration of haemoglobin and the hematocrit index. With the data obtained from this experiment, the authors of the study established a NOAEL for the derivative of the enzymatic hydrolysis of rutin of 539 mg/kg b.w./day (1 % of the diet) in male *Wistar* rats and 3,227 mg/kg b.w./day (5 % of the diet) in females (Hasumura et al., 2004).

There are no clinical tests directed at establishing the safety of the oral use of rutin, however, from the studies performed on humans to confirm the efficiency in the prevention and treatment of different diseases, it appears that doses of more than 500 mg/day do not induce adverse effects on the blood parameters or on the hepatic function (Boyle et al., 2000).

8.5.5 Conclusion

There are few scientific studies available guaranteeing the safety of the rutosid in humans.

Nevertheless, taking into account its presence in numerous foods, its low bioavailability when administered orally and considering that it is a source of quercetin, the Scientific Committee estimates that the daily amount of 150 mg of rutin, equivalent to 75 mg of quercetin, is not likely to have toxic effects on humans.

In accordance with the AFSSA, this Committee indicates that in the case of the use of a mixture of both substances, the total intake quercetin and rutin, must be equivalent to an intake less than or equal to the 75 mg referred to quercetin.

Due to the non-existence of scientific studies that guarantee the safety of its use in special population groups, in accordance with the AESAN proposal, it is not recommended for use in pregnant or nursing women.

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9. Nucleotides

9.1 Adenosine, guanosine, uridine, cytidine, inosine 5-monophosphate and their sodium salts

9.1.1 Proposal

The AESAN has proposed a maximum daily amount of 0.45 g for the sum of adenosine, cytidine, guanosine, inosine and uridine. This proposal has been referred to the industry.

Regulation (EC) No 953/2009 includes the nucleotides (adenosine-5'-phosphoric acid, citidine-5'-monophosphoric acid, guanosine-5'-phosphoric acid, inosine-5'-phosphoric acid, uridine-5'-phosphoric acid and their sodium salts) among the substances that may be added for specific nutritional purposes in foods for particular nutritional purposes (EU, 2009). In particular, it may be added to dietary foods intended for particular nutritional purposes, including foods intended for special medical use.

Nucleotides are also included in Directive 2006/141/EC (EU, 2006) relating to the milk and follow-on milk formulae and its transposition in Spain to Royal Decree 867/2008 (BOE, 2008) permit, on establishing the basic composition of infant formulae when reconstituted in accordance with the manufacturer's instructions, the use of certain quantities of cytidine 5'-monophosphate, uridine 5'-monophosphate, adenosine 5'-monophosphate, guanosine 5'-monophosphate and inosine 5'-monophosphate, provided that the total concentration of nucleotides does not exceed 1.2 mg/100 kJ (5 mg/100 kcal).

In Italy, nucleotides are authorised without the establishment of a maximum daily amount (Italy, 2012).

9.1.2 Characteristics and sources

Nucleotides are structured on nitrogen heterocyclic, puric or pyrimidinic bases, which are the fundamental molecules of nucleotides and nucleic acids, DNA and RNA. The principal pyrimidines forming part of the nucleotides are cytosine, uracil and thymine and the purines are adenine and guanine. These nitrogen-containing bases are transformed into nucleosides, when they bond with a pentose, ribose or 2-deoxyribose, with glycosidic bonds. Subsequently, the formation of the nucleotide occurs after the esterification of the nucleoside pentose with, at least, one molecule of phosphoric acid, which is easily converted into di- or tri-phosphorylated forms depending on the cell energy or metabolic function (Rudolph, 1994) (Hess and Greeenberg, 2012).

Nucleotides are naturally present in all foods of animal and plant origin, mainly as components of the nucleoproteins, although also as free nucleotides and nucleic acids. There are huge differences in the total content depending on the type of food (Gil, 2002).

Although *de novo* synthesis and salvage pathways exist in humans (Traut, 2002), and they cannot be recognised as essential nutrients, the existence of nutritional requirements for these compounds was recognised some time ago. Thus, a number of authors (Van Buren and Rudolph, 1997) (Carver, 1999), based on these requirements, gave them the category of "conditionally essential" in certain special situations such as rapid growth, malnutrition, infection or injury (Rudolph et al., 1990) (Uauy et al., 1996) (Carver, 1999) (Fontana et al., 2010).

The intake of exogenous nucleotides is the result of their wide distribution in the food we consume in our normal diet, especially those foods which contain cellular elements and nucleoproteins. For example, the viscera (heart, liver, etc.) and shellfish and molluscs are good sources of nucleotides (Kojima, 1974) (Clifflord and Story,

1976) (Barness, 1994). The total content of RNA in viscera ranges between 50 and 400 mg/100 g, from 80 to 350 mg/100 g in sea food and from 140 to 490 mg/100 g in dry pulses. Milk also contains significant quantities of nucleotides, especially human milk (Gil, 2002) (Verkerk and Gil, 2009). Table 3 lists the nucleotide content of different foods (Verkerk and Gil, 2009), although this content may vary among individuals and within the same individual at different moments.

Table 3. Nucleotide content of different foods (mg/100 g)

Food	Purines			Pyrimidines		Reference
	AMP	GMP	IMP	CMP	UMP	
Dried whey	19	0	4	270	1	(Mateo, 2005)
Protein isolated from whey	0	0	159	34	89	(Mateo, 2005)
Shangai crab	75	2	34	-	-	(Chen and Zhang, 2007)
Beef	-	2.2	163	-	-	(Sugita, 1990)
Pork	-	3.7	186	-	-	(Sugita, 1990)
Chicken	-	2.2	115	-	-	(Sugita, 1990)
Whale	-	5.3	326	-	-	(Sugita, 1990)
Horse mackerel	-	0	323	-	-	(Sugita, 1990)
River fish or Ayu	-	0	287	-	-	(Sugita, 1990)
Sea bass	-	0	188	-	-	(Sugita, 1990)
Sardine	-	0	287	-	-	(Sugita, 1990)
Bream	-	0	421	-	-	(Sugita, 1990)
Pike	-	0	227	-	-	(Sugita, 1990)
Chub mackerel	-	0	286	-	-	(Sugita, 1990)
Chum salmon	-	0	235	-	-	(Sugita, 1990)
Tuna	-	0	286	-	-	(Sugita, 1990)
Pufferfish	-	0	287	-	-	(Sugita, 1990)
Eel	-	0	165	-	-	(Sugita, 1990)
Dried Bonito fish	-	0	630 -1,310	-	-	(Sugita, 1990)

It is important to note that the proteins of unicellular organisms have concentrations of nucleic acids that are seven times higher than those in meat (Ingledew, 1999). Thus, yeasts are an excellent source of nucleotides (Tibbets, 2002) (Li et al., 2007). Therefore, the yeasts produced for the manufacture of bread and beer are a good source of nucleotides for use in the preparation of supplements.

9.1.3 Nutrition and metabolism

Few studies are available on the intake of nucleotides in adult diets. If an intake of 500 g/day or more of any product of animal origin is assumed (terrestrial or aquatic), and that this is prepared with permitted flavourings containing concentrated sources of nucleotides, intakes will be 1,000-1,500 mg/kg b.w./day, equivalent to between 17 and 25 mg/kg b.w./day. These figures are estimates and no data has been published to date. Therefore, a maximum dietary intake of nucleotides of 25 mg/kg b.w./day may be inferred.

Nucleotides, as mentioned, are synthesised *de novo* from metabolites such as L-glutamine, aspartate and glycine, mainly in the liver, they are recovered from the degradation of RNA and DNA and we consume them in our diet in the foods containing them (Grimble and Westwood, 2001). The relative importance of these processes for the maintenance of a corporal reserve of nucleotides and nucleosides varies with both the tissue and the cell cycle phase (Fairbanks et al*.,* 1999) (Grimble and Westwood, 2001). Exogenous intake acquires special relevance in the case of tissues and cells with a high "turnover", such as those that grow quickly or those linked to immunity. The enterocyte is particularly dependent on exogenous nucleotides.

Nucleotides are not absorbed as such but, after dephosphorylation, as nucleosides through active and passive mechanisms (Ngo et al., 2001) (Scharrer et al*.,* 2002) and in this way they are metabolised. The enterocyte captures more than 90 % of the exogenous and endogenous nucleosides and the bases. The metabolites are used by the salvage pathways, the nucleotides resynthesising and therefore meeting the body's requirements.

The degradation of the nucleotides depends on whether the constituent bases are puric or pyrimidinic. Nucleotides are purines, after the separation of the phosphate and ribose, the nitrogen-containing bases are oxidised. In humans, the final product of the metabolism is uric acid which is excreted in urine and that, on reaching serum levels of more than 7 mg/dl, may precipitate forming urate crystals in the articulations, in different tissues and in blood. The pyrimidines, unlike the purines, undergo the break of the ring, the end products of the catabolism are beta-amino acids (beta-alanine), some dipeptides such as anserine and carnosine and beta-amino isobutyrate, in addition to ammonia and carbon dioxide (Verkerk and Gil, 2009).

9.1.4 Safety

In this section we list the information contained in a report conducted in May 2009 by Verkerk and Gil on the safety of supplementation with nucleotides in humans.

In 1974, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) approved the use of 5'-ribonucleotides as food additives (JECFA, 1975). This assessment was the basis for the approval of 5'-ribonucleotides for inclusion in the list of foods for particular nutritional use (PARNUTS) listed in Directive 2001/15/EC (EU, 2001).

Based on the toxicological studies available on reproduction, teratogenicity and short- and long-term toxicity and observation studies in humans, it has not been thought necessary to establish an ADI (JECFA, 1975). That is, on the basis of all the available data, the total daily intake of these substances in foods does not pose a risk to health.

One of the key aspects studied for the assessment refers to the uric acid excretion levels following the intake of purine nucleotides. The administration of disodium 5'-ribonucleotide (DSRN) in doses of up to 67 mg/kg b.w., permits a NOAEL to be established for purine nucleotides of 33 mg/kg b.w. (2,000 mg/day in an adult). Logically

and given the end products of its metabolism, the pyrimidine nucleotides will have a significantly higher LOAEL (Verkerk and Gil, 2009).

Studies available on the administration of nucleotides, to healthy humans (McNaughton et al., 2006, 2007) and to patients (Uauy et al., 1996) (Carver, 1999) (Schlimme et al., 2000) (Grimble and Westwood, 2001) (Schaller et al., 2007), did not describe any adverse effects.

The intake of nucleotides in adults is very variable and ranges depending of the different cultures and also on the time. From the maximum intake studies, some 25 mg/kg b.w. (1,500 mg/day) have been estimated although there are no precise estimations. At present, the safety of the total intake of nucleotides depends more on the intake of purines than of pyrimidines due to their catabolism to urate (Verkerk and Gil, 2009).

In the United Kingdom, supplements of nucleotides on the market recommend a dose, depending on the individual, of between 2.25 and 6.75 mg/kg b.w.

The European Union has authorised nucleotides as food additives that enhance the *umami* flavour and has not established a maximum level although it establishes the principle of *quantum satis* (Fuke and Konosu, 1991) (EU, 1995) (Oruna-Concha et al*.,* 2007).

9.1.5 Conclusion

The data available at present establish a very low toxicity for exogenous nucleotides, which is supported by the non-specification of an ADI for them.

For the intake of purine nucleotides, a NOAEL has been established of approximately 33 mg/kg b.w. (~2,000 mg/day), given their possible influence on uric acid blood levels (Verkerk and Gil, 2009). A NOAEL has not been established for the pyrimidine nucleotides.

The Scientific Committee concludes that the maximum daily amount proposed by the AESAN of 0.45 g/day (equivalent to 7.5 mg/kg b.w. for an adult of 60 kg) for the sum of adenosine, cytidine, guanosine, inosine and uridine, is acceptable from the safety point of view for use as a food supplement.

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10. Polysaccharides and Oligosaccharides

10.1 Beta-glucans

10.1.1 Proposal

The AESAN has proposed the inclusion of the beta-glucans from oat and barley in Royal Decree 1487/2009 (BOE, 2009) with a maximum daily amount of 4 g per day. This proposal is based on the existence of two authorised health claims for beta-glucans derived from positive opinions of the EFSA in which it is established that it has been proved that beta-glucans at a dose of 3 g per day contribute to maintaining normal blood cholesterol levels and at doses of 4 g/30 g of carbohydrates, they contribute to reducing postprandial glycaemia (EFSA, 2011).

In Italy, beta-glucan is authorised in food supplements without the establishment of a maximum daily amount (Italy, 2012).

In Denmark it is authorised in food supplements with a total maximum amount that must not exceed 200 mg per daily recommended dose of 1.3-1.6 beta-glucan (Denmark, 2011).

10.1.2 Characteristics and sources

The beta-glucans are a heterogeneous group of non-amylase polysaccharides, a D-glucose monomer compound linked through β glycosidic bonds (Zekovic et al., 2005). The macromolecular structure of the beta-glucan depends on the source and the method of extraction. The simplest structure is in the form of linear unbranched β-(1,3)-D-glucan, which is found in prokaryotes and eukaryotes (McIntosh et al., 2005). Another relatively simple structural time is mainly found in the non-lignified cell walls of cereal grain, consisting of linear β-(1,3;1,4)-D-glucan structures (Wood, 2007). The glucans of barley, oats or wheat are found in the cell walls of the endosperm, whereas in the case of sorghum or other cereals they are concentrated in the aleurone layer. The branched structures of beta-glucans consist of β-(1,3)- or β-(1,4) glucan molecules bonded in side branches to (1,2)- or (1,6)-β-glucopyranosyl groups (Barsanti et al., 2011). The branched structures are the main structural components of the cell walls of yeast, fungi and some bacteria or algae (Volman et al., 2008).

Sources of beta-glucans are very varied. One of the most common sources of β-(1,3) D-glucans for use as a supplement comes from the cell wall of the yeast *Saccharomyces cerevisiae*. However, the β-(1,3) or β-(1,4) glucans are also extracted from the bran of some cereals such as oats and barley, and to a lesser degree from rye and wheat. The β-(1,3)-D-glucans from yeast are often insoluble. Those extracted from cereals may be soluble or insoluble. Other sources include some types of algae and various species of fungi, including *reishi* (*Ganoderma lucidum*), *shiitake* (*Lentinus edodes*), *maitake* (*Grifola frondosa*), *Schizophyllum commune*, *Trametes versicolor*, *Inonotus obliquus* (chaga mushroom) and *enokitake* (*Flammulina velutipes*).

The NDA Panel of the EFSA has favourably assessed different health claims relating to the beta-glucans:

- Beta-glucans contribute to the normal maintenance of plasma cholesterol levels**.** The EFSA indicates that this claim may be used when the food contains at least 1 g of beta-glucans from oats, oat bran, barley, barley bran, or a mixture of these sources per measured portion. The EFSA indicates that to be able to use this claim, consumers must be informed of the beneficial effect obtained from the daily intake of 3 g of beta-glucans from the above sources.
- The intake of beta-glucans from oats or barley as part of a meal contributes to reducing the postprandial glucose peak after the intake of this food. According to the EFSA, the claim may only be used for those

foods that contain at least 4 g of beta-glucans from oats or barley per 30 g of carbohydrates available in a measured portion as part of a meal. To be able to make this health claim, the consumer must be informed of the beneficial effect obtained from the intake of beta-glucans from oats or barley in the same meal.

- Beta-glucan from wheat has been demonstrated to reduce blood cholesterol levels. The EFSA rules that for the use of this claim, the consumer must be notified of the beneficial effect obtained from a daily intake of 3 g of beta-glucan from oats. This claim may be used in foods that provide at least 1 g of beta-glucan of oats per measured portion.

10.1.3 Nutrition and metabolism

Once consumed, the beta-glucans are practically not absorbed in the gastrointestinal tract due to their large molecular size and the inability of human digestive enzymes to hydrolyse them. Studies in animals have shown that a small fraction of beta-glucans can be taken up in the intestine by macrophages and internalised in the enterocyte in small fractions of beta-glucans that may pass to the general circulation and be transported to the endothelial reticular system, modulating immunity. However, the results of these studies must be interpreted with caution as the majority of the studies were *in vitro* and on animals used for research (Chan et al., 2009). There is not sufficient evidence to suppose that these mechanisms of action will occur in humans. Therefore, it is unlikely that the beta-glucans exercise direct systemic effects. There are no known adverse effects in relation to the possible interactions between the beta-glucans and the mucus-containing cells of the small intestine. On reaching the colon, the beta-glucans are fermented by intestinal microbiota producing H_2, CO_2, CH_4 and short-chain volatile fatty acids. These fatty acids acidify the intestinal content and act as modulators of the intestinal microbiota composition as they enhance the growth of certain microbial species. In addition, the volatile fatty acids resulting from fermentation are partly used as fuel in the enterocyte and partly they reach the general circulation producing potentially beneficial systemic effects.

Different foods contribute to the total intake of beta-glucans, including: oats, barley, wheat, rye and beans.

The intake of beta-glucans in Spain is, to a large extent, unknown. Recently, an estimate of intake was made in the United Kingdom, based on the beta-glucan content in different foods and the intake in that country (EFSA, 2011). It was estimated that an adult male consumes an average of 726 mg/day, where the 97.5th percentile is 4,674 mg/day (9 and 52 mg/kg b.w./day, respectively). For children aged 1.5 to 4.5, the mean intake of beta-glucans is estimated at 281 mg/day (20 mg/kg b.w./day), where the intake in the 97.5th percentile is 115 mg/kg b.w./day.

The intake of beta-glucans in food is traditionally very high in some countries such as those belonging to the Maghreb Union (Morocco, Algeria, Tunisia) in which barley is an ingredient in many traditional dishes and foods (bread, soup, porridge, etc.), and therefore the mean intake is estimated at 172 g/day for Morocco, estimating that this contributes to a mean intake of beta-glucans to the order of 6 g/day (Ferrante et al., 2001).

10.1.4 Safety

The FDA has assessed the safety of the use of beta-glucans from barley. In 2006, a product rich in beta-glucans isolated from barley was considered as a GRAS substance (FDA, 2006), for use in all foods except infant formulas.

Beta-glucan based products from oats (*Oatrim*®, *OatVantageT*®) have been marketed for more than 15 years in the United States and other countries as an ingredient to replace fats in foods without any undesirable effects

having been reported since their introduction. Following its safety assessment, in this country it is also authorised for use in many foods including meats, processed meats, sauces for dressings, mayonnaise, processed cheeses, pastries, yoghurt, ice-cream, frozen desserts, snacks, margarines, sauces for spreading, frozen starters, etc.

Two oral toxicity studies of 28 days, one on *Wistar* rats (Delaney et al., 2003a) and the other on mice (Delaney et al., 2003b), have not displayed adverse effects on the weight of the animal, the weight of the organs, the general condition of the animal, neurobehavioural changes, different biochemical determinations and haematological alterations, after the administration of beta-glucans from barley. In these studies, a NOAEL of 5.6 g of beta-glucans/kg b.w./day was observed in rats and between 19 and 23.6 g/kg b.w./day in mice (females and males, respectively).

An oral toxicity study for 28 days performed on rats has shown that high doses of beta-glucans from barley (5.9 g/kg b.w./day) are well tolerated as regards the general condition of the rats, the neurobehavioural effects, growth, intake of water and energy, haematology, clinical biochemistry, weight of the organs and apparent pathological alterations. This dose is 100 times higher than that suggested by the FDA to reduce cholesterol levels in humans (FDA, 2005).

There are no studies in humans with the specific purpose of assessing safety and tolerance to the use of beta-glucans from oats, barley or wheat. However, different beta-glucan concentrate-based products have been included in the food matrices (cup cakes, cereals, bread and drinks). Clinical tests on those foods that contain beta-glucan-based products also displayed a good level of acceptability and tolerance by participants.

More than 150 clinical tests have been reported on humans, administering products containing beta-glucans from oats, barley and other sources. The objective of the majority of these studies was to examine the effect of the intake of beta-glucans on cholesterol or glucose levels. Although the primary objective of these studies was to analyse the efficiency, some included clinical observations such as the appearance of adverse effects, providing the indirect opportunity to assess safety and tolerance. A number of the clinical tests were double-blind, placebo-controlled tests. These clinical tests are summarised in recent GRAS notifications from the FDA for beta-glucans from barley (FDA, 2011), the report on health claims from the EFSA (2010a, 2010b), the FDA health claim (1997, 2002) and various meta-analyses (Ripsin et al., 1992) (Brown et al., 1999) (Whitehead et al., 2008) (Othman et al*.,* 2011) (Tiwari and Cummins 2011). These reports and studies did not indicate the existence of problems of safety derived from the use of beta-glucans. Recently the studies were reviewed, and the conclusion reached that doses of beta-glucans of up to 10 g/day are well-tolerated. In these studies good long-term adscription to the treatment is observed, confirming the idea of good tolerance levels for this dose (Cloetens et al., 2012).

Given that the beta-glucans are fermented by bacteria in the colon, their intake may cause flatulence, diarrhoea, abdominal distension, abdominal colic pain, especially when the increase in intake is sudden (Beer et al., 1995) (Behall et al., 1997).

The EFSA has issued the following positive scientific opinions on the safety of different sources of beta-glucans as new ingredients:

- Beta-glucans from yeast of *Saccharomyces cerevisiae* (EFSA, 2011). In the form of food supplements at maximum doses of 375 mg/day, and of 600 mg/day in foods for particular nutritional uses. Its use is not authorised for use in infant and follow-on formulas.
- Chitin-glucan from *Aspergillus niger* (EFSA, 2010c). In the form of a supplement at a dose of 2 to 5 g/day.
- Mycelium extract of *Lentinula edodes* (EFSA, 2010d). Authorise as a supplement or ingredient in yoghurts, soft drinks, processed or cooked foods and baked products. In doses of 2.5 ml of *Lentinex*® which contain 1 mg lentinan (beta-glucan)/ml, that corresponds to 41.7 µg/kg b.w./day for an individual weighing 60 kg.

10.1.5 Conclusion

The EFSA has issued favourable reports on the use of various sources of beta-glucans as novel ingredients or as supplements, confirming the safety of its use at doses ranging from 375 mg/day to 5 g/day, depending on the beta-glucan source and the expected use (as an ingredient or as a supplement).

No adverse effects were indicated associated with the use of these compounds in animals used in research and the clinical tests performed on humans did not reveal any significant adverse effects either. It should be noted that many clinical studies in humans have been carried out with the objective of demonstrating the efficiency of the beta-glucans especially on the lipid profile and the glucose metabolism, and that only incidentally and following the intake of very high doses have any gastrointestinal effects such as flatulence, abdominal upset or diarrhoea been mentioned. These effects which are derived from the colonic fermentation of this type of fibre are produced briefly when there is a sudden increase in the intake of fermentable fibres.

The Scientific Committee concluded that the proposal of the AESAN for an intake of a maximum daily amount of 4 g de beta-glucans is acceptable from the safety point of view in its use as a food supplement.

In addition, taking into account that the dose that the EFSA considers efficient for reducing postprandial glycaemia for the beta-glucans from oats or barley is 4 g per intake, it is considered prudent that fibre supplements should not provide quantities of more than 4 g of beta-glucans per intake as higher doses have not been demonstrated to be more efficient in reducing postprandial glycaemia.

Therefore, the Scientific Committee of the AESAN recommends that the following warnings are displayed on the packaging of beta-glucan-based food supplements:

- When taking this type of preparation, the intake of other dietary fibre-based food supplements should be avoided.
- Given that the fibre may interact with some medicines, altering their efficiency, please seek medical advice if taken at the same time as other drugs.

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10.2 Chitosan obtained from shells of crustaceans

10.2.1 Proposal

The AESAN has proposed including chitosan obtained from the shells of crustaceans in Royal Decree 1487/2009 (BOE, 2009) at a maximum daily amount of 3 g with the warning that "excessive consumption may cause intestinal upset". This proposal is based on the existence of a health claim issued by the EFSA establishing that chitosan at a dose of 3 g per day contributes to maintaining normal blood cholesterol levels (EFSA, 2011). In addition, France has issued a favourable assessment for chitosan at a concentration of 2 g/day (AFSSA, 2008).

10.2.2 Characteristics and sources

Chitosan is a cationic polysaccharide consisting of deacetylated and acetylated units of glucosamine (units of β-(1→4)-2-acetamide-D-glucose and β-(1→4)-2-amino-D-glucose). It is not an intrinsic component of food and is obtained by deacetylation of the chitin from crustacean shells. It is also obtained from the fungi *Agaricus bisporus* and *Aspergillus niger*.

Chitosan has a wide range of applications in the cosmetic, pharmaceutical and food industries. In the food sector it is mainly used as a source of dietary fibre but, due to its bioavailability and non-toxic nature, it is used as a separating, absorbing and clarifying agent (Knorr, 1991) (Pinotti et al., 1997). Moreover, its potential for use in canning foods has been observed, particularly in food coverings or films (Tual et al., 2000). Other properties that favour its use are its antioxidant, antimicrobial and oxygen barrier functions (Jeon et al., 2000, 2001).

The European Union accepted chitosan from fungi as a novel food ingredient in 2008.

10.2.3 Nutrition and metabolism

Chitosan is not absorbed at intestinal level but it affects the metabolic responses and contributes to reducing the absorption of cholesterol and glycaemia (Kao et al., 2012). The positively charged amino groups of chitosan appear to interact with the negative charges of the biliary fatty acids or fatty acids, reducing their intestinal absorption, whereas the absorption of cholesterol, triglycerides and sterols us due to hydrophobic interactions with chitosan (Chen et al., 2011).

In addition, a study conducted to clarify the target organ and the action mechanisms of chitosan on transgenic mice has shown that chitosan, in the brain and stomach, activates the peroxisome proliferator-activated receptors (PPAR), key receptors for regulating the metabolism of fatty acids and glucose (Kao et al., 2012).

The EFSA considers that it has been scientifically proven that daily intakes of 3 g of chitosan "contribute to maintaining normal blood cholesterol levels" (EFSA, 2011).

10.2.4 Safety

The toxicity of chitosan has been widely studied.

In studies performed on mice and rats fed with 2 and 5 % chitosan, a negative effect was observed in growth and weight gain (AFSSA, 2008) together with a reduction in the absorption of vitamins B_2 and B_{12} (Rodrigues et al., 2011, 2012), a decrease in the intestinal absorption of calcium, magnesium and iron (Deuchi et al., 1995)

and an increase in the loss of calcium in the urine, and consequently a loss in bone mass (Wada et al., 1997) (Yang et al., 2002).

The studies in animals also suggested that chitosan reduces the absorption of the liposoluble vitamins, A and E (AFSSA, 2008).

In a study lasting four weeks on mice with polyhypovitaminosis, fed a diet containing 0.4 and 0.9 % chitosan, no significant effects were observed in the liver content of vitamins C, B_1, B_2 and A in the animals treated, in the blood concentration of vitamin B_2, or in the urinary excretion of thiamine and riboflavin. However, a higher dose of chitosan produced a decrease in the plasma levels of vitamin E (Vrzhesinskaia et al., 2011).

Studies in humans

In random controlled studies on overweight and obese adults, given chitosan for 4 weeks, in comparison with the control group, statistically significant weight losses, decreases in total cholesterol and falls in systolic and diastolic blood pressure were observed (Mhurchu et al., 2005) (Jull et al., 2008). However, on reviewing the quality of the tests, it was observed that the strictest tests suggested a weak effect of chitosan on weigh loss (Mhurchu et al., 2005).

According to the bibliographic review carried out by the AFSSA in 2008, the clinical studies carried out did not display any harmful effects from chitosan at intake doses of between 1 and 3 g/day for a supplementation period of between 28 days and 1 year, except for some cases of digestive symptoms (AFSSA, 2008). It also warns that doses above 3 g/day taken continuously may reduce the bioavailability of liposoluble vitamins. However, in a later study carried out with oral doses of 6.75 g/day of chitosan administered for 8 weeks, no differences were observed in the levels of vitamin A, E, D and carotenes with respect to the group who received the placebo (Tapola et al., 2008).

In addition, the AFSSA indicates that the chitosan may increase the risk of allergies as it stimulates the intestinal absorption of the antigens, although this case has not been confirmed to date. Nevertheless, a case of an anaphylactic reaction to chitosan has been reported following the oral intake of this compound (Kato et al., 2005).

A case was also reported in which the anticoagulant effects of warfarin were increased with the intake of chitosan, making caution necessary when taking anticoagulants (Huang et al., 2007).

10.2.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum amount of 3 g/day of chitosan is acceptable from the safety point of view for use as a food supplement.

Nevertheless, the Scientific Committee of the AESAN recommends that the packaging contains the warning that excessive consumption of chitosan may cause intestinal upset.

Given that the fibre may interact with some medicines, altering their efficiency, please seek medical advice if taken at the same time as other medicines.

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10.3 Fructooligosaccharides (FOS)

10.3.1 Proposal

The AESAN proposes a maximum daily amount of 9 g of fructooligosaccharides (FOS) or the sum of FOS plus inulin, with the warning that "excessive consumption may cause intestinal upset".

This proposal is based on the existence of a favourable assessment for the FOS in France, indicating that "a daily intake of 8 g of the oligofructose mixture enriched with inulin (4 g of oligofructose and 4 g of inulin) is free of risk and there is no scientific evidence to oppose an additional intake of FOS in the diet (AFSSA, 2006, 2008). In Italy, fructooligosaccharides and inulin are authorised in food supplements without the establishment of a maximum daily amount (Italy, 2012).

The industry were consulted with regard to the proposal and indicated that the composition and effects of the FOS and inulin are similar and that both are often used in the same product. Therefore, the proposal for a maximum daily amount for the sum of FOS and inulin is justified.

According to Royal Decree 867/2008 (BOE, 2008), approving the specific Technical and Sanitary regulation regarding infant formula and follow-on formula, fructooligosaccharides and galacto-oligosaccharides may be added to infant formula. In this case, the content must not exceed 0.8 g/100 ml of the combination of 90 % of oligogalactosyl lactose and 10 % of oligofructosyl saccharose.

10.3.2 Characteristics and sources

The fructooligosaccharides or fructose oligosaccharides are fructose polymers with a degree of polymerisation of less than 10. They can be obtained by the hydrolysis of inulin, through the inulinase enzyme, producing in this case oligofructoses (n= 2 to 9), or by *trans*-fructosylation by the enzyme beta-fructosidase, able to bond traces of fructose to the sucrose molecule, with the resultant fructooligosaccharides (FOS). It should be noted that although strictly speaking there is a specific denomination according to the origin, in many cases both terms are used as synonyms. FOS may be accompanied, as in the case of inulin, by a small fraction of simple sugars, including glucose, fructose or sucrose, obtained as secondary compounds.

The FOS are a type of short-chain soluble dietary fibre, of low molecular weight, considered since 1995 by the European Union as food ingredients. In Europe, it is estimated that the average intake of fructans (inulin and/or FOS) per inhabitant is between 3 and 11 g per day, and in the United States, between 1 and 4 g (Van Loo et al*.,* 1995). According to the report issued by the AFSSA (2008) the average intake is around 5 g per day.

The FOS are used as food ingredients and are included in a large variety of foods, as dairy products, desserts, breads, cereals, fruit preparations, dietary products, meal replacements, meat products, etc. However, the use of FOS is not possible in acid products with a long shelf life such as soft drinks, fruit jams, as they hydrolyse slowly releasing fructose (Coussemment, 1999).

It is mainly added to foods to enrich the fibre content of the food with prebiotic effects, or as a low calorie load agent. It is normally added in quantities of between 3 and 6 g per portion although in exceptional cases it may reach 10 g (Coussement, 1999). When the addition of this type of fibre, alone or inulin enriched, is to promote a prebiotic effect, the quantities used are in the order of 1 and 6 %, equivalent to between 3 and 8 g per portion (Coussement, 1999).

On other occasions the FOS are added to the food as sugar substitutes. The oligomer chains forming the FOS have physiochemical properties similar to those of sucrose or glucose syrup, although they are more soluble and

have a lower sweetening effect than sucrose, approximately 30 to 50 % in comparison to sugar. Therefore, it is difficult to use FOS alone as substitutes for sugar and they are often combined with strong sweeteners to obtain the required level of sweetness.

10.3.3 Nutrition and metabolism

The FOS (as with inulin) resist enzymatic digestion in the upper gastrointestinal tract, arriving, almost intact, at the colon, where they are completely fermented by the colon microbiota. The bacterial fermentation of the FOS takes place in the proximal colon (unlike that of inulin which is metabolised in the distal part) producing gases and short-chain organic acids (lactic, acetic, propionic and butyric).

One of the main functions of the FOS in the body is its prebiotic effect, selectively stimulating the growth and activity of different species of bifidobacteria. The majority of studies carried out to determine this effect confirm a significant increase in bifidobacteria with respect to other species of intestinal microbiota (Gibson et al., 1995) (Bouhnik et al., 1996) (Kleessen et al., 1997) (Kruse et al., 1999) (Bouhnik et al., 2007). To achieve this effect in adults, an intake of between 2.5 and 10 g per day of fibre is required (Kelly, 2008). However, the interindividual response to the same dose of this fibre appears to be highly variable in terms of the increase of the population of bifidobacteria.

Other studies also indicate additional beneficial effects, including the improvement of the intestinal transit rate (Kleessen et al., 1997) (Coussement and Frank, 2001) (Kelly, 2008), the increase in the absorption of calcium and other minerals (Van den Heuvel et al., 1999) (Abrams et al., 2005) (Kelly, 2009), the improvement in the metabolism of circulating lipids (Meyer, 1999) (Van Dokkum et al., 1999) (Causey, 2000) (Kelly, 2009) and the prevention of certain types of cancer (Koo and Rao, 1991) (Roland et al., 1994) (Delzenne et al., 1995) (Gallaher et al., 1996) (Taper et al., 1997).

In 2006 the AFSSA, based on studies in animals used for research and in humans, accepted the claim that "8 g/day of inulin enriched oligofructose increases calcium absorption" although only under certain conditions. Subsequently in 2011, the EFSA issued a scientific report on health claims relating to the FOS (obtained by synthesis from sucrose) concluding that it was not possible to establish a cause and effect relation between the intake of these fructans and a fall in the pathogenic intestinal microbiota, changes in the production of short-chain fatty acids and pH in the intestinal tract, changes in the intestinal function, reduction of gastrointestinal upsets, increase in the absorption of calcium and magnesium, maintenance of normal blood concentrations of LDL cholesterol, maintenance of normal blood levels of triglycerides (EFSA, 2011).

The colonic metabolism of the FOS may cause intestinal upset and effects such as flatulence, increase of osmotic pressure, abdominal distension, diarrhoea, etc., in certain segments of the most sensitive population. Clinical studies carried out by Rumessen and Gudmand-Hoyer (1998) concluded that the FOS, with the shortest chain and metabolised in the proximal colon, are more related to the appearance of secondary intestinal effects than inulin (with a higher degree of polymerisation).

10.3.4 Safety

The safety of FOS for use as an ingredient has been assessed by the health authorities in a number of European countries and in the United States. As a result, the use of FOS as an ingredient is widely accepted without any restrictions in numerous food formulas (Pascal, 2008). An ADI has not been established for this type of substance and in the United States, it has been considered a GRAS substance since 1992 (Kolbye et al., 1992).

A number of acute and chronic toxicity, carcinogenicity and genotoxicity studies have been conducted on animals used for research and have demonstrated that the FOS, even at high doses, do not have consequences on mortality, morbidity, toxicity (on target organs, as regards development and reproduction) nor on carcinogenicity (Takeda and Niizato, 1982) (Clevenger et al., 1988) (Carabin and Flamm, 1999) (Pascal, 2008). Similarly, clinical studies carried out using inulin and/or FOS, both on normal subjects and on patients, agree on the safety of these compounds (Roberfroid, 1993) (Coussement, 1999) (Pascal, 2008).

In general, the intake of up to 20 g of fructans is not considered to produce significant secondary effects. Nevertheless, some people experience intestinal upset after the intake of small quantities of these fibres (Carabin and Flamm, 1999). There is a wide interpersonal variability in the doses at which the secondary effects appear and it also depends on the food in which the fibre is contained (Coussement, 1999). According to the study carried out by Cadranel and Coussement (1995) on FOS tolerance in children aged between 10 and 13 years old, doses of up to 9 g per day from drinks or confectionary did not cause secondary effects.

The Scientific Committee on Food (SCF, 1997) recognised that there may be laxative effects with doses of FOS of more than 32 g/day, but that it is unlikely that an intake of up to 20 g/day would induce any undesirable effects. Above 30 g/day, flatulence is observed and digestive upsets may be stronger and doses of 50 g/day produce abdominal pain and diarrhoea (Briet et al., 1995). Nevertheless, in individuals over the age of 60, an increase was observed in the digestive upsets after doses of 8 g/day of FOS (Bouhnik et al., 2007). In the case of patients with irritable bowel syndrome, daily doses of 20 g of FOS have been shown to cause symptoms of intestinal upset for the first days but these symptoms are reduced with continuous treatment for 12 weeks (Olesen and Gudmand-Hoyer, 2000). To the contrary, a supplementation study with FOS (*versus* placebo) in patients with intestinal adenoma did not produce any adverse intestinal effects (Boutron-Ruault et al., 2005).

To obtain FOS through enzyme hydrolysis from inulin, the inulinase enzyme, isolated from *Aspergillus niger* is normally used. This enzyme is also widely used by the food industry, for example in obtaining fruit juices. The JECFA (Joint FAO/WHO Expert Committee on Food Additives) has assessed the safety of this enzyme concluding that it is safe and that consequently it is not necessary to specify an ADI. Similarly, in the United States, inulinase from *Aspergillus niger* has been considered a GRAS substance since 1973. In 1990 in Denmark, the safety of inulinase was also assessed and its use was accepted for the production of oligofructose.

The EFSA, in 2004, in response to a request to assess the safety and the suitability of the addition of FOS in doses of 1.5 to 3 g/l to infant formulas, concluded that there was no evidence of benefits to the infants that could be attributed to the addition of FOS under the conditions specified and that, to the contrary, the risk of the appearance of dehydration could not be rejected due to an increase of the frequency of diarrhoea processes (EFSA, 2004).

10.3.5 Conclusion

Although in general, the FOS are usually well-tolerated, even at doses of 20 g/day, there is a wide interpersonal variability in the doses at which the secondary effects associated with colonic fermentation appear and some people may suffer at lower doses.

Different toxicity assessments of the FOS have been made using animal models, including acute and chronic toxicity and carcinogenesis without results that display a risk to consumer health. Nor do the clinical studies carried out reveal any toxic effects associated with the consumption of these compounds. Similarly, the safety assessment of inulin in different countries has concluded that there is an absence of undesirable effects associated with its use as a source for obtaining fructans.

In view of the above, the Scientific Committee of the AESAN considers that, based on the information available to date and taking into account the general considerations reflected in this report, the proposal of a maximum amount of 9 g/day of fructooligosaccharides (FOS) is acceptable from the safety point of view for use as a food supplement.

Nevertheless, the Scientific Committee of the AESAN recommends that the following warnings are displayed on the packaging of FOS-based supplements:

- Do not exceed a daily dose of 9 g of FOS/day, or the sum of FOS and inulin, as excessive consumption may cause stomach upsets.
- When taking this type of preparation, the intake of other dietary fibre-based food supplements should be avoided.
- Given that the fibre may interact with some medicines, altering their efficiency, please seek medical advice if taken at the same time as other medicines.

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10.4 Galacto-oligosaccharides (GOS)

10.4.1 Proposal

The AESAN has recommended the inclusion of the galacto-oligosaccharides in Royal Decree 1487/2009 without specifying a maximum daily amount.

In Italy, the GOS are authorised in food supplements without the establishment of a maximum daily amount (Italy, 2012).

10.4.2 Characteristics and sources

The galacto-oligosaccharides (GOS) are a mixture of di- to octosaccharides formed by 1 to 7 units of galactose bonded to the extreme glucose molecule reducer. In GOS preparations, the O-β-D-galactopyranosyl-(1-4)-O-ß-D-galactopyranosyl-(l-4)-β-D-glucose trisaccharide is the main saccharide. The molecular weight of the individual oligosaccharides ranges between 342 (disaccharide) and 1,315 (octasaccharide) daltons. The mean molecular weight of the GOS fraction is 522.28 daltons (FDA, 2008).

The GOS are produced by the action on the lactose of the beta-galactosidases with the activity of transgalactosylation. The glycosidic bonds between two units of galactose are principally bonds of the β(1-4) type (such as 4'-galactosyl-lactose, 4'-GOS) when the beta-galactosidases derived from *Bacillus circulans* are used (Mozaffar et al., 1984) or from *Cryptococcus laurentii* (Ozawa et al., 1989), and of the type β(1-6) (such as 6'-galactosyllactose, 6'-GOS) when the enzymes derived from *A. oryzae* or *Streptococcus thermophilus* are used (Matsumoto, 1990). Normally more than 55 % of the lactose which is used as a substrate is converted into GOS (Ishikawa et al*.,* 1995) (Pérez-Conesa et al*.,* 2004). The type of bonds (β(1-4) or β(1-6)) and therefore the microbial source of beta-galactosidase, that generates them, has an influence over the use of the substrate by the intestinal bacteria (Depeint et al*.,* 2008).

The GOS have been used for the last four decades as food ingredients in Europe and Japan (Crittenden and Payne, 1996) (Sako et al*.,* 1999) (Nakakuki, 2003). In 2003 the annual production of GOS in the world was around 15,000 tm, and in Japan alone the annual demand was estimated at 6,500 tm (Nakakuki, 2003).

The intake of oligosaccharides in the diet is difficult to estimate, although the contribution from normal foods is considered to be low. It includes oligofructoses from fruit, vegetables and cereals and oligosaccharides obtained from the biosynthesis of sugars or from the hydrolysis of polysaccharides, which are added to the foods to modify their nutritive properties or their organoleptic characteristics. The milk derivatives in which beta-galactosidases are used to reduce the lactose content, in order to make them suitable for individuals with lactose intolerance, contain small quantities of GOS. Delzenne (2003) indicates that the intake of fructooligosaccharides may range from 3 to 13 g/person/day depending on the population. In Australia and New Zealand the mean intake and the intake in the 95th percentile of derivatives derived from inulin and GOS oligosaccharides in the infant population has been estimated (FSANZ, 2008). In children aged 9 months, an intake of 5 and 12 g/person/day, respectively, has been estimated, increasing to 17 and 42 g/person/day in children from 1 to 3 years old; prior to weaning the intake of GOS and derivatives of inulin in children is zero (FSANZ, 2008).

The NDA Panel of the EFSA, on assessing the health claim applications, considers that the GOS foods in the claims are sufficiently characterised.

Legal situation

The galactooligosaccharides (GOS) are considered GRAS for use as ingredients in infant formula at a concentration of 5 g/l, and in other types of food (milk, yoghurt, frozen desserts, etc.) (FDA, 2008)

The GOS obtained from lactose via beta-galactosidase isolated from *B. circulans*, that comply with the food grade specifications and that have been obtained applying good manufacturing practices, are GRAS for the uses requested in infant and follow-on formulas. The GOS used in infant formulas and considered GRAS are mainly 4'-galacto-oligosaccharides (FDA, 2009).

10.4.3 Nutrition and metabolism

The human digestive tract may hydrolyse the glucose polymers with alpha-glycosidic bonds such as starch and glycogen; however, the sugars in the diet linked by beta-glycosidic bonds are not digested to a significant degree in the intestinal lumen (Wisker et al., 1985). The undigested fibre reaches the colon where it is fermented by the microbiota producing H_2, CO_2, CH_4 and short-chain fatty acids.

The GOS are not hydrolysed by the amylase of human saliva, the alpha-amylase of hog pancreas or the artificial gastric juices (Ohtsuka et al., 1990) (Chonan et al., 2004). Nor was it hydrolysed when it was incubated with human pancreatic juices and brush-border membranes (Engfer et al., 2000).

Studies relating to the absorption, metabolism and excretion of GOS in humans are scarce. Dietary oligosaccharides have been observed in infant stools (n= 16), infants who for the first two weeks of their life and for a period of 28 days were given GOS:FOS enriched formulas (8 g/l; ratio 9:1; 5 g/kg b.w.), whereas they were not found in the stools of the control group (Moro et al., 2005).

Studies in humans that support the non-digestibility of the GOS

After 0.5 g GOS/kg b.w. was administered to five healthy volunteers, it was confirmed that in a 4-hour period the hydrogen content in the air exhaled was higher at base levels, indicating the fermentation of significant quantities of GOS by the gastrointestinal bacteria (Tanaka et al., 1983). Similarly the hydrogen content was determined in the air exhaled by 16 healthy individuals given a mixture of GOS orally, obtained from lactose by a transgalactosylation reaction of *Sporobolomyces singularis* cells and *Kluyveromyces lactis* beta-galactosidase. The elimination of hydrogen, between 1.5 and 8 hours postprandial, was significantly higher in the subjects who consumed GOS wit respect to the control group (Chonan et al., 2004).

10.4.4 Safety

The Scientific Committee of the European Commission on Food reviewed the use of GOS as an ingredient in infant and follow-on formulas, concluding that the inclusion of up to 8 g/l of a mixture (90:10) of oligogalactosyllactose (GOS) and oligofructosyl-saccharose (from inulin) of high molecular weight in these products is safe (SCF, 2003). The Food Standards Australia New Zealand (FSANZ) reached the same conclusion on assessing the safety of the addition of GOS and substances from inulin in infant and follow-on formulas (FSANZ, 2008).

The use of GOS in infant formulas at concentrations of up to 5g/l was reported to the FDA in the United States, without receiving any objections from the Agency (FDA, 2008).

Toxicity studies

In animals

- Acute toxicity. It has been indicated that in rats, the LD_{50} of the GOS administered orally is higher at 15 g GOS/kg b.w.; however there is no information relating to the design of the study (Matsumoto et al., 1993).
- Short-term toxicity and subchronic toxicity. In a study conducted on *Sprague-Dawley* rats aged 6 weeks for 3 months, in those given 2,500 or 5,000 mg GOS/kg b.w./day, no significant adverse effects attributable to the GOS were observed, establishing a NOAEL of 5,000 mg/kg b.w./day (Anthony et al., 2006).

In humans

Nineteen studies, seventeen on adults and another two on children after weaning, provided interesting information for the safety assessment of the GOS. Although not the objective in any of the studies, they contained parameters relating to tolerance (flatulence, swelling, abdominal cramps etc.), and the monitoring of adverse effects. The majority of these studies were carried out on healthy adults, with GOS intakes between 5 and 15 g/person/day, for periods of 1 to 3 weeks. However there are also studies with intakes from 20 to 30 g GOS/day (Tanaka et al., 1983) (Van den Heuvel et al., 2000).

Three studies mentioned intakes of GOS of between 5.5 and 10 g/day which are tolerated well, without adverse effects in periods of between 1 and 2.5 months (Ito et al., 1990) (Shadid et al., 2007) (Silk et al., 2008) (Vulevic et al., 2008).

For some years infant formulas enriched with GOS and FOS have been widely used in Europe. The long-term effects from the consumption of GOS-enriched milk were studied in 634 children aged between 1 and 3 years old, selected from a peri-urban population in the south of Delhi. They were randomly divided into two groups who received non-enriched milk or GOS-enriched milk GOS (2.4 g/100 g) and *B. lactis* HN019 (from 10^7 to 10^8 CFU/100 g) every day for one year. During the study, the general health, growth, iron status and different haematological parameters were assessed. The children who consumed the GOS-enriched milk displayed higher growth rates at 6 and 12 months old, and their nutritional iron status improved, the incidence of bloody diarrhoea decreased although not the number of cases of diarrhoea (Sazawal et al., 2004).

In general terms in the studies published to date, no adverse effects attributable to the intake of GOS are mentioned. Only flatulence has been indicated as a secondary effect, when the GOS are consumed repeatedly in quantities of between 10 and 15 g (Ito et al., 1990) (Deguchi et al., 1997) (Teuri et al., 1998) (Alles et al., 1999) although this effect is not mentioned systematically in all the studies using this dose (Bouhnik et al., 1997) (Teuri et al., 1998) (Van Dokkum et al., 1999) (Bouhnik et al., 2004) (Shadid et al., 2007). Given that similar observations have also been made about the increase of flatulence after the intake of 15 g of fructooligosaccharides for 7 days (Alles et al., 1996), this is considered as an expected effect associated with the intake of non-digestible fibre in high quantities.

10.4.5 Conclusion

The bibliography does not contain references of adverse effects that can be attributed to the intake of GOS, except a possible increase of flatulence when consumed in very high doses. A NOAEL in *Sprague-Dawley* rats of 5,000 mg/kg b.w./day has been described.

The Scientific Committee of the European Commission on Food (SCF, 2003) considers the use of GOS as an ingredient in infant and follow-on formulas to be safe at concentrations of up to 8 g/l of a mixture (90:10) of oligogalactosyllactose (GOS) and oligofructosyl-saccharose (from inulin).

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10.5 Konjac glucomannan (*Amorphophallus konjac* K. Koch)

10.5.1 Proposal

The AESAN has proposed the inclusion of konjac glucomannan in Royal Decree 1487/2009 (BOE, 2009) at a maximum daily amount of 4 g/day and with the warning "Do not use in foods intended for rehydration at the time of intake". This proposal is based on the authorisation for food supplements in Denmark, Belgium and Italy.

In Denmark, konjak glucomannan is authorised in food supplements in a total maximum amount that must not exceed 5 g per daily dose with the warning "Do not use in foods intended for rehydration at the time of intake" (Denmark, 2011). In Belgium and in Italy it is authorised in food supplements without the establishment of a maximum daily amount (Belgium, 1992) (Italy, 2012).

In addition, there are two health claims authorised for konjac glucomannan derived from two positive opinions of the EFSA which established that it has been proved that konjac glucomannan at a dose of 4 g per day contributes to maintaining normal blood cholesterol levels and at doses of 3 g divided into three doses of 1 g together with one or two glasses of water before meals, helps weight loss when following a low calorie diet (EFSA, 2010).

10.5.2 Characteristics and sources

Konjac glucomannan comes from the tuberous roots of the konjac (*Amorphophallus konjac* K. Koch). Its structure is that of a polysaccharide with a high molecular weight (200-2,000 kDa), that depends on the variety of konjac, the preparation method and even the storage time without processing. It is formed of a lineal chain of D-mannose and D-glucose chains in a ratio of 1.6:1 linked by β-1→4 glycosidic bonds, with a small branched fraction/portion (8 %) through β-(1→6)-glycosyl bonds, and to random acetyl groups in a ratio of approximately 1 group per 9 to 19 units of sugar. The acetyl groups contribute to solubility and to the gelling properties, their elimination through mild alkaline hydrolysis produces heat stable gels. It is a hydrosoluble fibre similar to pectin in structure and function, that provides a high level of viscosity to water.

It is used as an emulsifier and thickening food additive, and also as a sufficiently characterised food supplement.

10.5.3 Nutrition and metabolism

Konjac gum is a water-soluble hydrocolloid obtained from the roots of the perennial *Amorphophallus konjac* cultivated in Asian countries. Although it is not an intrinsic component of food in Western diets, for centuries it has been used as a traditional food in the manufacture of gels and noodles, in the Far East (China and Japan).

The use of konjac gum has been proposed as a gelifier, thickener, emulsifier and stabiliser in food, for example: pasta, baked products, cold meats and sausages, salad dressings, ice creams, desserts, jams, mayonnaise, soups and drinks. Its use is proposed at concentrations ranging between 0.01 and 2.0 % (SCF, 1997). Its use as a food additive provides an estimated intake of approximately 3 g/person/day. Its intake as a component of traditional food in Japan and China may lead to intakes of up to 4 g/person/day (SCF, 1997).

In addition, it is not clear to what extent the main component; glucomannan is digested in the human intestine. It does not appear to be digested by the enzymes from the human intestinal tract, but is susceptible to fermentation by the colonic microflora, although it has not been clearly proven to what degree (SCF, 1997). As

regards the health claim relating to the maintenance of normal blood cholesterol levels, the EFSA indicates that at least 4 g per day of glucomannan must be consumed, where the target population is the general population (EFSA, 2009).

The NDA Panel of the EFSA has favourably assessed a health claim relating to glucomannan with respect to the maintenance of normal blood cholesterol levels, and to favouring weight loss as part of an energy-restricted diet (EFSA, 2010). To obtain the effect, the EFSA indicates that at least 3 g per day of glucomannan must be consumed, in doses of 1 g taken with 1-2 glasses of water before meals. The target population for glucomannan are overweight individuals following a hypocaloric diet.

However the Panel did not find a cause and effect relation between the intake of glucomannan and: 1) the decrease in the postprandial glycemic response; 2) maintenance of normal blood glucose concentrations; 3) maintenance of normal blood triglyceride levels; 4) maintenance of normal intestinal functions; and 5) reduction of potentially pathogenic microorganisms.

10.5.4 Safety

Legal situation

The use of glucomannan as a food additive is authorised in Europe and is called E-425. In Spain, Royal Decree 142/2002 includes E-425 (konjac, konjac gum and konjac glucomannan) in the group of "Emulsifiers, stabilisers, thickeners and gelifiers" (BOE, 2002). The use of E-425 konjac and E-425 konjac gum is permitted in foods, alone or in combination at a maximum dose of 10 g/kg of food (except in the foods listed in article 3.3. of said Royal Decree). The Royal Decree indicates that it may not be used in dehydrated food products that are rehydrated at the time of consumption, nor in gelatine-based confectionary products, including mini-capsules of gelatine. E-425 is also included in the group of media and solvents permitted in food.

In Canada the use of konjac glucomannan is also authorised as a food ingredient (Health Canada, 2012).

In the United States, the FDA classifies konjac flour as a GRAS substance for use as a food ingredient and it has been included in the fourth edition of the Food Chemical Codex (FCC, 1996).

In Denmark, the use of konjak glucomannan is authorised in food supplements at a maximum total amount that must not exceed 5 g/day.

The Scientific Committee on Food (SCF, 1997) issued an opinion on the safety of the use of konjac glucomannan as a food additive (emulsifier, stabiliser and gelifier) in baked products, meat and fish derivatives, pasta, jams and soups. This Committee suggests its use at concentrations ranging between 0.01 and 2.0 %, depending on the food considered, and a daily intake of approximately 4 g is considered realistic by the manufacturer, where this may be reached with the traditional use of konjac flour (SCF, 1997).

The available toxicity data for konjac glucomannan include information relating to: 1) acute oral toxicity studies on mice and rats; 2) a cutaneous sensitivity study on guinea pigs (Buehler test - maximization test); 3) a subacute nutritional study (28 days) on rats; 4) subchronic nutritional studies (90 days) on beagle dogs and rats, the latter combined with a toxicity study on reproduction and relating to specific aspects (for example, effects on the intestine, colonic microflora and protein absorption; 5) a study over 18 months on rats evaluating cell ageing; and 6) an embryotoxicity study on domestic cats. Studies for 90 days did not exhibit any significant toxic effects attributable to glucomannan. The decrease in the intake of foods and the reduction of body weight, together with the hypertrophy of the cecum/colon are effects that are usually observed in nutritional studies using non-

absorbable dietary fibres. The no observed effect level (NOEL), in the 90-day study was 2.5 % of konjac glucomannan in the diet, equivalent to 1.25 g/kg b.w./day (SCF, 1997).

Tests on genotoxicity in bacteria (Ames test and gene mutation test with *E. coli*) were negative (SCF, 1997), as was the lymphoma test on rats and the micronucleus test on mice bone marrow. According to the information available, the studies in humans using konjac flour did not show any toxic effects. A reduced number of studies in humans indicate that single doses of more than 5 g/person/day produce diarrhoea, flatulence and slight abdominal pain. A decrease in the absorption of liposoluble vitamins A & E has been indicated, while the absorption of hydrosoluble vitamins B_{12} and B_1, respectively, was not affected or only slightly affected. However, glucomannan does not appear to affect mineral absorption.

In Australia, seven cases of oesophageal obstruction were reported, caused by the intake of a single tablet of unhydrated glucomannan (500 mg) marketed as a food supplement. No cases of intestinal obstruction due to the use of hydrated konjac have been reported (SCF, 1997). The risk of oesophageal obstruction is due to the fact that the glucomannan absorbs a large amount of water and swells very fast. If this occurs in the oesophagus, it could cause an obstruction. This fact does not contradict the long history of the use of glucomannan in the form of the tuber of *A. konjac*, which goes back to the year 900 in Japan. In this country, it is used in the form of ground tuber and unpurified flour from the tuber as a gelifier, allowing it to absorb water and swell before consumption. Whereas in the West, glucomannan is used as a highly purified food supplement, mainly in the form of capsules which swell after intake. To prevent obstruction, it is recommended that the glucomannan is taken with 150 or 200 ml of water, to fluidify and facilitate its transit (Henry et al., 1986).

In the assessment of konjac glucomannan as a food additive, the Scientific Committee on Food concludes that the studies for 90 days on rats and *Beagle* dogs did not exhibit any significant toxic effects, and that a NOEL may be established of 2.5 % of glucomannan in the diet, corresponding to 1.25 g/kg b.w./day (SCF, 1997). As studies of gene mutation in bacterial have only been carried out with negative results, long-term toxicity/carcinogenicity tests are required for the correct safety assessment. In addition, given that there is insufficient information about the degree to which the glucomannan is digested in the human intestine, it is not possible to establish a value for the ADI.

In addition, the available experimental data, and experience in humans do not give cause for concern. Konjac glucomannan as a component of the flour has been consumed for many centuries as a traditional food in the Far East. Apart from diarrhoea, abdominal pain and the negative effect on vitamin absorption when taken in high doses, no adverse effects have been observed in humans. Therefore the Scientific Committee on Food considers that the use of konjac glucomannan as an additive at a concentration of 1 % in foods is acceptable provided that the total intake from the sources does not exceed 3 g/day. This maximum value must be considered when establishing the conditions for use. The Committee observed that Directive 95/2/EC (EU, 1995) includes a footnote in relation to similar products indicating that this product must not be used in dehydrated foods intended for rehydration at the time of consumption. The Committee considers that a similar observation is applicable to konjac glucomannan (SCF, 1997).

Results of tests on animals indicate that konjac glucomannan does not limit the absorption of minerals such as calcium, iron, copper or zinc.

Possible interactions of glucomannan with medicines

As it has been observed that glucomannan reduces the postprandial glycemic response, the use of glucomannan-based supplements may provoke hypoglycaemia in patients with diabetes who take hypoglycemics.

As glucomannan may absorb pharmaceutical active principles, carrying them to the colon and hindering or impeding their absorption, as a precautionary measure, it is recommended that a warning is given that, in the case of pharmacological treatment, the medicine and this supplement must not be taken in the same intake, unless there is an explicit reference that there is no interaction between the two.

10.5.5 Conclusion

The principal negative effects claimed for glucomannan are: a) a negative influence on the bioavailability of vitamins E and A which must be confirmed with well-designed studies and with an adequate sample size; b) diarrhoea, flatulence and slight abdominal pain at doses higher than 5 g/day; and c) possibility of oesophageal obstruction when consumed in the form of 500 mg tablets.

The Scientific Committee considers that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum amount of 4 g/day is acceptable from the safety point of view for use as a food supplement.

Moreover, as the dose that the EFSA considers efficient for maintaining cholesterol levels for glucomannan is 4 g/day, it considers that fibre complements should not provide quantities of more than 4 g of glucomannan as higher doses have not been shown to be more efficient.

Therefore, the Scientific Committee recommends that the following warnings are displayed on the packaging of konjac glucomannan-based food supplements:

- To prevent gastrointestinal obstruction, glucomannan must be taken with 150 or 200 ml of water.
- The glucomannan tablet must not be taken just before going to bed.
- When taking this type of preparation, the intake of other dietary fibre-based food supplements should be avoided.
- Given that the fibre may interact with some medicines, altering their efficiency, please seek medical advice if taken at the same time as other medicines.
- Patients with diabetes must seek medical advice before taking this food supplement.

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10.6 Guar gum

10.6.1 Proposal

The AESAN has proposed the inclusion of guar gum dose in Royal Decree 1487/2009 (BOE, 2009) at a dose of 10 g/day and with the warning "Do not use in dehydrated foods intended for rehydration at the time of intake". This proposal is based on a favourable assessment of the use of this substance as a food supplement in France (AFSSA, 2008). In addition, there is an authorised health claim for guar gum derived from a positive opinion of the EFSA in which it establishes that it has been proven that guar gum at a dose of 10 g per day contributes to maintaining normal blood cholesterol levels (EFSA, 2010).

10.6.2 Characteristics and sources

Commission Directive 2009/10/EC, dated 13 February 2009, laying down specific purity criteria on food additives other than colours and sweeteners, defines guar gum as the ground endosperm of the seeds of natural strains of the guar plant *Cyamopsis tetragonolobus* (L.) *Taub*. (family *Leguminosae*). It consists mainly of a high molecular weight hydrocolloidal polysaccharide, composed of galactopyranose and mannopyranose units combined through glycosidic links, which may be described chemically as a galactomannan. The gum may be partially hydrolysed, by either heat treatment, mild acid or alkaline oxidative treatment for viscosity adjustment" (EU, 2009).

The guar plant is a legume originally from India, which is grown in intertropical regions. As indicated above, the main component of guar gum is a polysaccharide fraction formed by galactomannan polymers (>92 % of dry material), also including a protein fraction (5 %) (AFSSA, 2008).

Chemically, it is a high molecular weight hydrocolloid consisting of a principal chain of ß-D-mannopyranose units with (1→4) bonds with branches in position 6 to which units of α-D-galactose are bonded (for example 1→6- bond α-D-galactopyranose). It contains between 1.5 and 2 mannose residues per galactose residue (EFSA, 2007). It is a water soluble fibre but it is not digested in the human intestine.

Intact or partially-hydrolysed guar gum is not an intrinsic component of foods, it is used as a food additive and also consumed as a food supplement.

In the European Union the use of guar gum (E-412) as a food additive is authorised by Directive 95/2/EC (EU, 1995). There is no specified ADI, it may be used *quantum satis* in all applications to foods. It is normally used as a thickener, emulsifier and stabiliser in a wide range of food groups (EFSA, 2007).

According to the European Union and the FAO/WHO/JECFA, the galactomannan content of guar gum must not be less than 75 % and the molecular weight of the food grade product will be between 50,000 and 8,000,000 g/mol (JECFA, 1975) (EU, 2009).

Exposure

Guar gum is used in different concentrations as an emulsifier or stabiliser, either alone or in combination with other thickeners or stabilisers. It is normally used in sauces, salad dressings, instant noodles, processed meats, flour improvers and drinks.

The thickening properties, the hydration kinetics and the synergy with other colloids are the key underlying properties in the functioning of guar gum in foods (Ellis et al., 2001). The properties of guar gum in aqueous

solutions depend on the molecular size. The partial depolymerisation by hydrolysis affects its thickening properties and permits better control of viscosity, flow characteristics and the properties of stabilisation, without affecting the chemical nature of the gum, thus meeting the industry's requirements in a wide range of product functions (Ellis and Dawoud, 1991) (Blake et al., 1997) (Evans and Marrs, 1997) (Kök et al., 1999).

With respect to the use of guar gum as a food supplement or ingredient for particular nutritional use, the NDA Panel of the EFSA has assessed the health claims for intact/native guar gum and also for partially hydrolysed guar gum (EFSA, 2010, 2011).

In the case of native guar gum (not hydrolysed) the Panel concluded that the data submitted did not permit the establishment of a cause and effect relation between the consumer of guar gum and: 1) long-term maintenance of normal blood glucose levels; 2) the increase of satiety. However a cause and effect relation has been established between the intake of guar gum and the reduction in blood cholesterol levels. The claim could be made for foods that provide at least 10 g of guar gum per day in one or more portions (EFSA, 2010).

As regards the partially hydrolysed gum, the Panel concluded that the data submitted did not permit the establishment of a cause and effect relation between its intake and: 1) the decrease of gastrointestinal pathogenic microorganisms; 2) a beneficial physiological effect related to changes in the production of short-chain fatty acids and/or the pH in the gastrointestinal tract; 3) changes in the intestinal function and 4) reduction of intestinal disturbance (EFSA, 2011).

The estimates of mean exposure to partially hydrolysed guar gum in France, the United Kingdom and the United States give values of 3.45; 2.92 and 2.46 g/person/day respectively. The AFC Panel (Food Additives, Flavourings, Processing Aids and Materials in Contact with Food) of the EFSA concluded that in the worst case scenario, the daily mean exposure of a consumer may be estimated between 41 and 57 mg/kg b.w./day (EFSA, 2007).

10.6.3 Nutrition and metabolism

Guar gum is a plant polymer that is not digested by the enzymes in the stomach or the small intestine; but it is metabolised by the microbial flora in the colon. It has a low energy value, less than 4 kcal/g (AFSSA, 2002).

The digestive process in the intestinal tract of the partially hydrolysed guar gum is identical to that of intact guar gum, in both cases the metabolism is based on the fermentation of mannose and galactose by the colonic flora (Nyman and Asp, 1982). The initial partial hydrolysis only represents a pre-digestive stage that also occurs in the digestion of guar gum in the body.

10.6.4 Safety

The safety of guar gum as a food additive was assessed for the first time by the JECFA in 1969 and subsequently by the Scientific Committee on Food in 1978 (JECFA, 1970, 1974, 1975) (SCF, 1978).

In the United States, the FDA classifies guar gum as a GRAS substance for numerous applications in foods (CFR, 1974).

Toxicological studies have been carried out with native and partially hydrolysed guar gum.

Native (not hydrolysed) guar gum

Studies in animals

A carcinogenicity study has been published (103 weeks) on F344/N rats and B6C3F1 mice administered diets with contents of 25 or 50 g of guar gum/kg, (equivalent to approximately 1,250 or 2,500 mg/kg b.w./day in the case of the rats and to 3,600 and 7,200 mg/kg b.w./day in the case of the mice), confirming that these doses did not induce cancer (NTP, 1982a) (Melnick et al*.,* 1983).

In another subchronic study doses of up to 100 g of guar gum/kg of diet were administered, and in a toxicity study on developing rats, up to 150 g of guar gum/kg of diet (Melnick et al., 1983) (Track et al*.,* 1984). In these studies, the NOAEL corresponds to the highest tested dose.

Studies in humans

A clinical study on humans examined the effects of a daily dose of 30 g of guar gum for 16 weeks on non-insulin dependent diabetic patients. In line with previous reports, no adverse effects derived from the use of guar gum were observed. No alterations were observed in the hematopoietic function, assessed using complete blood counts, nor changes to the renal function measured through urea nitrogen and serum creatinine, nor hepatotoxicity determined with the serum enzyme levels nor electrolytic imbalances. The nutritional status assessed with anthropometric, protein and serum transferrin parameters and lymphatic count did not reveal any changes. The metabolism of lipids, vitamins and minerals was not modified. It was concluded that a daily intake of at least 30 g of guar gum for 16 weeks did not exhibit toxicity. Although the individuals in the group exposed to guar gum exhibited secondary effects such as gastrointestinal upset, flatulence and an increase in the frequency of bowel movements, the effects disappeared after a few days (McIvor et al., 1985).

Guar gum did not trigger measurable mutagenic responses in the host mediated test hat used *Salmonella* (SRI, 1972) and it was not carcinogenic in any species or gender (NTP, 1982b).

Partially hydrolysed guar gum

In Japan, it has been used since 1987 as a dietary fibre in different foods (Seon-Joo et al*.,* 2008).

The FDA has considered it as a GRAS substance since 1995 (Angels, 1995). When guar gum is hydrolysed, a simple shortening of the mannose chain takes place, and the structural relation between the mannose chain and the side galactosyl group continues to be the same as in the intact guar gum. There is no evidence that the changes in viscosity have any influence on the safety of the hydrolysed galactomannan molecules (Seon-Joo et al., 2008).

The toxicity of the partially hydrolysed guar gum was assessed in male and male *Sprague-Dawley* rats, at doses of 0, 0.5 and 2.5 g/kg b.w./day for 28 days. Tolerance was good and the intake of food and body weight were not affected by the treatment. Urine, blood and biochemical tests did not exhibit any alterations that might be attributed to the treatment, nor were there any changes in the general state of the rats nor any deaths (Takahashi et al., 1994a).

In a subchronic study (13 weeks) performed on rats, no signs of toxicity were observed with intakes of up to 10 % of partially hydrolysed guar gum in the diet (Takahashi et al., 1994b).

Mutagenicity was studied in a bacterial reverse mutation test with the TA100 and TA98 strains of *Salmonella typhimurium*, in which concentrations of up to 5 mg/plate did not have any effect on the rates of reverse mutation.

The administration of doses of 36 g/day of partially hydrolysed guar gum, for 4 weeks, to adult human volunteers, did not cause secondary effects (Takahashi et al., 1993), nor did a daily intake of 20-40 g of this substance (Meier et al., 1993).

A toxicity study has also been carried out (90 days) on weaned rats using two types of guar gum depolymerised by alkaline hydrolysis. Following the addition to the diet of different concentrations of these gums, (0, 20 or 50 g/kg food; corresponding, respectively to doses of 0, 1,000 or 2,500 mg/kg b.w./day), the growth, dietary intake, biochemical, clinical and hystiopathological tests on the exposed animals indicate the absence of adverse effects attributable to the substances tested (EFSA, 2007).

10.6.5 Conclusion

Cases of respiratory allergies have been reported in individuals in repeated contact due to inhalation of guar gum powder; and it has been indicated that a high consumption of the product may produce adverse gastrointestinal secondary effects (abdominal distension, flatulence, etc.), intestinal tolerance to guar gum is good at doses of less than at 40 g/day (AFSSA, 2002).

The Scientific Committee considers that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum daily amount of 10 g/day of intact guar gum or partially hydrolysed guar gum is acceptable from the safety point of view for use as a food supplement.

It is recommended that the following warnings are shown on the packaging:

- Given the increase in volume that is produced when guar gum is hydrated, the warning “Do not use on foods that must be rehydrated at the time of intake”.
- When taking this type of preparation, the intake of other dietary fibre-based food supplements should be avoided.
- It must be mentioned that the product must not be taken together with medicines or fibre complements, to prevent the risk of loss of absorption of the pharmacological active principle.

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10.7 Inulin

10.7.1 Proposal

The AESAN proposes a maximum daily amount of 9 g of inulin or the sum of inulin plus fructooligosaccharides (FOS), with the warning that "excessive consumption may cause intestinal upset".

The industry were consulted with regard to the proposal and indicated that the composition and effects of the FOS and inulin are similar and that both are often used in the same product. Therefore, the proposal for a maximum daily amount for the sum of FOS and inulin is justified.

There is a favourable assessment of this substance in France that indicates that "a daily intake of 8 g of the inulin-enriched oligofructose mixture (4 g of oligofructose and 4 g of inulin) is free of risk" (AFSSA, 2006). In a later assessment, the AFSSA established a safe dose of 9 g/day with the warning that "inulin may pose a risk for allergic subjects" (AFSSA, 2008).

Inulin is listed in the European Report on substances present in food supplements in the European Union (DG SANCO, 2008).

10.7.2 Characteristics and sources

Inulin is a lineal polymer formed by units of fructose bonded together by a β-(2-1) bond that ends in a glucose unit. Its structure can be represented with the formula GFn, where G is a glucose unit, F is a fructose unit and n the number of fructose units (n= 10 to 60).

Inulin is found in a wide range of vegetables, including roots (chicory), bulbs (onion, garlic), vegetables (salsify, leeks, artichokes) and fruit (bananas). It is a soluble food fibre (AFSSA, 2002). Several grams of inulin may be consumed per day in food (Van Loo et al., 1995). In Europe, it is estimated that the average intake of fructans (inulin and/or FOS) per inhabitant is between 3 and 11 g per day, and in the United States, between 1 and 4 g (Van Loo et al., 1995).

In Europe, the main industrial source of inulin is chicory root (*Cichorium intybus*), from which it is obtained by extraction with hot water. Chicory root inulin has a degree of polymerisation (DP)>10 (DP refers to the number of fructose units). In some cases, the inulin contains a fraction of free sugars (8-10 %). Oligofructose or fructooligosaccharides (FOS) with a DP<10 (n= 2 to 9) are obtained from the inulin by enzymatic hydrolysis.

Inulin is considered an ingredient (not an additive) that is used in the preparation of various foods, especially bakery and dairy products. It is mainly added to food as a dietary fibre due to its prebiotic properties. As a result of its inclusion, the fibre content is increased without unpleasant flavours or modification to the product viscosity, permitting the preparation of fibre-rich foods with an appearance and flavour similar to conventional foods. To obtain a prebiotic effect that enhances the growth of the intestinal bifidobacteria, the quantities of insulin (alone or with FOS) usually added to the foods comes to 3-8 g per portion.

On occasions inulin is added to the food as a fat substitute. The potential to substitute fat with inulin was discovered and patented by *Beneo Orafti*® in 1992. Inulin is combined with water to produce the same texture and sensation in the mouth as fat. This is only possible in foods with a high water contents such as dairy products. In general, 1 g of fat is replaced by 0.25 g of inulin. Therefore, fat substitution may lead to concentrations of inulin of approximately 2-6 g per portion (Coussemment, 1999). The use of inulin is not possible in acid products with a long shelf life such as soft drinks or fruit jams, as the inulin hydrolyses slowly releasing fructose (Coussemment, 1999).

10.7.3 Nutrition and metabolism

Inulin (as with FOS) resists enzymatic digestion in the upper gastrointestinal tract, arriving, almost intact, at the colon, where it is completely fermented by the microbiota. The bacterial fermentation of inulin takes place in the distal colon (unlike that of FOS which is metabolised in the proximal part) producing gases, lactate and short-chain fatty acids (acetate, propionate and butyrate).

Tolerance to inulin is on the whole good. The effects resulting from its colonic metabolism (flatulence, increase of osmotic pressure, abdominal distension, diarrhoea, etc.) usually only affect a small fraction of the most sensitive population. The majority of people are able to consume up to 20 g of inulin without notable secondary effects, whereas some people experience intestinal discomfort after the intake of small quantities of the substance (Carabin and Flamm, 1999). There is a wide interpersonal variability in the doses at which these effects appear and it also depends on the food in which the fibre is contained.

One of the main effects of inulin, and of the FOS, in the body is its prebiotic activity, selectively stimulating growth and the activity of different microbial species. *In vitro* studies have demonstrated that inulin is an excellent and selective growth medium and energy substrate for the bifidobacteria. Similarly, in clinical studies it has been confirmed that inulin causes a significant increase in bifidobacteria with respect to other species of intestinal microbiota (Kleesen et al., 1997) (Kruse et al., 1999) (Tuohy et al., 2001). In general, it is thought that the daily intake of between 2.5 and 10 g per day of inulin and/or other oligofructosaccharides may have a bifidogenic activity in adults, but there are notable differences between individuals in the response to the same dose, such that huge differences can be observed in the increase of the total number of bifidobacteria.

In addition to its prebiotic effect, various studies suggest other possible nutritional and physiological effects for inulin (Koo and Rao, 1991) (Roland et al., 1994) (Delzene et al., 1995) (Gallaher et al., 1996) (Kleessen et al., 1997) (Taper et al., 1997) (Meyer, 1999) (Van den Heuvel et al., 1999) (Van Dokkum et al., 1999) (Causey, 2000) (Coussement and Frank, 2001) (Abrams et al., 2005) (Kelly, 2008).

At present there is wide agreement with respect to the positive effect of inulin and/or the FOS in the bifidogenic function and in the metabolism of the lipids in hyperlipidemic individuals (Kelly, 2009). With respect to the role of inulin and/or FOS in the absorption of the calcium, differences have been observed depending on the populations studied (Kelly, 2009). In 2006 the AFSSA, based on studies in animals used for research and on humans, accepted, with certain conditions, the claim that “8 g/day of inulin enriched oligofructose increases calcium absorption”. The scientific evidence for the remaining metabolic functions attributed to inulin has not yet been fully defined or more studies are required in order to establish a clear dose and effect relation (Kelly, 2009).

In 2011, the NDA Panel of the EFSA considered that the fructans (mixtures of inulin and oligofructoses from chicory), were not sufficiently characterised in relation to the proposed claims (EFSA, 2011). The Panel concluded that based on the information provided, it was not possible to establish a cause and effect relation between the intake of insulin-type fructans and the effects on the intestinal function, defence against gastrointestinal pathogens, increase in the absorption and retention of calcium, increase in bone mineral density, maintenance of normal blood glucose levels and satiety.

10.7.4 Safety

The safety of inulin for use as an ingredient has been assessed by the health authorities in a number of countries, including European countries and the United States. As a result, the use of inulin as an ingredient is widely accepted without any restrictions in numerous food formulas (Pascal, 2008).

Although it cannot be considered as absolute proof of its safety, the long history of exposure to inulin in the diet, even in significant quantities in the case of certain specific diets (up to 20 g), without observing adverse effects confirms its safety (Coussement, 1999).

The majority of safety studies in animal models have been carried out with FOS but, given the structural similarities and the physiological effects to which they apply, the toxicological results obtained for FOS may also be extrapolated to inulin. Acute, chronic toxicity studies, carcinogenicity and genotoxicity studies conducted on animals (Hussein et al., 1999) (Hughes and Rowland, 2001) (Coudray et al., 2003) (Roller et al., 2004) (Rehman et al., 2007) (Van Loo, 2007) (Pascal, 2008) (Tako et al., 2008) have demonstrated tat the intake of inulin and its derivatives (FOS), even administered in high doses, does not have toxicological consequences.

All the clinical studies conducted with inulin and/or oligofructose, both in normal subjects and in patients (Roberfroid, 1993), provide evidence of its safety. For example, inulin has been used as a standard procedure for measuring the glomerular filtration rate after intravenous injection since 1931, without a registered history of toxic effects (Price et al., 1978).

The AFSSA report dated 22 December 2000 specifies that there may be a "possible appearance of intestinal disturbances in the event of an intake of more than 20 g/day" and that “cases of sensitivity to polysaccharides have been observed with the generation of anti-glucide antibodies" and that, consequently, “there is a risk that inulin-energies appear" (AFSSA, 2000). Some cases were described of a reaction to inulin; one of these was the anaphylactic shock observed in a patient allergic to artichokes (Franck et al., 2005). It may have been that a protein bonded to the inulin was responsible for the clinical manifestations observed. Another case, described by Gay-Crosier in 2000, refers to a man whose allergy symptoms appeared after the intake of artichoke leaves and salsify (Gay-Crosier et al., 2000).

In the United States, inulin and FOS have been considered as GRAS substances since 1992 (Kolbye et al., 1992).

10.7.5 Conclusion

Different toxicity assessments of inulin have been made using animal models, including acute and chronic toxicity and carcinogenesis without results that display a risk to consumer health. The clinical studies that assessed the safety of inulin and/or oligofructose did not exhibit toxic effects due to the intake of these compounds.

Although tolerance to inulin is usually good, it has indicated that there is a wide interindividual variability in the doses at which the undesired effects appear, including flatulence, an increase in osmotic pressure, abdominal distension, etc., derived from its bacterial fermentation in the colon. In view of these effects, the AFSSA considers that the consumer should be informed that an excessive intake may cause intestinal disturbances and does not recommend the use of food supplements with a high content of inulin. Nevertheless, it also indicates that there is no scientific evidence to oppose a supplementary intake of up to 9 g per day of inulin (food supplements and enriched foods), while observing that inulin may pose a risk for allergic subjects (AFSSA, 2000).

In view of the above, the Scientific Committee of the AESAN considers that, based on the information available to date and taking into account the general considerations reflected in this report, the proposal of a maximum amount of 9 g/day of inulin or the sum of inulin plus fructooligosaccharides (FOS) is acceptable from the safety point of view for use as a food supplement.

Nevertheless, the Scientific Committee of the AESAN recommends that the following warnings are displayed on the packaging of inulin-based supplements:

- Do not exceed a daily dose of 9 g of inulin/day, or the sum of FOS and inulin, as excessive consumption may cause stomach upsets.
- When taking this type of supplement, the intake of other dietary fibre-based food supplements should be avoided.
- Given that the fibre may interact with some medicines, altering their efficiency, please seek medical advice if taken at the same time as other medicines.

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10.8 Pectins

10.8.1 Proposal

The AESAN has recommended the inclusion of pectins in Royal Decree 1487/2009 with a maximum daily amount of 10 g.

There are two authorised health claims for pectins derived from two positive opinions of the EFSA which establish that it has been proven that pectins at doses of 6 g/day contribute to maintaining normal blood cholesterol levels and at doses of 10 g/day contribute to reducing the rise of blood glucose after eating (EFSA, 2010).

10.8.2 Characteristics and sources

Pectic substances include a wide group of complex plant polysaccharides with a basic structure formed by molecules of D-galacturonic acid linked by glycosidic α-D-(1,4) bonds, and in which some of the carboxyls may be esterified with methyls or in the form of salt. This lineal chain is generally bonded to neutral polysaccharides (arabinans, arabinogalactans and galactans) bonded to regions of rhamnogalacturonans which have a skeleton of L-rhamnose units linked by α-(1,2) bonds alternating with molecules of D-galacturonic acid linked by α-D-(1,4) bonds. Other structures can also be observed in the pectins including the xilogalacturonans, rhamnogalacturonans II and apiogalacturonans.

Pectins are naturally found in plant products, as they form part of the middle laminar of the walls of plant cells. Some fruits such as apples, red and blackcurrants, guavas, quince, plums, oranges and other citrus fruit are especially rich in pectins. They are also found in abundance in vegetables and pulses.

Pectins are also used as ingredients to enrich foods with plant fibre, as a substitute for fats or sugars in products with a low energy value and as texturing additives to give consistency to certain foods forming gels and other textures. Some of these additives are amidated pectins, in which some of the carboxyl groups of native pectins have been amidated. The pectins produce thermo-reversible gels in the presence of sucrose at low pH (pectins with a high degree of methylation, between 58 and 77 % which are used for example in the manufacture of jams) or in the presence of calcium ions (pectins with a low degree of methylation ≤50 %, used in low calorie gelatinous products, gelified milk, yoghurt, etc.). Due to its optimum gelling capacity, pectin is one of the main components responsible for the texture of vegetable products and the viscosity of their juices, and is of great technological interest in the food industry. It is used as a gelling, thickening, emulsifying and stabilising agent, in the preparation of jams, jellies and preserves, canned fruit, bakery and pastry products, drinks and other foods, as it gives them rheological characteristics and turbidity, properties which are sought by the manufacturer and the consumer. The list of additives published in annexe I of Council Directive, of 29 June 1978, on the "approximation of the laws of the Member States relating to emulsifying, stabilising, thickening and gelling agents which may be used in food products" classes non-amidated pectins under code E-440a and amidated pectins, generally with a low methoxyl index, with the number E-440b (EU, 1978).

Pectins are also used in the pharmaceutical industry. In Spain, for example Dr Manceau's syrup, an apple-based fluid extract, 10 g; fluid extract of avocado, 8 g; fluid extract of coriander, 1 g; and simple syrup q.s.f. 100 ml, used for constipation. In France a pectin-based preparation has been marketed for the treatment of reflux in newborns at doses of between 360 and 600 mg/100 ml). Exceptionally cases of renal lithiasis or bowel occlusion have been indicated as secondary effects.

The principal raw materials used for the production of pectin are sugar beet pulp, citrus peel and apple pomace. On many occasions, these are sub products from the sugar or fruit juice industry. The pectin is extracted from these materials by hot acid hydrolysis (pH 1.5 to 3.5). There are many other possible sources for obtaining pectins including grapes, carrots, peaches, pulses, potatoes, onions, tobacco and the waste from tropical fruit juices.

Recently the EFSA gave an opinion with respect to pectins, indicating that there is sufficient scientific evidence of a cause and effect relation between the consumption of pectins and: a) a reduction in the postprandial glycemic response, and b) the maintenance of normal blood cholesterol levels (EFSA, 2010).

To make these claims, the EFSA requires that the food provides at least 10 g of pectin (to reduce the glycemic response) or 6 g of pectins (for the maintenance of cholesterol) per intake.

10.8.3 Nutrition and metabolism

It is a type of water soluble fibre, which is almost totally degraded by human colonic bacteria (prebiotic fibre). During fermentation by the colonic bacteria, volatile fatty acids with a low molecular weight are produced (acetic, propionic and butyric, mainly) that the colon may use as an energy source. Pectins have considerable capacity for water retention, forming viscous gels able to delay gastric drainage, and to fix divalent cations, bile acids and other organic substances. Pectins are not noted for their ability to produce particular flatulence in humans after intake.

There is no estimate of the usual pectin intake by the population. However, to obtain a daily intake of 6 g of pectin (dose per intake considered effective for reducing postprandial glycaemia by the EFSA) 3 kg of oranges or apples or 1.5 kg of tinned apricots or pumpkin in syrup must be consumed (Marlett, 1992). Therefore, it is unlikely that the effective dose required for reducing postprandial glycaemia or cholesterol levels can be reached through normal diet without eating foods to which pectin has been added as an ingredient or without the intake of pectin supplements.

The amount of pectin used as an additive largely varies according to the product used. Therefore, for example, normally between 0.1 and 0.3 % is used in the case of fruit juices, between 0.3 and 1.2 % in the case of jams and preserves and between 1.5 and 2.0 % in the case of chewable sweets. Therefore, the amount of pectin included as an additive is not suitable for achieving the beneficial effects, given a reasonable intake of the food to which it has been added.

10.8.4 Safety

With respect to the ADIs of pectins, the Joint FAO/WHO Expert Committee on Food Additives concluded in 1973, after analysing short-, medium- and long-term toxicity studies, that there was no need to establish an ADI value for the E-440a additives (JECFA, 1973). In the case of amidated pectins (E-440b), based on the long-term complementary toxicity studies, an ADI was provisionally established in 1975 of 25 mg/kg b.w. (WHO, 1975) due to the trophic effect in the cecum. For a person weighing 60 kg, this value corresponds to a maximum intake of 1,500 mg of these pectins.

Since 1978, amidated pectins have been included in the United States GRAS list, implying that there are no maximum limits associated to their use. Along the same lines, in 1998, thee Scientific Committee on Food established for pectin (E-440a) and for amidated pectin (E-440b) an "unspecified" ADI (EU, 1998). Consequently,

pectins may be used in *quantum satis* in the majority of foods, except in those specifically restricted by virtue of Directive 95/2/EC dated 20 February 1995, on food additives other than colorants and sweeteners (EU, 1995).

In 2008, the AFSSA issued a ruling relating to the use of pectins in the manufacture of food supplements after assessing toxicity studies in animals and humans. The conclusions of this ruling may be summarised as follows:

- With the publications available to date a supplement in pectin cannot be justified for healthy human beings with a varied and balanced diet with an energy input suitable for covering their requirements.
- The principal negative effect that may arise from the intake of pectins comes form the capacity of these substances to chelate minerals, reducing their bioavailability as observed *in vivo* in animal models.

Due to the limited number of studies conducted on humans on the impact of a high intake of pectins on mineral bioavailability, the AFSSA considered that the available data was insufficient to propose a dose that would guarantee the safety of the intake of pectin-rich food supplements (AFSSA, 2008). Given the negative effect that the high intake of pectins would eventually have on the bioavailability of nutrients and as the AFSSA report was limited as regards the number of studies it included, it was considered necessary to make an in-depth review of *in vitro* studies in animals and on humans in relation to the bioavailability of minerals and micronutrients.

In general, it has been observed that the addition of pectins to the diet does not alter the absorption of the majority of minerals, except magnesium (Baig et al., 1983) (Van der Aar, 1983) (Ink, 1988) (Greger, 1999). Kim et al. in 1996 even demonstrated that some types of pectin (for example, those with a low molecular weight and a high degree of esterification) might increase the absorption of iron in animal models. Demigné et al. in 1989 demonstrated an increase in the flow of potassium, magnesium and calcium from the cecum, in rats with a pectin-rich diet in comparison to those with a diet that has not been enriched with this component. In a study on growing pigs, an undesired effect was observed from apple pectin with a low degree of methylation, at doses of 2.5 %, on the balance of calcium, magnesium and zinc (Bagheri and Gueguen, 1985). This was not observed for highly methylated apple pectin. However, an *in vitro* study carried out using newborn formulas supplemented with pectins with a high degree of methylation (3 g of pectin/100 g of dry material) demonstrated a reduction of only 10 % in the bioavailability of the calcium (Bosscher et al., 2003).

10.8.5 Conclusion

The principal negative effect claimed for pectins, a negative influence on mineral bioavailability, has not been confirmed as the results of *in vitro* tests and studies in animals and humans produced contradictory results. To assess whether a high pectin intake has a significant effect on mineral bioavailability, well-designed studies in humans with an adequate sample size are required.

The Scientific Committee considers that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum amount of 10 g/day is acceptable from the safety point of view for use as a food supplement.

Given that the dose that the EFSA considers to be efficient for reducing postprandial glycaemia for pectin is 10 g per intake, it considers that fibre complements should not provide quantities of more than 10 g of pectin per intake as higher doses have not been shown to be more efficient in reducing postprandial glycaemia.

It also recommends that the following warnings are shown on the packaging:

- When taking this type of preparation, the intake of other dietary fibre-based food supplements should be avoided.

- Given that the fibre may interact with some medicines, altering their efficiency, please seek medical advice if taken at the same time as other medicines.

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11. Other substances

11.1 Choline (as choline, choline chloride, citrate or bitartrate)

11.1.1 Proposal

The AESAN has proposed a maximum daily amount of choline of 1,500 mg, using sources of choline, and choline chloride, citrate or bitartrate salts. This proposal is based on the existence of legal limits in Belgium (75 mg/day at minimum and 1,500 mg/day at maximum) (Belgium, 2009).

Regulation (EC) No 953/2009 (EU, 2009) includes choline, choline chloride, citrate and bitartrate among the substances that may be added for specific nutritional purposes in foods for particular nutritional purposes. Specifically, it may be added to diet foods for particular nutritional purposes, including foods intended for special medical purposes, and excluding milk and follow-on milk formulae, processed cereal-based foods and baby food for infants and young children.

Directive 2006/141/EC (EU, 2006) relating to the milk and follow-on milk formulae and its transposition in Spain to Royal Decree 867/2008 (BOE, 2008) permit, on establishing the basic composition of infant formulae when reconstituted in accordance with the manufacturer's instructions, the use of choline in a minimum concentration of 1.7 mg/100 kJ (7 mg/100 kcal) and a maximum of 12 mg/100 kJ (50 mg/100 kcal). This authorisation includes choline and choline chloride, citrate and bitartrate.

In Italy, a maximum daily amount of 1,000 mg of choline is authorised in food supplements (Italy, 2012).

11.1.2 Characteristics and sources

Choline, a quaternary amine, is an essential nutrient for the normal function of all cells (Zeisel and Blusztajin, 1994). There is no doubt that cells require choline and die from apoptosis when deprived of this nutrient.

Choline is found in a wide variety of foods (Zeisel et al., 2003) of animal origin (including liver, eggs, pork, beef, salmon) and plant origin (including Brussel sprouts, broccoli, cauliflower). Excellent sources of choline in the diet include liver, eggs and wheat germ in which choline is found in free and esterified form (as phosphocholine, glycerophosphocholine, phosphatidylcholine and sphingomyelin). Human milk is rich in choline compounds.

The United States Department of Agriculture has included the content of food choline in its database (USDA, 2012).

Choline is absorbed by transport through the small intestine. Phosphatidylcholine is absorbed in the small intestine. The water-soluble choline compounds are absorbed via presystemic circulation.

Another source of choline, in addition to diet, is from the biosynthesis of phosphatidylcholine, catalysed by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT) (Zhu et al., 2004). This enzyme uses *S*-adenosyl methionine as a methyl donor and forms a new half of choline (Blusztajn et al., 1985).

Studies in animal models demonstrate that mice fed with a choline-deficient diet develop fatty liver disease, acute liver damage with mortality; a choline-rich diet may prevent this effect and even, if caught in time, revert the liver damage (Walkey et al., 1998) (Waite et al., 2002). Mice with PEMT deficiency have lower choline liver contents even if they receive choline supplements. Therefore, the production of choline by PEMT is admitted as a significant source of choline if compared with the dietary intake.

In humans, a choline-deficient diet causes, in the majority of adult males and post-menopausal females, signs of organic dysfunction (principally muscular and liver damage) (Zeisel et al., 1991) (Da Costa et al., 2004). Only 44 % of pre-menopausal females developed these signs, this may be because the oestrogens induce the expression of the PEMT gene, permitting a greater production of endogenous choline in pre-menopausal females. The significant interindividual variability in the dietary requirements of choline can be explained by genetic polymorphism. Choline is critical during fetal development, and may affect brain structure and function (Loy et al., 1991) (Albright et al., 1999) (Craciunescu et al., 2003).

11.1.3 Nutrition and metabolism

Choline, or its metabolites, guarantees the structural integrity and functions of the cell membranes, it is essential for normal cholinergic neurotransmission, for muscular function and lipid transport from the liver, and is the main source of methyl groups in diet (one of the choline metabolites, betaine, takes part in the methylation of homocysteine to form L-methionine. In the majority of mammals, the prolonged ingestion (weeks to months) of a choline-deficient diet (with adequate though limited folate and L-methionine content) causes hepatic, renal, pancreatic, memory and growth disorders (Zeisel and Blusztajn, 1994).

Several reviews on the metabolism and functions of choline have been published (Kuksis and Mookerjea, 1978) (Zeisel and Blusztajn, 1994).

Only a small fraction of dietary choline is acetylated, catalysed by the activity of choline acetyltransferase, an enzyme highly concentrated in the terminals of cholinergic neurons, but also present in other tissues such as the placenta. It is interesting that the placenta is one of the few tissues to store large quantities of choline as acetylcholine, probably to guarantee choline for the foetus (Leventer and Rowell, 1984).

The formation of betaine involves oxidation to betaine aldehyde in the innermost layer of the mitochondrial membrane (Lin and Wu, 1986) and the oxidation of betaine aldehyde forms betaine, both steps catalysed by the enzyme choline dehydrogenase (CHDH). The liver and kidney are the main places for the oxidation of choline.

The major use of choline is as a precursor for the synthesis of membrane phospholipids. Phosphatidylcholine is the predominant phospholipid (>50 %) in the majority of mammal membranes. The mechanisms controlling the biosynthesis of phosphatidylcholine (Kent, 1990) occur in two phases. In the first, choline is phosphorylated and converted into cytidine diphosphocholine, an intermediate that in combination with diacylglycerol forms phosphatidylcholine and cytidine monophosphate. In the second phase, the phosphatidylethanolamine is methylated sequentially to form phosphatidylcholine using 5-adenosylmethionine as the methyl donor. This last stage is more active in the liver, but has also been identified in many other tissues including the brain and mammary gland.

The metabolism of choline, folate and L-methionine are linked, interacting at the point at which the homocysteine is converted to L-methionine. Therefore, any requirement for choline in diet must be considered in relation to these other nutrients. The homocysteine may be methylated to form L-methionine in two parallel phases; in the first vitamin B_{12} and folic acid are involved in a reaction catalysed by the L-methionine synthetase. The deficiency of these nutrients or polymorphism in genes may result in high plasma concentrations of homocysteine. High levels of plasma homocysteine represent a risk factor for cardiovascular disease (Glueck et al., 1995) (McCully, 1996). The deficiency of folate and choline lead to effects on the brain apoptosis (Craciunescu et al., 2003, 2004).

Women during pregnancy and nursing require higher quantities of choline; the transport of choline to the foetus depletes the plasma choline (McMahon and Farrell, 1985). Therefore, although the capacity to synthesise

choline is increased, the demand for this nutrient is so high that it results in the depletion of its storage sites. In general, females are less susceptible to a deficiency in dietary choline and have adequate stores of choline prior to pregnancy. However, they are at greater risk of a choline deficiency during pregnancy. An ingestion in the diet of choline of 300 mg/day to >500 mg/day is admitted to prevent the risk of a defect in the newborn baby (Shaw et al., 2004). The intake of dietary choline during pregnancy is important due to its influence on the development of the fetal brain and because it is important to maintain normal plasma levels of homocysteine during pregnancy. High concentrations of homocysteine in the mother are associated with an increase in the incidence of defects at birth (Hobbs et al., 2005) (Velzing-Aarts et al., 2005).

11.1.4 Safety

One of the functional consequences of choline deficiency in the diet in humans is the development of fatty liver (Zeisel et al., 1991) (Buchman et al., 1995) due to a lack of phosphatidylcholine. Choline deficiency in humans is also associated with liver damage (high serum aminotransferases). In addition, it has been recognised that choline deficiency in humans may also present muscular injury (Da Costa et al., 2004).

Numerous studies in humans have been published that, although not strictly directed at assessing the safety of choline, can serve as a base for accepting the safety of its use. The majority of these studies are directed at assessing the efficiency of choline in fetal viability and embryogenesis of the brain, in memory, cholinergic function and neuronal disorders, in adults. None of these studies, with doses ranging from 1 g/day for 3 months to 25 g/day for 6 months, have found evidence of adverse effects attributable to choline (Levy, 1982) (Little et al., 1985) (Spiers et al., 1996).

In 1998, the Nutrition Service for the United States Institute of Medicine established an adequate intake (AI) and UL for choline (Table 4). The UL was derived from the LOAEL (hypotension) in humans.

Table 4. Reference intake values in diet for choline

Population	Age	Adequate Intake (AI)	Tolerable Upper Intake
Children	1-3 years old	200 mg/day	1,000 mg/day
	4-8 years old	250 mg/day	1,000 mg/day
	9-13 years old	375 mg/day	2,000 mg/day
Males	14-18 years old	550 mg/day	3,000 mg/day
	≥19	550 mg/day	3,500 mg/day
Females	14-18 years old	400 mg/day	3,000 mg/day
	≥19	425 mg/day	3,500 mg/day
Pregnant women	For all ages	450 mg/day	UL approximate age
Nursing women	For all ages	550 mg/day	UL approximate age

Source: (IMNAS-USA, 1998).

11.1.5 Conclusion

There is no evidence of adverse effects in the different assessments made nor in the various clinical studies conducted to assess the efficiency of choline in different pathological situations in humans. However, in 1998 the United States Institute of Medicine established for adults an UL for choline of 3 to 3.5 g/day, derived from the lowest level of observable adverse effect (hypotension).

Therefore, the Scientific Committee of the AESAN concludes that, based on the information available to date and taking into account the general considerations reflected in this report, the proposal of a maximum amount of 1,500 mg/day of choline is acceptable from the safety point of view for use as a food supplement.

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11.2 Chondroitin sulfate

11.2.1 Proposal

The AESAN has recommended a maximum daily amount of 500 mg of chondroitin sulfate. This proposal is based on the existence of a favourable assessment of this substance in France considering 500 mg/day (AFSSA, 2008) and on the authorisation in Italy in food supplements of 500 mg/day (Italy, 2012).

11.2.2 Characteristics and sources

Chondroitin is a polysaccharide included in the group of glycosaminoglycans. From the chemical point of view it is formed of a chain of disaccharides of alternating N-acetylgalactosamine and glycuronic acid. It is usually found bonded to proteins forming aggregates with a high molecular weight called proteoglycans. It is a natural constituent of the articular cartilage, where it is found in the form of sulfate giving it mechanical and elastic properties (CGCOF, 2011).

Chondroitin sulfate is mainly extracted from bovine, porcine or marine (shark) cartilage, although recently methods have been developed to obtain it from vegetable sources (Hathcok and Shao, 2007). The best-known chondroitin sulfate, as regards its efficiency and safety, is that extracted from the bovine trachea and this is therefore, the most used in the majority of studies. Depending on the origin (land or marine) it contains different proportions of sulfate in position 4 or sulfate in position 6, and has different values of molecular weight.

11.2.3 Nutrition and metabolism

Chondroitin is not a nutrient, but a normal component of diet. It is marketed and used as a food supplement to improve the health of articulations, having acquired huge popularity among the general public, and there are large number of commercial preparations that have not been sufficiently studied as regards their effectiveness and safety (Vista and Lau, 2011). Both chondroitin and chondroitin sulfate have been the object of various applications for health claims to the EFSA (ID 1504, ID 1505) in relation to the improvement of health and mobility of the articulations. However, the NDA Panel of the EFSA considers that in accordance with the data available, it is not possible to establish a cause and effect relation between the intake of chondroitin or chondroitin sulfate and the maintenance of normal articulations (EFSA, 2009). This report highlights that all the studies in humans of the effects of chondroitin and chondroitin sulfate on the health of the articulations have been carried out in patients diagnosed with arthritis who, in the opinion of the EFSA, are not representative of the general population. Two recent publications (Sawitzke et al., 2010) (Vista and Lau, 2011) have revised the information existing on clinical efficiency and safety in the use of glucosamine and chondroitin sulfate, and the possible benefits of its use as a food supplement. In the review of Sawitzke et al. (2010) none of the treatments used, including chondroitin sulfate, obtained significant clinical differences with respect to the placebo, nor were any differences observed as regards the adverse effects observed in all the treatments. Vista and Lau (2011) studied, in a more specific manner, the possible effect of food supplements on osteoarthritis and in their conclusions they indicate that, although various supplements for arthritis in certain combinations and doses may play a role in some patients in the reduction of moderate or severe pain, the routine use of food supplements is not recommended for the treatment of arthritis. Similarly, they concluded that, the long-term use of supplements for osteoarthritis is on the whole safe. Wandel et al. (2010) in a meta-analysis in which 3,803 patients were included, did not find any differences between the placebo and the administration of chondroitin and glucosamine, alone or in combination.

Chondroitin is absorbed orally, its bioavailability is from 15-24 % and the maximum concentration is reached in 4 hours. At least 90 % of the dose is metabolised first by lysosomal sulfatases, to then be polymerised by hyaluronidases, beta-glucuronidases and beta-N-acetylhexosaminidases. The liver, kidney and other organs take part in the biotransformation. No interactions with other medicines have been described (Bioibérica, 2003) (CGCOF, 2011).

11.2.4 Safety

The catalogue of pharmaceutical specialities of the Pharmaceutical College General Council (CGCOF, 2011) lists precautions in the use of medicines containing chondroitin in patients with renal or heart failure, as on very rare occasions, there may be cases of oedema and/or water retention. This effect can be attributed to the osmotic effect of chondroitin sulfate.

In animals used for research, a possible interaction has been described with platelet antiaggregants, however, in the clinical research and in the pharmacosurveillance carried out at the recommended doses, no effects were detected at this level.

No adequate or controlled clinical trials have been carried out on pregnant women and it is not known whether this substance is excreted in breast milk or its effects on the newborn child. Intake is not recommended during nursing and nor is it recommended for use in children as sufficient clinical experience is not available. Adverse reactions include nausea and/or gastrointestinal upsets (with a frequency of 1/10,000 to 1/1,000) that generally do not require the suspension of the treatment.

In any case, several European countries have used, over 20 years, chondroitin sulfate for the symptomatic treatment of arthritis and pharmacosurveillance studies have not detected any significant toxic effect during this period. The European League Against Rheumatism (EULAR) recommends this medicine for the treatment of arthritis of the knee due to its high safety profile. On a scale of 0-100, they give it a toxicity of 6, and it is one of the safest products for the treatment of arthritis together with glucosamine sulfate (Jordan and Arden, 2003).

The total absence of toxicity of chondroitin sulfate has been demonstrated in the clinical studies published (Monfort et al., 2008). Studies lasting from 6 to 40 months in which doses of 1-2 g/day were administered demonstrated a total absence of toxicity. Similarly, the latest reviews published (Sawitzke et al., 2010) (Vista and Lau, 2011) also confirm the absence of adverse effects in the prolonged use of these medicines.

Chondroitin sulfate does not posses toxicity in itself. The exogenous administration of chondroitin sulfate of natural origin is not thought to cause systemic or genetic toxicity, given that it forms part of the physiological components of the connective tissues, and its structure is identical to that of endogenous chondroitin sulfate, a natural component of the human connective tissue.

In its report, the AFSSA (2008) also recognises the lack of toxicity of this substance, indicating an oral LD_{50} in rats and mice >10 g/kg.

Acute, subacute and chronic toxicity studies and mutagenicity, genotoxicity, carcinogenesis and toxicity studies on reproduction with chondroitin sulfate have all been negative (Bioibérica, 2003). The most recent study on the risk assessment of chondroitin sulfate (Hathcock and Shao, 2007) indicates that the highest dose of chondroitin sulfate used in clinical trials is 1,200 mg/day. At these doses, there was only one case of adverse effects (gastritis) in a study conducted with 165 patients who received treatment for 3 years (Verbruggen et al., 2002).

The absence of adverse effects in the different tests has not permitted the establishment of a NOAEL/LOAEL value for chondroitin sulfate. The evidence indicates that a dose of 1,200 mg/day did not produce adverse effects,

but does not provide information about at which doses adverse effects may appear. Therefore, these authors (Hathcock and Shao, 2007) identify the dose of 1,200 mg/day as the OSL for the oral intake of chondroitin sulfate. Considering the relatively low intake of chondroitin sulfate in the diet, this risk assessment represents a good approximation to establish the ULs at 1,200 mg/day, which would represent the maximum intake dose in the framework of a food supplement.

11.2.5 Conclusion

As there is an authorised medicine with a dosage of 800 to 1,200 mg/day and in order not to interfere in the therapeutic use of this substance, the maximum daily amount of chondroitin in food supplements must be established at a lower level than the therapeutic level, as suggested in the proposal.

The Scientific Committee considers that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum amount of 500 mg/day of chondroitin sulfate is acceptable from the safety point of view for use as a food supplement.

The use of chondroitin sulfate as a food supplement is not recommended for children, pregnant or nursing women due to the lack of specific information for these population groups.

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11.3 Creatine monohydrate

11.3.1 Proposal

The AESAN has recommended a maximum daily amount of 3,000 mg of creatine monohydrate. This proposal is based on the existence of a favourable assessment of this substance in France considering 3,000 mg/day (AFSSA, 2008) and on the authorisation in Italy (legislative proposal) of 3,000 mg/day (Italy, 2012). In Italy a amount of 6,000 mg/day is specified for sports people for a maximum of one month with the warning that it is "not recommended for pregnant women and children" (Italy, 2012).

The EFSA has issued the opinion that there is no risk with dietary supplement of creatine monohydrate up to doses of 3,000 mg/day, a dose similar to the endogenous daily renewal of creatine (EFSA, 2004).

11.3.2 Characteristics and Sources

Creatine (N-(aminoiminomethyl)-N-methyl glycine) with the empirical formula $C_4H_9N_3O_2.H_2O$ and a molecular weight of 149.1 daltons is an endogenous substance that is synthesised in the liver, kidneys and pancreas from the essential amino acids L-arginine, glycine and L-methionine (Balsom et al., 1994). In mammals it is accepted that the main formation of guanidinoacetate takes place in the kidneys, it is transported in the blood and undergoes methylation to creatine in the liver. In humans it is found in high concentrations in the skeletal muscle (approximately 95 % of the total creatine). An individual weighing 70 kg presents approximately 120 g of total creatine with a renewal rate of 2 g/day. Part of this renewal can be replaced with exogenous sources of creatine in foods, mainly meat, fish and poultry. A normal diet provides 1-2 g of creatine per day (SCF, 2000).

11.3.3 Nutrition and Metabolism

The transfer of the amidine group from L-arginine to glycine forming L-ornithine and guanidine acetic acid represents the first of the two steps in the biosynthesis of creatine, catalysed by the AGAT (L-arginine:glycine amidinotransferase) and GAMT (S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase) enzymes; the formation of guanidinoacetate is the limiting step in the biosynthesis of creatine (Walker, 1979). There are high levels of the AGAT and GAMT enzymes in the pancreas and the liver only has GAMT. The principal path of biosynthesis of creatine in mammals involves the formation of guanidinoacetate in the kidney, its transport to the blood and methylation to form creatine in the liver. Creatine is exported from the liver and transported through the blood to the tissues that require creatine. The highest content of creatine is found in the skeletal muscle, heart, spermatozoa, and photoreceptor cells of the retina. Medium levels are found in the brain, adipose tissue, intestine, seminal vesicles, endothelial cells and macrophages and only low levels are found in the lung, spleen, kidney and liver. There appears to be a good correlation between the level of the mRNA transporter and the total activity of kinase creatine, which in turn is also correlated with the total concentration of creatine. Therefore, an increase in the serum concentration of creatine, whether due to an endogenous source or to an exogenous source (supplementation in diet), results in the associated fall in the mRNA content and in the activity of the AGAT enzyme, suggesting a regulation of the AGAT expression at pretranslational level (Wyss and Kaddurah-Daouk, 2000).

In humans, creatine has significant implications for different pathological conditions. It is assumed that the creatine kinase/ phosphorylcreatine/creatine system plays a major role in the energy metabolism of the skeletal muscle. There is scientific evidence to suggest a close correlation between changes in the creatine metabolism and various neuromuscular diseases (such as different muscular dystrophies, spinal muscular atrophy,

amyotrophic lateral sclerosis), heart diseases, cardiac ischemia, neurodegenerative disease associated to oxidative stress (Parkinson's, Alzheimer's). Creatine and similar compounds may emerge as a new class of neuroprotective agents (Wyss and Kaddurah-Daouk, 2000).

The kidney plays a crucial role in the metabolism of creatine, it is the principal organ contributing to the synthesis of guanidinoacetate. In cases of chronic renal failure, the activity of renal AGAT and the urinary excretion of guanidinoacetate is decreased (Takano et al., 1989) (Marescau et al., 1997).

In general, the level of creatinine in urine is used as a marker of the renal function, however those subjects, for example many athletes, who take creatine frequently as a food supplement have high contents of creatinine in the urine, representing a greater index of muscular conversion of creatine to creatinine, rather than a renal failure. In healthy subjects who take creatine, following its muscular storage, the additional creatine is converted to creatinine and it is excreted in the urine (Pline and Smith, 2005).

11.3.4 Safety

Toxicological information about creatine in animals is available. The EFSA (2004) indicates a LD_{50} in rats greater than 2 g creatine/kg b.w. and the absence of mutagenic effects. It also describes the absence of adverse effects in a toxicity study for 28 days on rats treated at doses of 2 g creatine/kg b.w./day. Several researchers (Ipsiroglu et al., 2001) (Taes et al., 2003) (Tarnopolsky et al., 2003) (Ju et al., 2005) at doses ranging from 0.05 to 2 g creatine/kg b.w. for 2 and 8 weeks did not observe any effects or alterations that compromised the renal function.

In view of the widespread use of creatine as an ingredient in dietary supplements, it is necessary to assess the safety of the use of creatine through a quantitative risk analysis. For creatine monohydrate, the risk analysis is derived from the data from clinical trials conducted on humans and published in scientific literature. This data did not reveal any observed adverse effects, therefore the highest intake level with a sufficient margin of confidence is identified as the OSL (Hathcock, 2004) or HOI (WHO, 2006). The OSL identified from clinical tests on humans does not require correction for dietary intakes or substances from endogenous synthesis, thus the OSL for creatine monohydrate is identified as a safe ULS.

The scientific data published on humans relating to safety is described below.

There are more than 70 published studies of clinical tests on humans involving creatine, the majority of which were on healthy adults, random, controlled and aimed at assessing safety. The size of the sample, dose and duration, control of diet, physical exercise and clinical measures vary significantly among the different tests. Research on the safety and toxicity of creatine mainly focuses on its effect on the renal function, based on the fact that excess creatine is eliminated via renal glomerular filtration either as creatine or as its metabolite creatinine (Ropero-Miller et al., 2000). In clinical practice, several markers are used to assess the renal function, levels of creatinine and serum urea, and levels of albumin or urinary inulin (Farquhar and Zambraski, 2002). High levels of serum creatinine (normal value is 50-115 µmol/l) may indicate a compromised renal function. In these clinical tests, the studied form of creatine is "creatine monohydrate", each gramme corresponding to 0.879 g of creatine. Therefore, when creatine is mentioned in these clinical tests, it refers to "creatine monohydrate". In general, the population may consume approximately 1 g creatine/day in diet, equivalent to the amount produced endogenously (Balsom et al., 1994), the clinical tests published present intakes which are 3 to 12 times higher and are therefore suitable for assessing the safety of orally administered creatine.

Various clinical tests have been described (double blind, random and controlled tests) with doses of up to 26 g creatine/day (loading dose) followed by up to 6 g creatine/day (maintenance dose) (Chrusch et al., 2001); other

tests involve doses of 30 g creatine/day up to 5 years (Kreider et al., 1998) (Poortmans and Francaux, 1999) (Robinson et al., 2000) (Schilling et al., 2001) (Mayhew et al., 2002) (Potteiger et al., 2002) (Greenwood et al., 2003a) (Greenwood et al., 2003b) (Kreider et al., 2003) (Rosene et al., 2004) (Groeneveld et al., 2005). Only two of these tests described gastrointestinal effects (Chrusch et al., 2001) (Groeneveld et al., 2005) and another two studies observed an increase in serum creatinine (33 % with levels of 90 µmol/l) in subjects who had received 20 g creatine/day (5 days) followed by 3 g creatine/day (8 weeks) (Robinson et al., 2000) and an increase of serum creatinine (22.5 % with levels of 125 µmol/l) in subjects who received 15.75 g creatine/day for 8 days (Kreider et al., 1998), but both values fall within the normal range. However, these clinical studies did not permit the identification of an OSL as they had been conducted on a relative small sample size (n= 14).

Two clinical tests were described on healthy adults at doses of 20 g creatine/day (for 5 and 7 days) followed by a dose of 10 g creatine/day (28 days) (Arciero et al., 2001) (Watsford et al., 2003) and one test on patients with amyotrophic lateral sclerosis (ALS) with doses of 10 g creatine/day (310 days) (Groeneveld et al., 2005). No adverse effects or alterations in the levels of serum urea or urinary albumin were observed in these tests. However these tests cannot be used to identify an OSL either as, although the sample size was greater (n= 88), they involved patients suffering from amyotrophic lateral sclerosis.

Four clinical tests conducted by Chrush et al. (2001) have been described with doses of 26 g creatine/day, (7 days) followed by 6 g creatine/day (84 days); Bennett et al. (2001) with doses of 20 g creatine/day, (6 days) followed by 6 g creatine/day (28 days); Powers et al. (2003) with doses of 25 g creatine/day, (7 days) followed by 5.7 g creatine/day (21 days); Kilduff et al. (2003) with doses of 22.8 g creatine/day (7 days) followed by 5.7 g creatine /day (21 days), where in all these tests no adverse effects or increase in urinary creatinine levels were observed. These studies cannot be used to identify an OSL either due to the small sample size and short duration of the test.

Other clinical tests described (Walter et al., 2000) (Hespel et al., 2001) (Wilder et al., 2001) (Stevenson and Dudley, 2001) (Derave et al., 2003, 2004) (Eijnde et al., 2003) (Louis et al., 2003) (Van Loon et al., 2003) (Verbessem et al., 2003) (Tarnopolsky et al., 2004a, 2004b) (Escolar et al., 2005) with maintenance doses of 5 g creatine/day and a duration of up to 1 year on different populations serve to identify an OSL (Shao and Hathcock, 2006). No adverse effects were observed in any of these studies. The clinical test conducted by Derave et al. (2004), although the sample size is small (n= 8), is important and has also been included in the identification of the OSL as it tested healthy adults with a loading dose of 20 g creatine/day (7 days), followed by a maintenance dose of 5 g creatine/day (19 weeks).

To conclude, an OSL of 5 g creatine/day has been identified from the data on healthy individuals who consume a wide variety of diets, apart from the endogenous synthesis of creatine itself (Shao and Hathcock, 2006). Therefore, an ULS based on toxicity data from clinical trials on humans is also 5 g creatine/day, with a UF (Uncertainty Factor) of 1.0.

Summary of the results of the risk analysis of creatine monohydrate considering data from clinical tests on humans described in scientific literature

- NOAEL or LOAEL in humans: > 10 g/day.
- OSL: 5 g/day.
- ULS: as 5 g/day was the dose administered to healthy adults with a normal diet, the OSL does not require correction, and therefore the OSL= ULS= 5 g/day.

11.3.5 Conclusion

The Scientific Committee considers that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum amount of 3,000 mg/day of creatine monohydrate is acceptable from the safety point of view for use as a food supplement.

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11.4 Glucosamine (as sulfate or hydrochloride)

11.4.1 Proposal

The AESAN has recommended a maximum daily amount of 500 mg of glucosamine. This proposal is based on the existence of a favourable assessment in France considering 500 mg/day (AFSSA, 2008) and on the authorisation in Italy of 500 mg/day (Italy, 2012).

11.4.2 Characteristics and sources

Glucosamine is a water soluble monosaccharide amino acid (2-amino-2-deoxy-D-glucose). It is the principal component of the glycosaminoglycans forming the matrix of all the connective tissues including the cartilage (Harris et al., 2005). Evidence exists, obtained over 30-40 years, to confirm that glucosamine may be efficient in relieving pain occurring in cases of arthritis (Deal and Moskowitz, 1999). In general, dietary supplements of glucosamine are presented in the forms of glucosamine hydrochloride, glucosamine sulfate or N-acetyl glucosamine. Historically, the raw material for the supplements of glucosamine is obtained from chitin (long chain polymer of *N*-acetylglucosamine), a component present in shellfish (Almada, 2003). In terms of structure, chitin is similar to the polysaccharide, cellulose, and in terms of function to the protein, keratin. Nowadays it can be produced by fermentation from plant sources. Numerous clinical tests have investigated the efficiency of glucosamine compounds administered orally, often in combination with chondroitin sulfate, on individuals with arthritis.

11.4.3 Nutrition and metabolism

Glucosamine is assumed to be limiting factor in the synthesis of the glycosaminoglycan component of the cartilage. Glucosamine acts by suppressing or reducing the expression of various cytokine mediators of the degradation of articular cartilage, mediators such as matrix metalloproteinase, interleukins (IL) 1β and 8, cyclooxygenase (COX)-2, tumour necrosis factor (TNF)-α. Glucosamine acts synergically together with these cytokine mediators modulating the metabolism of the articular cartilage matrix (Lippiello, 2003). Glucosamine presents an adequate oral bioavailability, in the sulfate form it exceeds 90 % and in the hydrochloride form it reaches 100 % (Deal and Moskowitz, 1999) (Matheson and Perry, 2003) (Anderson et al., 2005) (IoM, 2005) (Persiani et al., 2007).

The possible effects of glucosamine on the insulin function and on the glucose metabolism have been studied. Mounauni et al. (2000) suggest that glucosamine may affect the homeostasis of glucose, based on the causal observation of an activation of the hexosamine phase. The activation of hexosamine leads to a deterioration of the pancreatic β-cell function, and therefore it is possible that glucosamine may increase the risk of diabetes (Kaneto et al., 2001) (Yoshikawa et al., 2002). This possible diabetogenic effect of glucosamine has been investigated in various clinical tests with daily doses of 1,500 mg glucosamine hydrochloride for 90 days (Scroggie et al., 2003) and with daily doses of 1,500 mg of glucosamine sulfate for 12 weeks (Tannis et al., 2004) concluding that no adverse effects are caused in relation to the glucosamine and the glucose metabolism.

11.4.4 Safety

There are numerous publications of clinical tests on humans to assess the efficiency of glucosamine in arthritis that also contain useful information regarding safety. None of these clinical tests has found significant adverse effects relating to the intake of glucosamine (Deal and Moskowitz, 1999) (Reginster et al., 2001) (Pavelka et al., 2002) (Richy et al., 2003) (Zerkak and Dougados, 2004) (Clegg et al., 2006). Nor did clinical tests lasting 3 years provide evidence of adverse effects among the groups treated with the placebo and those treated with glucosamine (Reginster et al., 2001) (Pavelka et al., 2002). The conclusion of these studies is the absence of significant adverse effects (Drovanti et al., 1980) (Pujalte et al., 1980) (Muller-Fassbender et al., 1994) (Noack et al., 1994) (Braham et al., 2003) (McAlindon et al., 2004) (Clegg et al., 2006). Other clinical tests on humans concluded that it is safe to use glucosamine (Matheson and Perry, 2003) (Bruyere et al., 2004).

A large number of studies in animals used for research and *in vitro* studies aimed at assessing safety and the metabolism and metabolic effects of glucosamine have been reviewed in detail by Anderson et al. (2005). The LD_{50} of glucosamine hydrochloride is higher than 5,000 mg/kg b.w., and the NOAEL is 2,700 mg/kg b.w. in rats and 2,149 mg/kg b.w. in dogs (Anderson et al., 2005). Assuming a body weight in humans of 60 kg, a daily dose of 1,500 mg in humans is equivalent to 25 mg/kg b.w., and a daily dose of 2,000 mg to 33 mg/kg b.w. Therefore, the extrapolation of numerous data collected in *in vivo* and *in vitro* toxicity studies suggest the low probability of adverse effects appearing in humans.

In the studies conducted on humans, none of the clinical tests detected adverse effects relating to the administration of glucosamine (either in its sulfate form or hydrochloride form), therefore by definition there is no basis for identifying a LOAEL for humans. In the absence of a LOAEL, a NOAEL is not normally established. Without these two values, it is not appropriate to establish an UL (IoM, 1998).

The glucosamine dose used in the majority of clinical tests published is 1,500 mg/day. There is only one clinical test with a dose of 2,000 mg of glucosamine hydrochloride and no adverse effects were observed in this test either. There is considerable data to permit the identification of 1,500 mg of glucosamine sulfate as the OSL, but a clinical test with 2,000 mg of glucosamine hydrochloride also permits the identification of an OSL of 2,000 mg glucosamine hydrochloride, which may also be used for glucosamine sulfate. The OSL for glucosamine established at 2,000 mg is also identified as the ULS. The warning of allergic reactions is appropriate and necessary only for those products that include glucosamine extracted from shellfish.

Due to the large amount of data from existing clinical tests on humans, the value of the OSL can be identified without needing to use the data obtained from animals used for research.

For the glucosamine food supplement, based on the published data obtained in well-designed, random and controlled clinical tests on humans with meta-analysis and a high confidence level in the analysis of the results, Hathcok and Shao (2007) suggest the following risk assessment:

- Absence of a well-defined critical effect for the selection of a NOAEL or LOAEL.
- OSL: 2,000 mg glucosamine (in its sulfate form, and hydrochloride form).
- ULS: 2,000 mg/day.

11.4.5 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the general consideration reflected in this report, the proposal for a maximum daily amount of glucosamine as a food supplement of 500 mg, submitted by the AESAN and based on the prior reports from France and Italy, and considering the bibliography consulted and described in this report, is acceptable from the safety aspect for use as a food supplement

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11.5 Inositol hexaphosphate

11.5.1 Proposal

The AESAN has recommended a maximum daily amount of 2,000 mg for inositol hexaphosphate. This proposal is based on the existence of a favourable assessment of this substance in France considering 2,000 mg/day of inositol hexaphosphate (AFSSA, 2008) and on the authorisation in Italy of 2,000 mg/day (Italy, 2012).

Regulation (EC) No 953/2009 (EU, 2009) includes inositol among the substances that may be added for specific nutritional purposes in foods for particular nutritional purposes. Specifically, it may be added to diet foods for particular nutritional purposes, including foods intended for special medical purposes, and excluding milk and follow-on milk formulae, processed cereal-based foods and baby food for infants and young children.

Directive 2006/141/EC (EU, 2006) relating to the milk and follow-on milk formulae and its transposition in Spain to Royal Decree 867/2008 (BOE, 2008) permits, on establishing the basic composition of infant formulae when reconstituted in accordance with the manufacturer's instructions, the use of inositol in a minimum concentration of 1 mg/100 kJ (4 mg/100 kcal) and a maximum of 10 mg/100 kJ (40 mg/100 kcal).

Inositol is listed in the European Commission Report on substances present in food supplements in the European Union (DG SANCO, 2008).

In Denmark inositol is authorised in food supplements with a total maximum amount that must not exceed 50 mg per daily recommended dose (Denmark, 2011).

11.5.2 Characteristics and sources

Inositol is a cyclic polyol, with molecular formula $C_6H_{12}O_6$, whose active isomer is myo-inositol or inositol monophosphate. Inositol and its phosphate derivatives are physiologically inter-convertible and are present in an interval of 0.01-1.0 mM in the majority of cells.

In 1914, Anderson presented the molecular structure of myo-inositol hexaphosphate also known as phytic acid, a structure that was later confirmed by more precise analytical techniques (Johnson and Tate, 1969) (Barrientos and Murthy, 1996).

Phytate, the salt from phytic acid, is widely found in plants, it is a form of storage for phosphorus and minerals, and approximately 75 % of the total phosphorus is mainly present in grains or seeds (Raboy, 2003). Other parts of the plants, including the roots and tubers are phytate-poor (approximately 0.1 % of the total) (Phillippy et al., 2003). In addition to phytate, other inositol phosphates such as pentaphosphate inositol and tetraphosphate inositol are also present in the seeds, but in lower proportions (approximately 15 %). During the germination of the seeds, the phytate is hydrolysed and releases minerals such as calcium and magnesium for germination and the development of the seed (Tabekhia and Luh, 1980) (Beal and Mehta, 1985).

Phytate is mainly found in unprocessed foods. The daily intake of phytate and other inositol phosphates is estimated to vary between 0.3 and 2.6 g, and in the case of a vegetarian diet, it may reach values of 4.6 g (Reddy, 2002). The majority of cereals, pulses, nuts, seed and soybean oils contain phytate in a proportion of 0.5 to 6.4 % of dry weight or even more; hexaphosphate inositol is mainly bonded to calcium and magnesium ions. Phytate is stable at cooking temperature but may be hydrolysed during the process due to the action of phytases (Schlemmer et al., 1995) (Sandberg and Andlid, 2002).

For decades, phytate has been considered as an anti-nutrient, as in its gastrointestinal passage it may inhibit the absorption of certain essential trace elements and some minerals, which under certain dietary circumstances may result in calcium, iron and zinc deficiency (McCance and Widdowson, 1942) (Halsted et al., 1972). In this respect, numerous investigations have been carried out with the object of releasing phytate from food and thus preventing mineral or essential trace element deficiency.

However, in the last 30 years, studies have been made of the beneficial properties of phytate which include antioxidant and anti-cancer activities (Graf et al., 1987) (Shamsuddin, 1995), the inhibition of calcium salt crystallisation and the prevention of kidney stone formation (Grases and Costa-Bouza, 1999), and the reduction in blood cholesterol and glucose levels (Jariwalla et al., 1990) (Lee et al., 2006, 2007). All these discoveries have resulted in numerous discussions in the scientific community regarding the role of phytate and other inositol phosphates in human nutrition and their beneficial properties for health. The current demand of the population in the improvement of the bioavailability of minerals and trace elements to help prevent cancer, the formation of kidney stones or other diseases typical of today's society, means that nowadays phytate is viewed as a food supplement and that the term "anti-nutrient" has been left in the past.

11.5.3 Nutrition and metabolism

It has been demonstrated in humans that 37-66 % of phytate is degraded during digestion in the stomach and small intestine when the diet is rich in food of plant origin containing phytases (Sandberg and Anderson, 1988). However, considering that the majority of cereals and pulses are ingested after processing or cooking, processes which inactive most phytases, the degradation of phytate in the stomach and small intestine due to the presence of phytases is limited. There is evidence that the principal hydrolysis of phytate in humans occurs in the large intestine thanks to the microbial phytases.

Different studies carried out on cell cultures, on rats and humans showed the absorption of phytate, although the absorption mechanism is not clear. Phytate absorption in humans is correlated to the amount of phytate consumed and the increase observed in the blood concentrations and the urinary excretion of phytate (Grases et al., 2000a, 2001c, 2006). Similar results were observed in rats (Grases et al., 2001a, 2001b). Given that the absorption of phytate present in the diet is independent of how full the stomach is (Grases et al., 2006), it is assumed that phytate absorption probably takes place in the small intestine, although some studies show that absorption in the intestine is very low and does not exceed a small percentage ≤2 % (Grases et al., 2000b). In any case, there are a large number of *in vitro* and *in vivo* studies that show certain activities after the application of sodium phytate or calcium-magnesium phytate, from which it can be deduced that phytate or its degradation products are absorbed in the intestine (Grases and Costa-Bouza, 1999) (Vucenik and Shamsuddin, 2006). Nevertheless, further studies are required to explain the fact that phytate may be hydrolysed completely in the intestine followed by intracellular re-phosphorylation after absorption, or the fact that it may be absorbed via active transport, by pinocytosis or other paths of absorption or a combination of the same.

The data collected from the different reviews on the average daily intake of phytate in humans in different countries shows differences according to gender and age, and according to whether they are from urban or rural areas, developed or developing countries.

In general, for adults, the different daily intake values of phytate suggested are as follows (Schlemmer et al., 2009):

- Low intakes of 200 to 350 mg/day, probably due to western-style diets low in phytate-rich plant-based foods.

- Medium intakes of 500 to 800 mg/day, probably due to western-style diets with portions enriched with cereals, seeds or other phytate-rich foods.
- High intakes >1,000 mg/day, probably due to diets rich in vegetables and foods containing phytates such as vegetarian diets.

In the developing countries, due to the high content of cereals and pulses consumed in the traditional diet, the daily intake of phytate reaches high values of up to 2,000 mg/day and even higher.

The data collected from various publications on the daily intake of phytic/phytate acid in the European Union reaches the following values:

- In the United Kingdom (adults, 40 years old), 500-1,436 mg/day.
- In Italy, 112-1,367 mg/day.
- In Sweden (vegetarian diets), 500-2,927 mg/day.

11.5.4 Safety

There are numerous studies in humans that, although aimed at assessing the beneficial properties and not the safety, may serve as abase for accepting the safety of its use (Jenab and Thompson, 1998) (Shamsuddin, 2002) (Grases et al., 2004, 2007a, 2007b) (Engelman et al., 2005) (Lee et al., 2005, 2006) (Vucenik and Shamsuddin, 2006).

The different assessments made for phytate (inositol hexaphosphate) have not revealed risks for the health of consumers. Various clinical studies carried out to assess the efficiency of inositol in different pathological situations have not provided evidence of acute adverse effects attributable to phytate.

Studies on animal models

Studies have been carried out on animals on the effects of myo-inositol on the lipid metabolism (Yagi and Kotaki 1969) (Shepherd and Taylor, 1974) (Okazaki et al., 2006), on lung cancer (Estensen et al., 2004) (Witschi et al., 2004), on neuropathy (Nakamura et al., 1997) and on diabetic cataracts (Beyer-Mears et al., 1989), when included in the diet at 0.2 to 3 %. No adverse effects were observed in any of these studies.

Studies in humans

Studies of supplementation in humans have been carried out with respect to diabetic neuropathy (Agostini et al., 2006), mood changes, specific premature retinopathy (Friedman et al., 2000) or on the prevention of lung cancer (Lam et al., 2006). No adverse effects were observed in any of these studies.

In two studies carried out by Lam et al. (2006), the effect was assessed of myo-inositol, administered in increasing doses from 12 to 30 g/day, to 16 subjects for one month. The only adverse effects observed were slight gastrointestinal upset. The maximum daily dose without adverse effects was established at 18 g/day. In a second test, the effects myo-inositol were assessed on 10 subjects at a dose of 18 g/day for 3 months, and no adverse effects were observed.

11.5.5 Conclusion

The Scientific Committee considers that, based on the information available to date and taking into account the general considerations reflected in this report, the AESAN proposal of a maximum amount of 2,000 mg/day of inositol (hexaphosphate) is acceptable from the safety point of view for use as a food supplement.

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