Report of the Scientific Committee of the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN) on the conditions of use of certain substances to be used in food supplements-4

Section of Food Safety and Nutrition

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Abstract

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

In Spain, food supplements are regulated by Royal Decree 1487/2009, which transposed Directive 2002/46/EC on the approximation of the laws of the Member States relating to food supplements into Spanish law. However, only the use of vitamins and minerals is currently regulated. Therefore the Scientific Committee has been asked to make an assessment of the proposal to authorise certain substances other than vitamins and minerals in the manufacture of food supplements. The substances proposed by the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN) are L-aspartic acid, L-citrulline, glycine, L-proline, L-serine, L-arginine-L-aspartate, L-lysine-L-aspartate, L-lysine-L-glutamate, N-acetyl-L-cysteine, N-acetyl-L-methionine, hidroxymethylbutyrate, lipoic acid, *Monascus purpureus*, activated carbon and lactulose.

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

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case individuals undergoing medical treatment must seek medical advice as to the suitability of taking food supplements, given the possibility of interactions in certain cases. In addition, in the case of food supplements with an antioxidant effect, it should be noted that in certain conditions and at high doses, these compounds may behave as pro-oxidants.

Key words

Food supplements, L-aspartic acid, L-citrulline, glycine, L-proline, L-serine, L-arginine-L-aspartate, L-lysine-L-aspartate, L-lysine-L-glutamate, N-acetyl-L-cysteine, N-acetyl-L-methionine, hidroxymethylbutyrate, lipoic acid, *Monascus purpureus,* activated carbon, lactulose.

1. Introduction

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

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

Royal Decree 1487/2009, of 26 September, relating to food supplements currently only considers vitamins and minerals among the substances authorised for use in the manufacture of food supplements in Spain. 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. Therefore, AECOSAN has drawn up several requests for evaluation relating to 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.

To continue the work to update Royal Decree 1487/2009, of 26 September, relating to food supplements, AECOSAN has asked the Section of Food Safety and Nutrition of the Scientific Committee to assess the suitability of certain substances as food supplements in order to include them in Annex III of this Royal Decree.

2. Proposal

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

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

Proposed substance	Proposed maximum daily quantity
L-aspartic acid	-
L-citrulline	-
Glycine	-
L-proline	-
L-serine	-
L-arginine-L-aspartate	-
L-lysine-L-aspartate	-
L-lysine-L-glutamate	-
N-acetyl-L-cysteine	300 mg
N-acetyl-L-methionine	-
Hydroxymethylbutyrate	3 g
Lipoic acid	-
Monascus purpureus	10 mg (Monacolin K)
Active carbon	2 g
Lactulose	10 g

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 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 it is 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 their efficiency 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 or lactating 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. Individuals receiving treatment with medicines containing substances present in food supplements should seek medical advice to avoid the possibility of an overdose.

The consumption of supplements containing substances found naturally in foods may result in a total intake which is higher than the maximum quantity established for these substances in their use as a food supplement. 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.

References

BOE (2008). Real Decreto 867/2008, de 23 de mayo, por el que se aprueba la reglamentación técnico-sanitaria específica de los preparados para lactantes y de los preparados de continuación. BOE Nº 131 de 30 de mayo de 2008, pp: 25121-25137.

BOE (2009). Real Decreto 1487/2009, de 26 de septiembre, relativo a los complementos alimenticios. BOE Nº 244 de 9 de octubre de 2009, pp: 85370-85378.

EU (1997). Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1991 concerning novel foods and novel food ingredients. OJ L 43 of 14 February 1997, pp: 1-6.

EU (2002a). Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31 of 1 February 2002, pp: 1-24.

EU (2002b). Directive 2002/46/CE of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. OJ L 183 of 12 July 2002, pp: 51-57.

EU (2006a). Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. OJ L 404 of 30 December of 2006, pp: 9-25.

EU (2006b). Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC. OJ L 401 of 30 December 2006, pp: 1-33.

EU (2009). Commission Regulation (EC) No 953/2009 of 13 October 2009 on substances that may be added for specific nutritional purposes in foods for particular nutritional uses. OJ L 269 of 14 October 2009, pp: 9-19.

EU (2012). Commission Regulation (EU) No 432/2012 of 16 May 2012, establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children's development and health. OJ L 136 of 25 May 2012, pp: 1-40.

FVO (2011). Food and Veterinary Office. Report on a desk study on official controls in the field of food supplements. Health & Consumer Directorate-General. European Commission.

4. Amino acids

4.1 Proposal

The AECOSAN has recommended the inclusion of different amino acids in Royal Decree 1487/2009 without specifying a maximum daily quantity, except in the case of N-acetyl-L-cysteine. The application is based on the fact that they are accepted for use in the preparation of foods intended for special medical purposes, as established in the Annex to Regulation (EC) No 953/2009.

4.2 L-aspartic acid

L-aspartic acid is a non-essential amino acid, which means that it can be synthesised in the body. The body is able to synthesise 80 % of all the amino acids and the remaining 20 % must be obtained from the diet.

L-aspartic acid is present in frequently consumed foods of animal or plant origin and is a metabolite of the sweetener aspartame.

4.2.1 Characteristics and sources

L-aspartic acid has a molecular weight of 133.1 g/mol. It has the following molecular structure (Figure 1):

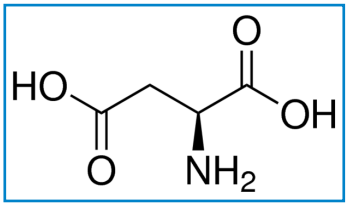

Figure 1. Molecular structure of L-aspartic acid.

The blood plasma concentration ranges between 0.01 and 0.3 mg/100 and in urine (mg/24 hours) the concentration is 165 mg, where 2 mg are in free form and 163 mg in conjugated form (Hernández and Sastre, 1999). Aspartic acid is found in cereals and pulses (chick peas, lentils and soya), tubers such as the potato, nuts including peanuts and seafood such as salmon and prawns.

The additive aspartame, used as a sweetener, is an exogenous source of L-aspartic acid, as it metabolises in aspartic, phenylalanine and quantities of methanol (Gil, 2005).

4.2.2 Nutrition and metabolism

L-aspartic acid is reversibly linked to oxaloacetate via the enzyme aspartate aminotransferase. This amino acid intervenes in the synthesis of purine and pyrimidine bases and in ureogenesis (Gil, 2005).

As all amino acids it suffers two biotransformation reactions. One transamination reaction, catalysed by transaminases, in which one amino acid is converted into another. This type of reaction takes place in the cell cytosol and in the mitochondrions. Essentially the transaminases

are alanine aminotransferase and aspartate aminotransferase, both of which require pyridoxal phosphate as a cofactor. This is derived from vitamin B_6 or pyridoxin.

The second reaction is the oxidative deamination, which takes place in the mitochondrions. Here, the enzyme glutamate-dehydrogenase acid eliminates the amino group from glutamic acid. Consequently urea is formed and the carbonated chains are products of glycolysis and the Krebs cycle. The product of the deamination of aspartic is fumarate.

Aspartic acid also intervenes in the DNA metabolism and has neurotransmission functions (Gil, 2005).

4.2.3 Safety

Excess L-aspartic acid may cause neurotoxicity in animals, in particular hypothalamic lesions in rats (Schnainker and Olney, 1974). In humans, according to the Food and Nutrition Board (FNB/IOM, 2002), no adverse effects have been reported when supplements of 8 g per day are administered in addition to dietary aspartic.

Tada et al. (2008), carried out a subchronic toxicity study in rats, in which the authors established a NOAEL (No Observed Adverse Effect Level) for female rats of 715.2 mg/kg/day and of 696.6 mg/kg/day for male rates. Considering the lowest NOAEL of 696.6 mg/kg/day (700 mg/kg/day) and taking an uncertainty factor of 100, the ADI (Acceptable Daily Intake) is 7 mg/kg/day.

For an individual weighing 70 kg, this would permit a daily intake of 490 mg of L-aspartic acid.

It should be noted that this amino acid forms part of the basic dietary food and is eaten as a metabolite of the sweetener aspartame.

4.2.4 Conclusion

The Scientific Committee concludes that, based on the information available to date and taking into account the general considerations reflected in this report, there are subchronic toxicity trials in rats from which it is possible to estimate that a maximum daily quantity of 490 mg of L-aspartic is acceptable from the safety point of view for use as a food supplement.

In addition, it should be noted that this amino acid forms part of the basic dietary food and is eaten as a metabolite of the sweetener aspartame.

References

FNB/IOM (2002). Food and Nutrition Board/Institute of Medicine. Dietary Reference Intakes. Available at: http://www.nal.usda.gov/fnic/DRI/DRI_Energy/energy_full_report.pdf [accessed: 22-09-15].

Gil, A. (2005). In book: *Tratado de Nutrición* (tomo I). Madrid. Editorial Acción Médica.

Hernández, M. and Sastre, A. (1999). In book: *Tratado de Nutrición*. Madrid. Editorial Díaz de Santos.

Schnainker, B. and Olney, J.W. (1974). Glutamate-type hypothalamic-pituitary syndrome in mice treated with aspartate or cysteate in infancy. *Journal of Neural Transmission*, 35, pp: 207-215.

Tada, Y., Yano, N., Takahashi, H., Yuzawa, K., Ando, H., Kubo, Y., Nagasawa, A., Uehara, S., Ogata, A. and Nakae, D. (2008). Toxic effect of l-aspartic acid at high dose levels on kidneys and salivary glands in Fischer 344 rats detected in a 90-day feeding study. *Food and Chemical Toxicology*, 46 (8), pp: 2789-2795.

4.3 L-citrulline

4.3.1 Characteristics and sources

L-citrulline is an α-amino acid belonging to the group of non-protein amino acids. It corresponds to (2S)-2-amino-5-(carbamoylamino) pentanoic acid and its chemical formula is $C_6H_{13}N_3O_3$ (Figure 2):

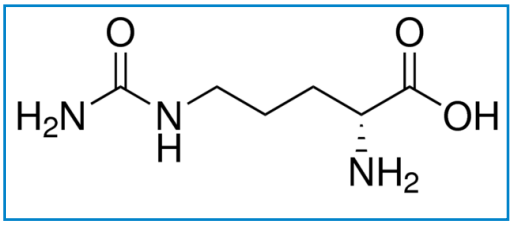

Figure 2. Molecular structure of L-citrulline.

It is found in high levels in certain cucurbits including water melon, cucumber, pumpkin, courgette, etc. (Kaore and Kaore, 2014), and in certain algae such as *Grateloupia vulgaris* (Curis et al., 2005).

It is also present in vegetables including onions and garlic, and in protein foods such as fish, meat, pulses and milk.

4.3.2 Nutrition and metabolism

L-citrulline is a non-essential amino acid and a precursor to L-arginine which forms part of the urea cycle.

Three enzymes intervene in the metabolism of free citrulline: nitric oxide synthase (NOS) and ornithine carbamoyltransferase (OCT) which produce citrulline, and argininosuccinate synthase (ASS) which transform citrulline into arginine. The tissue distribution of these enzymes results in three metabolic routes for citrulline. Firstly, in the liver citrulline is synthesized by the OCT and metabolised by the ASS for the production of urea. Secondly, in the majority of tissues that produce NO, citrulline is recycled to arginine via ASS to increase the availability of arginine for the production of NO. Lastly, in third place, citrulline is synthesised in the intestine from glutamine (through the OCT), released to the blood stream and converted into arginine in the kidney (by the ASS). In this way, the circulating citrulline is in fact a disguised form of arginine to prevent capture by the liver (Curis et al., 2005).

Various proteins contain citrulline as a result of a post-translational modification. These citrulline residues are generated by a family of enzymes called Peptidyl-Arginine Deiminases (PAD), which convert arginine into citrulline in a process called citrullination or deimination. Proteins which normally contain citrulline residues include: myelin basic protein (MBP), filaggrin, and several histone proteins, while other proteins such as fibrin and vimentin may be citrullinated during cell-death and tissue inflammation.

4.3.3 Safety

There are few available toxicity studies with L-citrulline. Pradilla et al. (2012) did not find symptoms of systemic or neuronal toxicity in a histopathological analysis on mice exposed intraperitoneally to up to 200 mg/kg every 8 hours for 90 days.

Studies conducted on humans do not reveal any toxic effects from citrulline. The highest oral doses used are 0.18 g/kg/day (approximately 12.6 g/day considering 70 kg weight) for 7 days (Thibault et al., 2011) and 15 g in a single dose (Moinard et al., 2008). Nevertheless, the latter advised a dose of 10 g for use in clinical practice as higher doses did not imply a greater benefit. Longer-term studies of supplementation, for example Figueroa et al. (2015) do not mention adverse effects after oral administration of 6 g/day of L-citrulline for 8 weeks to elderly women. Similarly, Rajantie et al. (1980) did not observe signs of toxicity in children treated for 2 years with 2-2.8 mg/day.

EFSA has published a scientific opinion regarding the verification of health claims relating to citrulline-malate: faster recovery from muscular fatigue after exercise, with negative results (EFSA, 2014). In the application, a daily dose of 2-3 g for children and 3-6 g for adults is recommended, where the target population is healthy children above the age of 6 years and adults. It should be taken with meals, and must not be taken during pregnancy and breastfeeding. It must not be taken for more than 4 weeks.

As indicated above, L-citrulline is a precursor of L-arginine. Unlike arginine which is extensively captured by the liver and metabolised to urea, the citrulline synthesised in the intestine passes freely through the liver and is captured by the kidney (Osowska et al., 2004). In this organ, approximately 80 % of citrulline is transformed to arginine (Kaore and Kaore, 2014). A number of authors consider L-citrulline is a good alternative to supplementation with arginine (Hartman et al., 1994) (Osowska et al., 2004) as the latter is an ureagenesis catalyst (Curis et al., 2005). For L-arginine, AECOSAN recommended a maximum daily quantity of 3 g for use as a food supplement (AECOSAN, 2012) although an Observed Safe Level (OSL) could be established of 20 g/day with a sufficient level of confidence.

4.3.4 Conclusion

No data has been found in the scientific bibliography of adverse effects or clinical alterations due to the oral intake of L-citrulline, therefore it is not possible to establish a NOAEL/LOAEL for its oral administration. In 2012, AECOSAN recommended a maximum daily intake of 3 g for L-arginine which was considered acceptable by the Scientific Committee given that there is an Observed Safe Level (OSL) of 20 g/day. Given that L-citrulline is a precursor of this, the same value of 3 g is acceptable from the safety point of view for use as a food supplement, as studies of supplementation exist with higher long-term doses that do not give signs of toxicity.

References

AECOSAN (2012). Agencia Española de Consumo, Seguridad Alimentaria y Nutrición. Informe del Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) sobre condiciones de uso de determinadas sustancias distintas de vitaminas, minerales y plantas para ser empleadas en complementos alimenticios-1. *Revista del Comité Científico de la AECOSAN*, 17, pp: 11-234.

Curis, E., Nicolis, I., Moinard, C., Osowska, S., Zerrouk, N., Benazeth, S. and Cynober, L. (2005). Almost all about citrulline in mammals. *Amino Acids*, 29, pp: 177-205.

EFSA (2014). European Food Safety Authority. Scientific Opinion on the substantiation of a health claim related to citrulline-malate and faster recovery from muscle fatigue after exercise pursuant to Article 13(5) of Regulation (EC) No 1924/20061. *The EFSA Journal*, 12 (5), pp: 3650.

Figueroa, A., Álvarez-Alvarado, S., Ormsbee, M., Madzima, T.A., Campbell, J.C. and Wong, A. (2015) Impact of L-citrulline supplementation and whole-body vibration training on arterial stiffness and leg muscle function in obese postmenopausal women with high blood pressure. *Experimental Gerontology,* 63, pp: 35-40.

Hartman, W.J., Torre, P.M. and Prior, R.L. (1994). Dietary citrulline but not ornithine counteracts dietary arginine deficiency in rats by increasing splanchnic release of citrulline. *Journal of Nutrition*, 124, pp: 1950-1960.

Kaore, S.N. and Kaore, N.M. (2014). Capítulo 53. In book: *Citrulline: pharmacological perspectives and role as a biomarker in diseases and toxicities. Biomarkers in Toxicology* (Elsevier). pp: 883-905.

Moinard, C., Nicolis, I., Neveux, N., Darquy, S., Benazeth, S. and Cynober, L. (2008). Dose-ranging effects of citrulline administration on plasma amino acids and hormonal patterns in healthy subjects: the Citrudose pharmacokinetic study. *British Journal of Nutrition,* 99, pp: 855-862.

Osowska, S., Moinard, C., Neveux, N., Loï, C. and Cynober, L. (2004). Citrulline increases arginine pools and restores nitrogen balance after massive intestinal resection. *Gut,* 53, pp: 1781-1786.

Pradilla, G., Garzon-Muvdi, T., Ruzevick, J., Bender, M., Edwards, L., Momin, E.N., Thompson, R.C. and Tamargo, R. (2012). Systemic L-citrulline prevents cerebral vasospasm in haptoglobin 2-2 transgenic mice after subarachnoid hemorrhage. *Neurosurgery*, 70 (3), pp: 747-756.

Rajantie, J., Simell, O., Rapola, J. and Perheentupa, J. (1980). Lysinuric protein intolerance: A two-year trial of dietary supplementation therapy with citrulline and lysine. *The Journal of Pediatrics*, 97, pp: 927-932.

Thibault, R., Flet, L., Vavasseur, F., Lemerle, M., Ferchaud-Roucher, V., Picotf, D. and Darmaun, D. (2011). Oral citrulline does not affect whole body protein metabolism in healthy human volunteers: Results of a prospective, randomized, double-blind, cross-over study. *Clinical Nutrition*, 30, pp: 807-811.

4.4 Glycine

Glycine is classified as a non-essential amino acid for mammals due to the fact that it is synthesized endogenously. However, the quantity of glycine synthesized *in vivo* is known to sometimes be insufficient and in the event of chronic glycine deficiency the adverse effects described include effects on growth and the immune system (De Koning et al., 2003) (Lewis et al., 2005). Consequently, the classification of glycine as a conditionally essential amino acid in humans is currently under consideration mainly to ensure optimum growth (Wu et al., 2013).

4.4.1 Characteristics and sources

Glycine is a small amino acid found in the hydrophobic interior of proteins. The rest is polar, and not charged. Glycine has the simplest chain, one hydrogen atom. The molecular weight of this amino acid is 75.07 g/mol and its molecular structure is as follows (Figure 3):

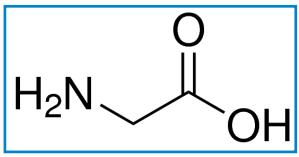

Figure 3. Molecular structure of glycine.

Its isoelectric point is 5.97. It is found in food of animal origin such as meat, fish, eggs and dairy products and in food of plant origin including pulses, vegetables, potatoes, fruit and wholegrain cereals.

Glycine has multiple physiological functions. It is the main component of collagen and elastin which are the most abundant proteins in the body. Glycine is the precursor to various important metabolites of low molecular weight including porphyrins, purines, glutathione, the heme group and creatine.

Glycine is important as an inhibitory neurotransmitter in the Central Nervous System (CNS), and has a significant role in the nervous tissue, and also in epigenetic regulation. Consequently, some authors have described glycine as a functional amino acid in the field of nutrition (Zhong et al., 2003).

Three phases have been identified for the synthesis of glycine in animals and in humans (Wang et al., 2013). Glycine is synthesized from: 1) serine, via serine hydroxymethyltransferase (SHMT); 2) choline, via the formation of sarcosine; and 3) threonine, via the threonine dehydrogenase pathway.

4.4.2 Nutrition and metabolism

The metabolism of glycine occurs in three pathways: 1) decarboxylation and deamination of the glycine by a complex enzymatic system, 2) conversion into serine by the enzyme SHMT, and 3) conversion into glyoxylate by D-amino acid oxidase (Figure 4).

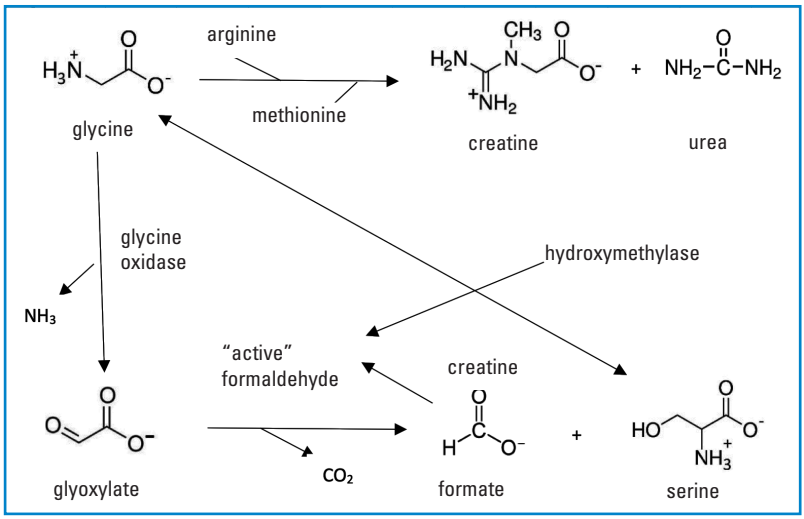

Figure 4. Biochemical pathways for glycine metabolism. **Source:** (Wang et al., 1985).

Glycine and serine have a related metabolism, as both are derived from glyceric acid-3-P, which is an intermediary of glycolisis. This acid is oxidised and then transaminated to form the 3-P-serine, which forms serine as a result of hydrolysis of the phosphate group. Glycine is synthesized from serine, in a reaction in which tetrahydrofolate is converted to N5,N10-Methylene tetrahydrofolate, leaving a remainder of glycine (Gil, 2005).

Glycine has a non-GABAergic receptor, which is common with other amino acids including β-alanine, taurine, L-alanine, L-serine and proline. This receptor is found in the postsynaptic neuronal membranes (Matilla et al., 2002).

Glycine is also an amino acid which forms part of the conjugation or Phase II reactions in the biotransformation of toxins and drugs. Via the enzyme glycine N-acetyltransferase, it forms conjugates with xenobiotics that are easily eliminated. In this way, benzoic acid and salicylic acid are conjugated by glycine (Repetto and Repetto, 2009).

4.4.3 Safety

At present there are not enough toxicity studies of this amino acid in animals and humans to provide an analysis in order to establish a NOAEL. Future studies may help to clarify the nature of the morphological alterations in brain cells induced by the administration of glycine. The Federation of American Societies for Experimental Biology for the Food and Drug Administration (FDA/FASEB, 1993) indicates that the consumption of amino acids in the form of dietary supplements may pose a risk for various population groups including women of reproductive age, children, adolescents, the elderly and the sick. It should be noted that glycine is synthesised by the body and is also present in food of animal and plant origin. From a safety point of view there is not data that permits the establishment of a daily intake for humans. The

EFSA (2014) indicates that high concentrations of glycine to the order of 20 g/kg of food is safe for dogs and cats.

The role of glycine and other excitatory amino acids is queried in the production of neurodegeneration in humans. The application of long-term therapies with glycine in cases of schizophrenia is associated with neurotoxic effects. Neuronal death has been observed induced by ischemia with high levels of glutamate, glycine and γ-aminobutyric acid (Baker et al., 1991) (Globus et al., 1991).

The first studies conducted on animals used in research with the aim of collecting data on the neurotoxic potential of glycine were carried out by Shoham et al. (1999). Rats treated for 2 weeks with a glycine dietary supplement, doses of 0.8 g/kg/day and four times higher, 3.2 g/kg/day, than the highest glycine dose employed in clinical trials did not demonstrate effects on rat brain cell morphology. Nevertheless, in a more prolonged treatment, with regimens of 1 g/kg/day or 5 g/kg/day for 5 months, although there was no evidence of neuronal degeneration, a reduction of the voltage-sensitive Ca^{++} channels (Class B, N-type) was observed in specific regions of the brain that may be due to a general adaptation to long-term treatment with glycine (Shoham et al., 2001).

4.4.4 Conclusion

The Scientific Committee considers that the available toxicological information is insufficient to determine the maximum daily quantity of glycine that might be considered safe in its use as a food supplement.

References

Baker, A.J., Zernon, M.H., Sheller, M.S., Yaksh, T.L., Skilling, S.R., Smullin, D.H., Larson, A.A. and Kuczenski, R. (1991). Changes in extracellular concentration of glutamate, aspartate, glycine, dopamine, serotonin, and dopamine metabolites after transient global ischemia in the rabbit brain. *Journal of Neurochemistry,* 57, pp: 1370-1379.

De Koning, T.J., Snell, K., Duran, M., Berger, R., Poll-The, B.T. and Surtees, R. (2003). L-serine in disease and development. *Biochemical Journal*, 371 (3), pp: 653-661.

EFSA (2014). European Food Safety Authority. Scientific Opinion on the safety and efficacy of the use of amino acids (chemical group 34) when used as flavourings for all animal species. *The EFSA Journa*l, 12 (5): 3670, pp: 1-19.

FDA/FASEB. (1993). Food and Drug Administration. Center for food Safety and Applied Nutrition. Illnesses and injuries associated with the use of selected dietary supplements. Available at: http://www.cfsan.fda.gov/-dms/ds-ill.html [accessed: 17-04-07].

Gil, A. (2005). In book: *Tratado de Nutrición* (tomo I). Editorial Acción Médica. Madrid.

Globus, M.Y.T., Ginsberg, M.D. and Busto, R. (1991). Excitotoxicity index -a biochemical marker of selective vulnerability. *Neuroscience Letters,* 127, pp: 39-42.

Lewis, R.M., Godfrey, K.M., Jackson, A.A., Cameron, I.T. and Hanson, M.A. (2005). Low serine hydroxymethyltransferase activity in the human placenta has important implications for fetal glycine supply. *Journal of Clinical Endocrinology and Metabolism,* 90 (3), pp: 1594-1598.

Matilla, B., Mauriz, J.L., Culebras, J.M., González-Gallego, J. and González, P. (2002). La glicina: un nutriente antioxidante protector celular. *Nutrición Hospitalaria,* XVII (1), pp: 2-9.

Repetto, M. and Repetto, G. (2009). In book: *Toxicología Fundamenta*l. Ediciones Díaz de Santos. Madrid.

Shoham, S., Javitt, D.C. and Heresco-Levy, U. (1999). High dose glycine nutrition affects glial cell morphology in rat hippocampus and cerebellum. *International Journal of Neuropsychopharmacology,* 2, pp: 35-40.

Shoham, S., Javitt, D.C. and Heresco-Levy, U. (2001). Chronic high-dose glycine nutrition: effects on rat brain cell morphology. *Biological Psychiatry,* 49, pp: 876-885.

Wu, G., Wu, Z., Dai, Z., Yang, Y., Wang, W., Liu, C., Wang, B., Wang, J.W. and Ying, Y. (2013). Dietary requirements of nutritionally non-essential amino acids by animals and humans. *Amino acids,* 44, pp: 1107-1113.

Zhong, Z., Wheeler, M.D., Li, X., Froh, M., Schemmer, P. and Yin, M. (2003). L-Glycine: a novel antiinflammatory, immunomodulatory, and cytoprotective agent. *Current Opinion in Clinical Nutrition & Metabolism Care*, 6, pp: 229-240.

4.5 L-proline

4.5.1 Characteristics and sources

L-proline is a protein amino acid whose α-amino is not a primary amine but a secondary amine. It corresponds to the (S)-pyrrolidine-2-carboxylic acid and the chemical formula is $C_5H_9NO_2$. It has the following molecular structure (Figure 5):

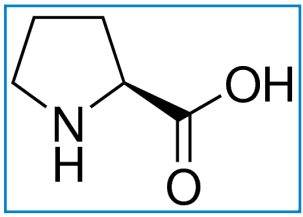

Figure 5. Molecular structure of L-proline.

Food rich in L-proline include gelatine, pork rind, soya protein, some cheeses and powdered milk.

4.5.2 Nutrition and metabolism

L-proline is a non-essential amino acid formed from glutamate and which can generate glutamate in its metabolisation.

The glutamic semialdehyde, formed reversibly from glutamate via glutamate kinase and dehydrogenase, can be reversibly transformed into pyrroline-5-carboxylate to connect with the formation of proline via the pyrroline carboxylate reductase (Lehninger et al., 1995).

It is a fundamental amino acid in the structure of collagen, especially after its post-translational formation in hydroxyproline, with the assistance of vitamin C (Sánchez de Medina, 2010).

Hyperprolinemia is a congenital metabolic disorder produced when the amino acid proline is not able to correctly degrade, resulting in an increase in its blood and urine levels. Two forms have been described, type I and type II, due to deficiencies in the activities of proline oxidase and 1-pyrroline-5-carboxylic acid dehydrogenase, respectively (Wise and Netto, 2011). In clinical practice, these patients show different phenotypes, certain neurological, renal and/or auditory defects while others are asymptomatic (Mitsubuchi et al., 2008) (Ferreira et al., 2014).

4.5.3 Safety

A number of toxicity studies for proline exists. Kampel et al. (1990) exposed rats to an oral dose of 50 mg/kg/day (~9 mg/day) of D-proline and L-proline for one month. Severe histopathological changes were observed in the liver (fibrosis and necrosis) and in the kidney (severe tubular lesions) in the group exposed to D-proline, while the group exposed to L-proline did not reveal any lesions. Serum parameters such as GOT, GPT, alkaline phosphatase, γGT, LDH, HBDH and creatine were also significantly high in comparison with the control group and the group exposed to L-proline. The toxicity therefore seems to be specific to the D isomer, although the authors concluded that the toxic effects were not due to D-proline, as it could not be found in the tissues and the serum, but to some metabolic intermediary of the conversion of the D to the L-proline.

Tada et al. (2010) conducted a subchronic study (90 days) administered orally to rats exposed to 0, 0.625, 1.25, 2.5 and 5 % of L-proline in the diet. No mortality or clinical signs were observed. Nevertheless significant changes were observed in certain parameters of the haematology and clinical biochemistry, in the final weight of females at 0.625 %, in the relative weight of the male spleen above 2.5 % and in the liver of the group at 5 %, etc. However, no damage was observed in the histopathological study that could be associated with the treatment. They concluded that the damage, although attributable to the treatment, was insignificant from a toxicological point of view. Therefore, a NOAEL was established as the dose administered to the group of 5 % of dietary L-proline, 2 772.9 mg/kg/day for males and 3 009.3 mg/kg/day for females (approximately 194 g/day in humans considering the worst case and a weight of 70 kg).

There has long been evidence to suggest that L-proline may act as a neuronal modulator or transmitter in the central nervous system (Takemoto and Semba, 2006). There appears to be a causal relation between hyperprolinemia type II and neurological manifestations in infancy (Phang et al., 2001). Proline has been observed to have adverse effects on the nervous system (Nadler et al., 1988) (Moreira et al., 1989). Nadler et al. (1988) observed that L-proline at a dose of 400 nmol or higher destroyed 32-66 % of the pyramidal and granular cells in rats injected in the hippocampus, considering this amino acid not only as a neuroexciter but also as a neurotoxin. Moreover, Delwing et al. (2003) observed oxidative stress in the neuronal cortex of rats exposed to a subcutaneous injection of 12.8 µmol/g (isomer not revealed), the dose selected to obtain plasma proline levels of 1-2 mM, similar to that found in patients with hyperprolinemia type II (Phang et al., 2001). Similarly, oxidative damage was observed in the DNA, proteins and blood lipids of rats in which chronic hyperprolinemia was induced experimentally, via the administration of between 12.8 and 18.2 µmol/g of proline (isomer not identified) between the sixth and twenty-eighth days of life (Ferreira et al., 2014).

Other authors however consider hyperprolinemia to be innocuous. Thus, Hayasaka et al. (1985), in different clinical cases administered a supplementation with proline over 5 years with doses of up to 10 g/day for 2 years, and did not make any reference to toxic effects.

Proline was not mutagenic in the Ames test (isomer not specified), 800 mg/kg (Green and Savage, 1978); 2mM (L) (Sargentini and Smith, 1986), but L-proline (10, 50, 100 µg/ml) did produce

an increase in the exchange of sister chromatids not dependent on the concentration in human lymphocytes (Xing and Na, 1996).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) assessed L-proline for use as a food additive and concluded that it did not give cause for concern as its intake through food (5 210 mg/day) was much higher (45 000 times) than as a food additive (JECFA, 2006).

4.5.4 Conclusion

Taking the study by Tada et al. (2010) as a reference, on oral administration in rats, in which the lower value of the NOAEL was established at 2 772.9 mg/kg/day (approximately 194 g/day in humans considering a weight of 70 kg) and using a corrective factor of 1 000, given that alterations were observed, a maximum quantity in food supplements of 2.8 mg/kg/day (approximately 200 mg/day) of L-proline is recommended for adults. This recommendation refers to L-proline and not to D-proline or racemic combinations for which the level of safety is lower. Proline must not be given to individuals with congenital hyperprolinemia, in particular to children.

References

Delwing, D., Bavaresco, C.S., Wannmacher, C.M., Wajner, M., Dutra-Filho, C.S. and Wyse, A.T. (2003). Proline induces oxidative stress in cerebral cortex of rats. *International Journal of Developmental Neuroscience*, 21, pp: 105-110.

Ferreira, A., Scherer, E., da Cunha, A., Manfredini, V., Biancini, G., Vanzin, C., Vargas, C. and Wyse, A. (2014) Hyperprolinemia induces DNA, protein and lipid damage in blood of rats: Antioxidant protection. *The International Journal of Biochemistry & Cell Biology*, 54, pp: 20-25.

Green, N.R. and Savage, J.R. (1978). Screening of safrole, eugenol, their ninhydrin positive metabolites and selected secondary amines for potential mutagenicity. *Mutation Research*, 57, pp: 115-121.

Hayasaka, S., Saito, T., Nakajima, H., Takahashi, O., Mizuno, K. and Tada, K. (1985) Clinical trials of vitamin B6 and proline supplementation for gyrate atrophy of the choroid and retina. *British Journal of Ophthalmology*, 69, pp: 283-290.

JECFA (2006). Joint FAO/WHO Expert Committee on Food Additives Safety evaluation of certain food additives and contaminants. Sixty-third meeting of the Joint FAO/WHO Expert Committee on Food Additives, *WHO Food Additives Series*: 54. IPCS, WHO, Geneva.

Kampel, D., Kupferschmidt, R. and Lubec, G. (1990). Toxicity of D-proline. In book: *Amino Acids: Chemistry, Biology and Medicine*. Lubec, G. and Rosenthal, G.A. (Ed.), ESCOM, Leiden, The Netherlands. pp: 1164-1171.

Lehninger, A., Nelson, D. and Cox, M. (1995). Biosíntesis de aminoácidos, nucleótidos y moléculas relacionadas. In book: *Principios de Bioquímica* (II Ed.). Ediciones Omega. Barcelona. pp: 688-735.

Mitsubuchi, H., Nakamura, K., Matsumoto, S. and Endo, F. (2008). Inborn errors of proline metabolism. *Journal of Nutrition*, 138, pp: 2016-2020.

Moreira, J.C.F., Wannmacher, C.M.D., Costa, S.M. and Wajner, M. (1989). Effect of proline administration on rat behavior in aversive and nonaversive tasks. *Pharmacology Biochemistry and Behavior*, 32, pp: 885-890.

Nadler, J.V., Wang, A. and Hakim, A. (1988). Toxicity of L-proline toward rat hippocampal neurons. *Brain Research*, 456, pp: 168-172.

Phang, J.M., Hu, C.A. and Valle, D. (2001). Disorders of proline and hydroxyproline metabolism. In book: *The Metabolic and Molecular Bases of Inherited Disease,* 8th Ed. Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (Eds.), McGraw-Hill, New York. pp: 1821-1838.

Sánchez de Medina, F. (2010). Metabolismo de los aminoácidos. In book: *Tratado de Nutrición*. Gil, A. (Ed.). pp: 451-484.

Sargentini, N.J. and Smith, K.C. (1986). Mutagenesis by normal metabolites in Escherichia coli: Phenylalanine mutagenesis is dependent on error-prone DNA repair. *Mutation Researc*h, 161, pp: 113-118.

Tada, Y., Yano, N., Takahashi, H., Yuzawa, K., Ando, H., Kubo, Y., Nagasawa, A., Ohashi, N., Ogata, A. and Nakae, D. (2010). Toxicological evaluation of L-proline in a 90-day feeding study with Fischer 344 rats. *Regulatory Toxicology and Pharmacology*, 58, pp: 114-120.

Takemoto, Y. and Semba, R. (2006) Immunohistochemical evidence for the localization of neurons containing the putative transmitter L-proline in rat brain. *Brain Research*, 1073-1074, pp: 311-315.

Xing, W. and Na, R. (1996). Amino acids excess increase SCEs in human lymphocytes. *Mutation Research,* 372, pp: 75-78.

Wise, A. and Netto, C. (2011). Behavioral and neurochemical effects of proline. *Metabolic Brain Disease*, 26, pp: 159-172.

4.6 L-serine

L-serine is classified as nutritionally non-essential amino acid. It is synthesized endogenously in sufficient quantities to maintain the nitrogen balance without requiring a dietary intake (Sucil, 1984). L-serine plays a versatile role in the intermediary metabolism in eukaryotic cells. L-serine does not only take part in the synthesis of proteins but is also a precursor of other amino acids and biomolecules which are essential for cell function, differentiation, survival and proliferation. L-serine participates in the synthesis of glycine, L-cysteine, phosphatidylserine, sphingolipids and D-serine. It also participates indirectly in the biosynthesis of purines and pyrimidines. The physiological role of *the novo* synthesized L-serine in mammalian neurobiology in health and disease is such that the established viewpoint of non-essential amino acids should be revaluated.

4.6.1 Characteristics and sources

Serine ($C_3H_7NO_3$) has a molecular weight of 105.09 g/mol; its chemical structure is shown in figure 6:

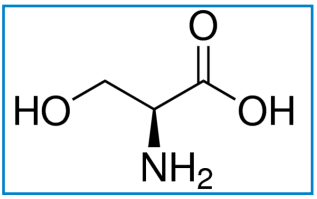

Figure 6. Molecular structure of serine.

L-serine is essential for the synthesis of phosphatidylserine and sphingolipids, basic components of the cell membrane, important for the development and functioning of the nervous system (Dos Santos Fagundes et al., 2001). Emerging evidence indicates that L-serine functions as a glia-derived trophic factor which strongly promotes the survival and differentiation of cultures neurons (Furuya and Watanabe, 2003). In humans, phosphatidylserine is mainly concentrated in

the brain where it represents 15 % of the total store of phospholipids. In general, it is linked to memory and is found in soya and other plant products, in dairy products, eggs, nuts and in meat.

4.6.2 Nutrition and metabolism

Biosynthesis of serine starts from the glycolytic intermediate 3-phosphoglycerate, with three sequential steps started by 3-phosphoglycerate dehydrogenase (3PGDH), known as the phosphorylated pathway (Figure 7). Serine can also be obtained from diet and from the breakdown of proteins and phospholipids. Nevertheless, given that the permeability of serine in the hematoencephalic phase is low, the phosphorylated phase is the principal pathway and is particularly important in the synthesis and metabolism of serine in the brain. It is known that patients with 3PGDH deficiency have low serine and glycine concentrations in plasma and suffer from severe neurodevelopmental defects (De Koning et al., 1999) (Furuya, 2008).

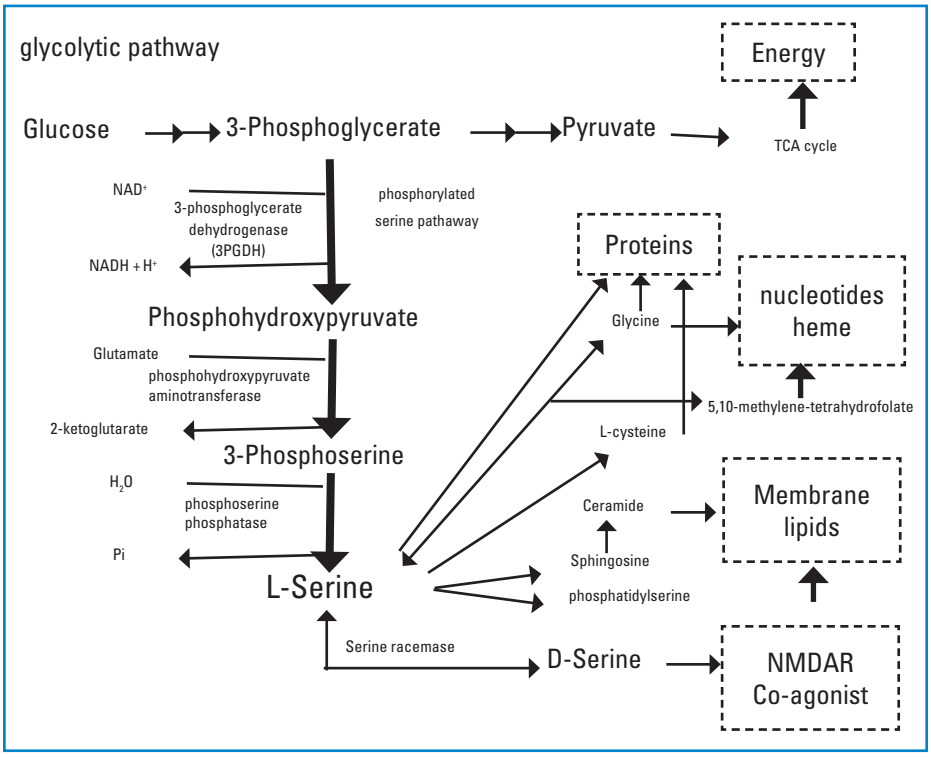

Figure 7. Synthesis of L-serine from the glycolytic intermediary (phosphorylated pathway) and its use in the synthesis of different biomolecules. **Source:** (Furuya and Watanabe, 2003).

4.6.3 Safety

The amino acids are mainly provided in the diet not as free amino acids but as protein constituents. The safety of amino acids consumed in the diet do not give cause for concern as they are nutrients required for the synthesis of the body's structural and functional components and are consumed in large quantities in food as an essential part of the diet. However, recently, there has been a growing interest in the consumption of individual amino acids as food supplements due to their beneficial effects on health and physical performance. This is the case with the amino acid L-serine. Little information is available regarding the safety of L-serine as a dietary supplement. Studies on humans indicate that four healthy subjects, treated with a single oral dose of 200 mg/kg b.w. of serine, did not reveal any adverse effects (Pepplinkhuizen et al., 1980). Another study conducted on a pregnant women diagnosed with a deficiency of the 3PGDH enzyme in the foetus, received treatment with doses of serine of 190 mg/kg b.w., 3 times a day, for the last 20 weeks of the pregnancy, without observing any adverse effects in the patient, the foetus or the newborn child (De Koning et al., 2004).

Jorissen et al. (2001, 2002) assess in a double-blind, placebo-controlled study on humans, healthy subjects with age-related memory changes, the safety of phosphatidylserine, derived from soya, at doses of 300 and 600 mg for 12 weeks of treatment. No signs of toxicity or effects on the biochemical and haematological parameters were observed and the authors concluded that the observed safe level of phosphatidylserine as a food supplement would be up to doses of 200 mg, three times a day.

More recently Vakhapova et al. (2011) assessed, in elderly people with memory problems, the safety of a treatment with phosphatidylserine (PS) in combination with an omega-3 fatty acid (docosahexaenoic acid, DHA) at doses of 300 mg PS and 79 mg DHA/day for 15 weeks, or 100 mg PS and 26 mg DHA/day for 30 weeks, in a double-blind clinical trial. No adverse effects were observed in the 157 participants who received 300 mg PS/day for 15 weeks. Of these patients, 121 participants continued treatment for a further 15 weeks at doses of 100 mg PS/day. A reduction in diastolic blood pressure and a slight increase in body weight were observed in some patients and considered to be minor effects. In conclusion, the results of this study indicate that the consumption of phosphatidylserine (PS) at doses of 300 mg PS/day for 15 weeks are safe, well-tolerated and do not produce adverse effects on the parameters studied (clinical signs, biochemical and haematological parameters). These results and doses are within the range of those described earlier by Jorissen et al. (2001, 2002), and by Richter et al. (2010, 2011).

With respect to safety studies on animals used in research, Kaneko et al. (2009) conducted a study of subchronic oral toxicity, for 13 weeks. In male and female rats, treated orally with doses of 500, 1 500 and 3 000 mg/kg b.w./day, no mortality, or any effect relating to the everyday clinical signs, body weight and consumption of food was observed. Nor were any adverse effects observed at the end of the test in the blood, serum biochemical and urine tests, in the weight of the organs or in the histopathological examinations of all the organs. From this study it was concluded that a NOAEL could be established of 3 000 mg/kg b.w./day. This relatively high NOAEL suggests that L-serine may be well-tolerated in its long-term use as a dietary supplement.

As the acetylated derivative of L-serine (NAS) is one of the most common N-acetylated amino acids of the proteins and given that it has been estimated that approximately 90 % of the proteins with serine residues are like N-acetylated derivatives (Driessen et al., 1985), Van de Mortel et al. (2010) recently assessed the toxicity of NAS in rats. The acute oral toxicity was assessed at a dose constraint of 2 000 mg/kg b.w., without observing mortality or any adverse effects. The subchronic oral toxicity was also assessed in rats for doses of NAS, over 28 days, of 100, 500 and 1 000 mg/kg b.w./day, observing a NOAEL for systemic toxicity of 839.7 mg/kg b.w. and of 893.6 mg/kg b.w. for males and females, respectively.

Lifshitz et al. (2015) conducted a toxicity test for reproduction and development, without observing any adverse effects. In another study, the genotoxicity and subchronic toxicity was assessed in rats (doses repeated at 90 days) preceded by an in-utero exposure phase with phosphatidylserine derived from fish phospholipids (with 49 % of phosphatidylserine); the tested dose levels were 1 100, 2 200 and 3 400 mg/kg b.w./day. The results showed that it is not genotoxic, it does not affect fertility or embryonic development. No clinical signs related to the treatment were observed, nor any effects on the body weight, intake of food and water, hematological parameters, clinical biochemistry, weight of organs and histopathological examinations for the 1 100 and 2 200 mg/kg b.w./day doses. In the group treated with the highest dose (3 400 mg/kg b.w./day equivalent to 1 666 mg/kg b.w./day of phosphatidylserine), an incident was observed in female rats in the histopathological examinations, classified as minimal-mild, of renal multifocal corticomedullary mineralisation without kidney degeneration, cellular necrosis or any other morphological, biochemical or physiological change. Five female rats also displayed this focal mineralisation. The results generated in this study give a NOAEL of 2 100 mg/kg/day equivalent to 1 029 mg/kg b.w./day of phosphatidylserine.

4.6.4 Conclusion

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 quantity of 200 mg of L-serine is acceptable from the safety point of view for use as a food supplement.

References

De Koning, T.J., Duran, M., Dorland, L., Gooskens, R., Van Shaftingen, E., Jacken, J., Blau, N., Berger, R. and Poll-The, B.T. (1999). Beneficial effects of L-serine and glycine in the management of seizures in 3-phosphoglycerate dehydrogenase deficiency. *Annales of Neurology*, 44, pp: 261-265.

De Koning, T.J., Klomp, L.W.J., Van Oppen, A.C.C., Beemer, F.A., Dorland, L., Van den Berg, I.E.T. and Berger, R. (2004). Prenatal and postnatal treatment in 3-phosphoglycerate-dehydrogenase deficiency. *Lancet,* 364, pp: 2221-2222.

Dos Santos Fagundes, J., Rotta, L.N., Schweigert, I.D., Valle, S.C., De Olivera, K.R., Huth Kruger, A., Souza, KB., Souza, D.O. and Perry, M.L. (2001). Glycine, serine and leucine metabolism in different regions of rat central nervous system. *Neurochemical Research*, 26, pp: 245-249.

Driessen, H.P., De Jong, W.W., Tesser, G.I. and Bloemendal, H. (1985). The mechanism of N-terminal acetylation of proteins. *CRC Critical Reviews in Biochemistry*, 18, pp: 281-325.

Furuya, S. and Watanabe, M. (2003). Novel neuroglial and glioglial relationships mediated by L-serine metabolism. *Archives of Histology and Cytology*, 66 (2), pp: 109-121.

Furuya, S. (2008). An essential role for de *novo* biosynthesis of L-serine in CNS development. *Asia Pacific Journal of Clinical Nutrition*, 17 (SI), pp: 312-315.

Jorissen, B.L., Brouns, F., Van Boxtel, M.P.J., Ponds, R.W.H.M., Verhey, F.R.J., Jolles, J. and Riedel, W.J. (2001). The Influence of Soy-derived Phosphatidylserine on Age-Associated Memory Impairment. *Nutritional Neuroscience*, 4, pp: 121-134.

Jorissen, B.L., Brouns, E., Van Boxtel, M.P.J. and Riedel, W.J. (2002). Safety of soy-derived phosphatidylserine in elderly people. *Nutricional Neuroscience*, 5 (5), pp: 337-343.

Kaneko, I., Han, L., Iu, T., LI, J., Zhao, Y., Li, C., Yi, Y., Liang, A. and Hayamizu, K. (2009). A 13-week subchronic oral toxicity study of L-serine in rats. *Food and Chemical Toxicology*, 47, pp: 2356-2360.

Lifshitz, Y., Levi, L., Eyal, I., Cohen, T. and Tessler, S. (2015). Sub-chronic (13-week) oral toxicity study, preceded by an in utero exposure phase and genotoxicity studies with fish source phosphatidylserine in rats, *Food and Chemical Toxicology,* 86, pp: 234-244.

Pepplinkhuizen, L., Bruinvels, J., Blom, W. and Moleman, P. (1980). Schizophrenia-like psychosis caused by a metabolic disorder. *Lancet, 1*, pp: 454-456.

Richter, Y., Herzog, Y., Cohen, T. and Steinhart, Y. (2010). The effect of phosphatidylserine containing omega-3 fatty acids on memory abilities in subjects with subjective memory complaints: a pilot study. *Clinical Interventions in Aging*, 5, pp: 313-316.

Richter, Y., Herzog, Y., Eyal, I. and Cohen, T. (2011). Cognitex supplementation in elderly adults with memory complaints: an uncontrolled open label trial. *Journal of Dietary Supplements*, 8, pp: 158-168.

Sucil, K. (1984). Enzymes of serine metabolism in normal, developing and neoplasic rat tissues. *Advances in Enzyme Regulation*, 22, pp: 325-400.

Van de Mortel, E.I.M., Shen, Z.A., Barnett, Jr.J.F. Krsmanovic, L., Myhre, A. and Delaney, B.F. (2010). Toxicology studies with N-acetyl-L-serine. *Food and Chemical Toxicology,* 48, pp: 2193-2199.

Vakchapova, V., Richter, Y., Cohen, T., Herzog, Y. and Korczyn, A.D. (2011). Safety of phosphatidylserine containing omega-3 fatty acids in non-demented elderly: a double blind placebo-controlled trial followed by an open-label extension. *BMC Neurology,* 11, pp: 79-89.

4.7 L-arginine-L- aspartate

4.7.1 Characteristics and sources

Also known as L-aspartate of L-arginine, this is L-aspartic acid salt (or (S)-2-amino-butanedioic) of L-arginine (or (S)-2,6-diaminohexanoic acid) (1:1). It is not naturally found in food. The molecular formula is $C_{10}H_{21}N_5O_6$ (Figure 8):

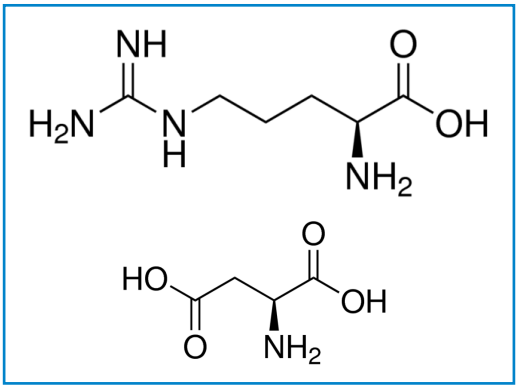

Figure 8. Molecular structure of L-arginine-L-aspartate.

The pair of amino acids remains linked by electrostatic forces through the negative charges of the carboxyl groups and the positive charges of the amino groups.

4.7.2 Nutrition and metabolism

This compound does not contain a peptide bond and does not require hydrolysis to produce individual amino acids. It separates in solution, as occurs in the stomach, and is therefore absorbed in the form of individual amino acids. Its bioavailability and metabolism is equivalent to that of individual amino acids (SCF, 2003). Figures for L-arginine are available in a preliminary report from the Scientific Committee of AECOSAN (2012) and L-aspartic acid is described in the same document in section 4.2.

4.7.3 Safety

The safety of L-arginine was assessed by AECOSAN (2012) and that of L-aspartic acid in the present report.

The use of L-aspartate of L-arginine is currently authorised in food for special medical uses (EU, 2013).

There are various studies in the bibliography on the beneficial effects derived from the administration of this substance, due to the synergic effect of both amino acids in the metabolism. However, in the majority of these the connection between both amino acids is not specified (Sallam and Steinbüchel, 2010), nor whether it is a mixture or the dipeptide, and the absorption of one or other takes place as a result of different mechanisms (Matthews, 1972), which might affect its bioavailability. Although the available studies do not focus on assessing safety, the majority do not refer to the detection of adverse effects due to treatment either. Nevertheless, Abel et al. (2005) and Colombani et al. (1999) concluded that there are no apparent reasons for using the supplement with arginine aspartate as an ergogenic, and Colombani et al. (1999) observed that the administration of 15 g/day for 14 days in long-distance runners led to a reduction in the total plasma amino acids level. Blazejewski et al. (2009) conducted a test on healthy volunteers, treated

with 30 g/day of arginine aspartate for 21 days and observed adverse reactions of a digestive nature (diarrhoea, flatulence and to a lesser extent headaches) together with a reduction in the secretion of IGF1 (insulin-like growth factor 1) and of IGFBP-3 (insulin-like growth factor-binding protein 3).

4.7.4 Conclusion

If the individual amino acids are considered, an Observed Safe Level (OSL) for supplementation with L-arginine was established at 20 g/day, and therefore the AECOSAN recommendation of a maximum daily quantity of 3 g of L-arginine was considered acceptable from a safety point of view in its use as a food supplement (AECOSAN, 2012).

With regard to L-aspartic acid the Scientific Committee concluded (section 4.2) that subchronic toxicity tests exist in rats from which it is possible to estimate that the daily intake of 490 mg of L-aspartic acid is acceptable from the safety point of view. In addition, it should be noted that this amino acid forms part of the basic dietary food and is also eaten as a metabolite of the sweetener aspartame.

Nevertheless, for L-arginine-L-aspartate, the information available regarding its safety is limited and in many cases lacks an adequate characterisation of the substance used in the tests. Therefore, the Scientific Committee considers that the available toxicological information is insufficient to determine the maximum daily quantity of L-arginine-L-aspartate that might be considered safe in its use as a food supplement.

References

Abel, T., Knechtle, B., Perret, C., Eser, P., Von Arx, P. and Knecht, H. (2005). Influence of chronic supplementation of arginine aspartate in endurance athletes on performance and substrate metabolism-a randomized double-blind, placebo-controlled study. *International Journal of Sports Medicine*, 26, pp: 344-349.

AECOSAN (2012). Agencia Española de Consumo, Seguridad Alimentaria y Nutrición. Informe del Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición sobre condiciones de uso de determinadas sustancias distintas de vitaminas, minerales y plantas para ser empleadas en complementos alimenticios-1. *Revista del Comité Científico de la AECOSAN*, 17, pp: 11-234.

Blazejewski, S., Georges, A., Forest, K., Corcuff, J.B., Abouelfath, A., Girodet, P.O., Kamagate, M., Jacquet, A., Pillet, O., Bordenave, L. and Moore, N. (2009). The chronic oral administration of arginine aspartate decreases secretion of IGF-1 and IGFBP-3 in healthy volunteers. *Fundamental & Clinical Pharmacology*, 23, pp: 339-344.

Colombani, P.C., Bitzi, R., Frey-Rindova, P., Frey, W., Arnold, M., Langhans, W. and Wenk, C. (1999). Chronic arginine aspartate supplementation in runners reduces total plasma amino acid level at rest and during a marathon run. *European Journal of Nutrition*, 38, pp: 263-270.

EU (2013). Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009. OJ L 181 of 29 June 2013, pp: 35-56.

Matthews, D.M. (1972). Intestinal absorption of amino acids and peptides. *Proceedings of the Nutrition Society*, 31, pp: 171-177.

Sallam, A. and Steinbüchel, A. (2010). Dipeptides in nutrition and therapy: cyanophycin-derived dipeptides as natural alternatives and their biotechnological production. *Applied Microbiology and Biotechnology,* 87, pp: 815-828.

SCF (2003). Scientific Committee on Food. Statement of the Scientific Committee on Food on L-serine and some amino acid-amino acid salts for use in foods for particular nutritional purposes. SCF/CS/ADD/NUT/55 Final. 2 April 2003.

4.8 L-lysine-L-aspartate

4.8.1 Characteristics and sources

Also known as L-aspartate of L-lysine, this is L-aspartic acid salt (or (S)-2-amino-butanedioic) of L-lysine (or (S)-2,6-diaminohexanoic acid) (1:1). It is not naturally found in food. The molecular formula is $C_{10}H_{21}N_3O_6$ (Figure 9):

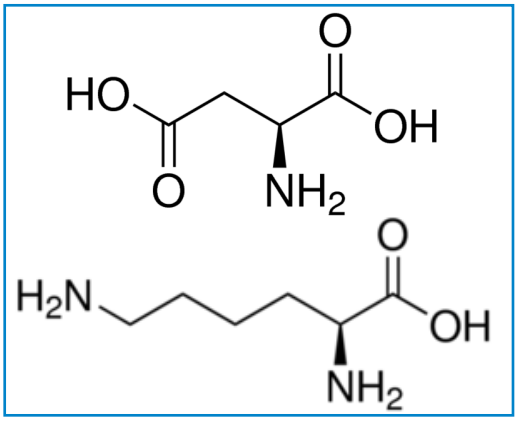

Figure 9. Molecular structure of L-lysine-L-aspartate.

The pair of amino acids remains linked by electrostatic forces through the negative charges of the carboxyl groups and the positive charges of the amino groups.

4.8.2 Nutrition and metabolism

This compound does not contain a peptide bond and does not require hydrolysis to produce individual amino acids. It separates in solution, as occurs in the stomach, and is therefore absorbed in the form of individual amino acids. Its bioavailability and metabolism is equivalent to that of individual amino acids (SCF, 2003). Figures for L-lysine are available in a preliminary report from the Scientific Committee of AECOSAN (2012) and L-aspartic acid is described in the same document in section 4.2.

4.8.3 Safety

The safety of L-lysine was assessed by AECOSAN (2012) and that of L-aspartic acid in the present report.

The use of L-aspartate of L-lysine is currently authorised in food for special medical uses (EU, 2013).

Studies of preparations composed by aspartate and lysine are very limited (Sallam and Steinbüchel, 2010). Only their use in the treatment of muscular fatigue and involuntary muscular contractions is known (Morelle and Lauzanne, 1984). No information is available regarding a possible interaction of effects after their joint administration.

4.8.4 Conclusion

If the individual amino acids are considered, with respect to L-lysine, the Scientific Committee concluded that, in line with the information available at that time, the AECOSAN recommendation (2012) of a maximum daily quantity of 2 250 mg of L-lysine, was acceptable from the safety point of view for its use as a food supplement.

With regard to L-aspartic acid, the Scientific Committee concluded (section 4.2) that subchronic toxicity tests exist in rats from which it is possible to estimate that the daily intake of 490 mg of L-aspartic acid is acceptable from the safety point of view. In addition, it should be noted that this amino acid forms part of the basic dietary food and is also eaten as a metabolite of the sweetener aspartame.

Nevertheless, for L-arginine-L-aspartate, the Scientific Committee considers that the available toxicological information is insufficient to determine the maximum daily quantity of L-arginine-L-aspartate that might be considered safe in its use as a food supplement.

References

AECOSAN (2012). Agencia Española de Consumo, Seguridad Alimentaria y Nutrición. Informe del Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) sobre condiciones de uso de determinadas sustancias distintas de vitaminas, minerales y plantas para ser empleadas en complementos alimenticios-1. *Revista del Comité Científico de la AECOSAN*, 17, pp: 11-234.

EU (2013). Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009. OJ L 181 of 29 June 2013, pp: 35-56.

Morelle, J.V. and Lauzanne-Morelle, E.M.T. (1984). Derivatives of lysine and aspartic acid. US Patent 4447366.

Sallam, A. and Steinbüchel, A. (2010). Dipeptides in nutrition and therapy: cyanophycin-derived dipeptides as natural alternatives and their biotechnological production. *Applied Microbiology and Biotechnology*, 87, pp: 815-828.

SCF (2003). Scientific Committee on Food. Statement of the Scientific Committee on Food on L-serine and some amino acid-amino acid salts for use in foods for particular nutritional purposes. SCF/CS/ADD/NUT/55 Final. 2 April 2003.

4.9 L-lysine-L-glutamate

4.9.1 Characteristics and sources

Also known as L-glutamate of L-lysine, this is L-glutamic acid salt (or 2-aminopentanedioic acid) of L-lysine (or 2.6-diaminohexanoic acid) (1:1). It is not naturally found in food. The molecular formula is $C_{11}H_{23}N_3O_6$ (Figure 10):

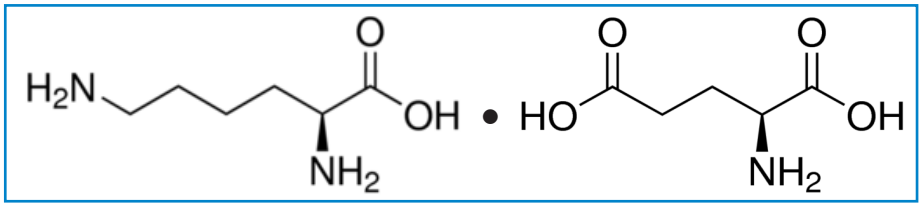

Figure 10. Molecular structure of L-lysine-L-glutamate.

The pair of amino acids remains linked by electrostatic forces through the negative charges of the carboxyl groups and the positive charges of the amino groups.

4.9.2 Nutrition and metabolism

This compound does not contain a peptide bond and does not require hydrolysis to produce individual amino acids. It separates in solution, as occurs in the stomach, and is therefore absorbed in the form of individual amino acids. Its bioavailability and metabolism is equivalent to that of individual amino acids (SCF, 2003). The data for both amino acids are available in a previous report published by the Scientific Committee of AECOSAN (2012).

4.9.3 Safety

The safety of both individual amino acids was assessed by AECOSAN (2012).

The use of L-glutamate of L-lysine is currently authorised in food for special medical uses (EU, 2013).

Studies of preparations composed by glutamate and lysine are very limited and there is no available information regarding the possible interaction of their effects.

4.9.4 Conclusion

If the individual amino acids are considered, with respect to L-lysine, the Scientific Committee concluded that, in line with the information available at that time, the AECOSAN recommendation (2012) of a maximum daily quantity of 2 250 mg of L-lysine, was acceptable from the safety point of view for its use as a food supplement.

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 quantity of 1 g/day is acceptable from a safety point of view for use in food supplements.

Nevertheless, for L-lysine-L-glutamate, the Scientific Committee considers that the available toxicological information is insufficient to determine the maximum daily quantity that might be considered safe in its use as a food supplement.

References

AECOSAN (2012). Agencia Española de Consumo, Seguridad Alimentaria y Nutrición. Informe del Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) sobre condiciones de uso de determinadas sustancias distintas de vitaminas, minerales y plantas para ser empleadas en complementos alimenticios-1. *Revista del Comité Científico de la AECOSAN*, 17, pp: 11-234.

EU (2013). Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009. OJ L 181 of 29 June 2013, pp: 35-56.

SCF (2003). Scientific Committee on Food. Statement of the Scientific Committee on Food on L-serine and some amino acid-amino acid salts for use in foods for particular nutritional purposes. SCF/CS/ADD/NUT/55 Final. 2 April 2003.

4.10 N-acetyl-L-cysteine

4.10.1 Proposal

The AECOSAN has recommended a maximum daily quantity for N-acetyl-L-cysteine in food supplements of 300 mg. The proposal is based on the fact that higher doses are considered to be medicinal in Spain.

4.10.2 Characteristics and sources

N-acetyl-L-cysteine, N-acetyl-cysteine or N-acetyl-3-mercaptoalanine is an acetylated derivative of cysteine. Cysteine is a proteinogenic sulphur-containing amino acid which is non-essential polar and hydrosoluble (Mataix, 2002).

It is a crystalline whitish powder which is highly soluble in water and other polar solvents such as ethyl alcohol. It has the following molecular structure (Figure 11):

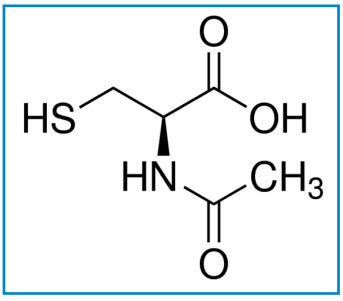

Figure 11. Molecular structure of N-acetyl-cysteine.

It has a molecular weight of 163.2 g/mol. It has an antioxidant action thanks to the presence of -SH groups in its molecule.

L-cysteine is considered as an "anti-ageing" supplement thanks to its antioxidant properties. It is an indirect antioxidant, facilitating the biosynthesis of glutathione. It is able to increase the activity of the glutathione S-transferase enzyme by providing glutathione for the detoxification of peroxides, a reaction catalysed by the glutathione-peroxidase enzyme (Moldeus and Cotgreave, 1994).

N-acetylcysteine is used as a mucolytic agent, as it is able to break the disulfide bridges of the proteins forming the respiratory secretions.

In addition, it is the antidote for paracetamol or acetominophen, as it acts by donating -SH groups, restoring reduced levels of glutathione and preventing damage to the hepatocytes as a result of the oxidative stress produced by a toxic metabolite of paracetamol (p-benzoquinone imine) (Brayfield, 2014).

N-acetylcysteine is obtained from the acetylation of L-cysteine hydrochloride with acetic anhydride in an aqueous alkaline medium. The compound is determined analytically by high-performance liquid chromatography (EFSA, 2003).

4.10.3 Nutrition and metabolism

Cysteine dioxygenase (CDO) is an important enzyme that contributes to the regulation of steady-state intracellular cysteine levels. It is expressed in high levels in the liver, with lower levels in the kidney, brain and lung; CDO is an iron metalloenzyme that catalyses the addition of molecular oxygen to the sulfhydryl group of cysteine, yielding cysteinesulfinic acid.

The oxidative catabolism of cysteine to cysteinesulfinate by CDO represents an irreversible loss of cysteine from the free amino acid pool; cysteinesulfinate is shuttled into several pathways including hypotaurine/taurine synthesis, sulfite/sulfate production, and the generation of pyruvate (Figure 12). *In vivo* data suggest that the liver is the organ with the highest amount of CDO protein expression and activity (Garcia and Stipanuk, 1992). The liver is a safeguard against the oral dietary intake of an excess of cysteine. Individuals with hepatic insufficiency or a deficit of the CDO enzyme are at greater risk from an excess intake of cysteine.

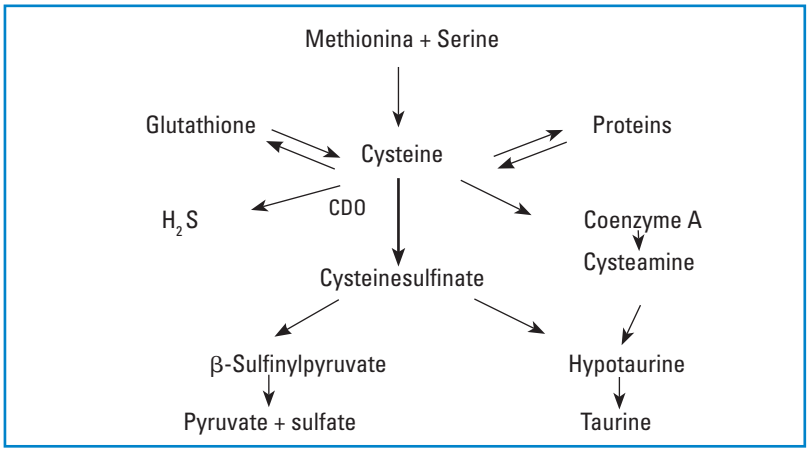

Figure 12. Principal pathways of cysteine metabolism. **Source:** (Stipanuk et al., 2006).

The oral administration of N-acetyl-L-cysteine to both rats and humans is rapidly absorbed. Next the compound is deacetylated and circulates in the blood stream bound to plasma proteins. Pharmacokinetic studies on patients with respiratory disorders (Rodenstein et al., 1978) show that maximum plasma concentrations are obtained 2-3 hours after oral administration and that between 13-38 % of the oral dose administered is recovered in urine within 24 hours. Other studies also describe in humans that following the oral administration of 600 mg of N-acetyl-L-cysteine, a bioavailability of 6-10 % is obtained and after the intravenous administration of 600 mg of N-acetyl-L-cysteine a plasma half-life of elimination of 2.27 hours was observed. Oral administration of N-acetyl-L-cysteine, in spite of its low bioavailability, is clinically efficient; it undergoes enterohepatic circulation (first-pass effect) and consequently the hepatocytes have thiol groups for the synthesis of glutathione (Borgstrom et al., 1986).

As N-acetyl-L-cysteine is a source of L-cysteine, from a nutritional point of view it is a precursor of L-cysteine and its nutritional value corresponds to this last amino acid. N-acetyl-L-cysteine is deacetylated to L-cysteine in tissue. The degree of this deacetylation may be affected in the presence of other N-acetyl derivatives as sources of their respective amino acids. The bioavailability of L-cysteine from N-acetyl-L-cysteine in the presence of other N-acetyl amino derivatives is unknown. This information is necessary to support the use of this product (EFSA, 2003).

4.10.4 Safety

The liver in mammals regulates the content of intracellular free cysteine. Levels of cysteine must be sufficiently high to permit the synthesis of proteins and the production of other essential molecules including glutathione, coenzyme A, taurine, and inorganic sulfur. At the same time, cysteine concentrations must be kept below the cytotoxicity threshold. High tissue levels of cysteine may result in an autoxidation of cysteine forming cystine, reactive oxygen species (ROS), oxidation of thiol groups, neurotoxicity mediated by NMDA-type glutamate receptors, and excess production of H_2S (Stipanuk et al., 2006).

Toxicity due to excess cysteine has been demonstrated in various animal models (Lehmann, 1987) (Andine et al., 1991) (Lehmann et al., 1993) and high levels of cysteine due to chronic administration are associated with rheumatoid arthritis (Bradley et al., 1994), Parkinson's and Alzheimer's (Heafield et al., 1990), lupus erythematosus (Gordon et al., 1992), cardiovascular effects (Ozkan et al., 2002) and complications during pregnancy (El-Khairy et al., 2003).

Bonamoni and Gazzamiga (1980) reviewed the acute and subchronic studies in *Sprague-Dawley* rats and *Beagle* dogs. They also collected embryotoxicity and reproduction studies in rats and rabbits and an *in vitro* mutagenicity test. In the studies of chronic toxicity in *Sprague-Dawley* rats doses of up to 1 000 mg/kg/day were observed to not affect behaviour, weight gain, haematology, liver and kidney function or coagulation and prothrombin time. Necropsies and histological examinations did not reveal any pathological lesions. Therefore, and considering the dose of 1 000 mg/kg as the maximum dose at which no adverse effect is observed in rodents, the NOAEL could be 1 000 mg/kg. Dividing the NOAEL by an uncertainty factor of 100, and considering 70 kg as the human body weight, we have an ADI of 700 mg/day.

Available studies on humans indicate that L-cysteine is well-tolerated, however it has not been possible to define the maximum dose for gastrointestinal effects such as vomiting and diarrhoea. Doses of 1.8 g/day in patients with chronic hepatitis generate gastrointestinal upsets (Beloqui et al., 1993). The therapeutic dose as a mucolytic agent is 0.4-0.6 g/day (EFSA, 2003).

4.10.5 Conclusion

The Scientific Committee concludes that, based on the information available to date that establishes a NOAEL and taking into account the general considerations reflected in this report, the AECOSAN proposal of a maximum daily quantity of 300 mg of N-acetyl-L-cysteine is acceptable from the safety point of view for use as a food supplement.

References

Andine, P., Orwar, O., Jacobson, I., Sandberg, M. and Hagberg, H. (1991). Extracellular acidic sulfur-containing amino acids and gamma-glutamyl peptides in global ischemia: postischemic recovery of neuronal activity is paralleled by a tetrodotoxinsensitive increase in cysteine sulfinate in the CA1 of the rat hippocampus. *Journal of Neurochemistry*, 57, pp: 230-236.

Beloqui, O., Prieto, J., Suarez, M., Gil, B., Qian, C.H., Garcia, N. and Civeira, M.P. (1993). N-acetyl cysteine enhances the response to interferon-a in chronic hepatitis C: a pilot study. *Journal of interferon & cytokine research*, 13, pp: 279-282.

Bonanomi, L. and Gazzaniga, A. (1980).Toxicological, pharmacokinetic and metabolic studies on acetylcysteine. *European Journal respiratory disease,* 61 (Suppl. III), pp: 45-61.

Borgström, L., Kagedal, B. and Paulsen, O. (1986). Pharmacokinetics of N-acetylcysteine in man. *European Journal of Clinical Pharmacology*, 31, pp: 217-222.

Bradley, H., Gough, A., Sokhi, R.S., Hassell, A., Waring, R. and Emery, P. (1994). Sulfate metabolism is abnormal in patients with rheumatoid arthritis. Confirmation by *in vivo* biochemical findings. *Journal of Rheumatology*, 21, pp: 1192-1196.

Brayfield, A. (2014). Martindale. The complete drug reference. Pharmaceutical Press. U.K.

EFSA (2003). European Food Safety Authority. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) on a request from the Commision related to

N-acetyl-L-cysteine for use in foods for particular nutritional uses and in foods for special medical purposes. *The EFSA Journal*, 21, pp: 1-8.

El-Khairy, L., Vollset, S.E., Refsum, H. and Ueland, P.M. (2003). Plasma total cysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine Study. *American Journal of Clinical Nutrition*, 77, pp: 467-472.

Garcia, R.A. and Stipanuk, M.H. (1992).The splanchnic organs, liver and kidney have unique roles in the metabolism of sulfur amino acids and their metabolites in rats. *Journal of Nutrition*, 122, pp: 1693-1701.

Gordon, C., Bradley, H., Waring, R.H. and Emery, P. (1992). Abnormal sulphur oxidation in systemic lupus erythematosus. *Lancet*, 339, pp: 25-26.

Heafield, M.T., Fearn, S., Steventon, G.B., Waring, R.H., Williams, A.C. and Sturman, S.G. (1990). Plasma cysteine and sulphate levels in patients with motor neurone, Parkinson's and Alzheimer's disease. *Neuroscience Letter*, 110, pp: 216-220.

Lehmann, A. (1987). Alterations in hippocampal extracellular amino acids and purine catabolites during limbic seizures induced by folate injections into the rabbit amygdala. *Neuroscience,* 22, pp: 573-578.

Lehmann, A., Hagberg, H., Orwar, O. and Sandberg, M. (1993). Cysteine sulphinate and cysteate: mediators of cysteine toxicity in the neonatal rat brain? *European Journal of Neuroscience*, 5, pp: 1398-1412.

Mataix, J. (2002). In book: *Nutrición y alimentación humana (Tomo I).* Editorial Ergon. Madrid.

Moldeus, P. and Cotgreave, I.A. (1994). N-Acetylcysteine. *Methods in enzymology*, 34, pp: 482-492.

Ozkan, Y., Ozkan, E. and Simsek, B. (2002). Plasma total homocysteine and cysteine levels as cardiovascular risk factors in coronary heart disease. *International Journal of Cardiology*, 82, pp: 269-277.

Rodenstein, D., de Coster, A. and Gazzaniga, A. (1978). Pharmacokinetics of oral acetylcysteine: Absorption, binding and metabolism in patients with respiratoriy disorders. *Clinical Pharmacokinetics*, 3, pp: 247-254.

Stipanuk, M.H., Dominy, J.E.Jr., Lee, J.I. and Coloso, R.M. (2006). Mammalian cysteine metabolism: new insights into regulation of cysteine metabolism. *Journal of Nutrition*, 136 (6), pp: 1652S-1659S.

4.11 N-acetyl-L-methionine

4.11.1 Proposal

N-acetyl-L-methionine is an essential source of methionine amino acid for use in food for special medical purposes in children over the age of 1 year and for adults.

The AECOSAN has recommended a maximum quantity of 2.5 mg/kg/day (175 mg/day for a person weighing 70 kg) of N-acetyl-L-methionine in adults from the safety point of view for use as a food supplement.

4.11.2 Characteristics and sources

The chemical synonyms of N-acetyl-L-methionine are: L-2-acetylamino-4-(methylthio)-butyric acid or 2-acetylamino-4-(methylthio)-butanoic acid. It has a molecular weight of 191.25 g/mol of which 78 % corresponds to L-methionine. It has the following molecular structure (Figure 13):

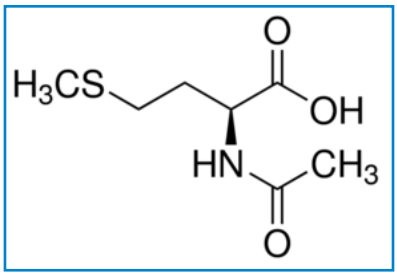

Figure 13. Molecular formula of N-acetyl-L-methionine.

It is a crystalline whitish powder with a slight odour. N-acetyl-L-methionine is obtained from the acetylation of L-methionine. L-methionine is an amino acid authorised for use in food with nutritional purposes. The modification of L-methionine to N-acetyl-L-methionine improves the palatability of the product in the diet without compromising its biological value. N-acetyl-L-methionine is recommended as a source of methionine in children and in adults.

4.11.3 Nutrition and metabolism

The metabolism of N-acetyl-L-methionine implies hydrolysis to L-methionine due to the action of the acylase-I enzyme, found in many mammal tissues (intestine, liver and kidney), able to use amino acids from endogenous and exogenous acyl derivatives (Giardina, 1997) (Baxter et al., 2002). This deacetylation has been demonstrated in both *in vivo* and *in vitro* studies (Francis and Smith, 1975). In humans, it has been observed that when orally administering equimolar quantities of L-methionine and N-acetyl-L-methionine to healthy male and female volunteers, the quantity of methionine found in plasma was similar 15 minutes after the administration of the two compounds (Stegink et al., 1980). These facts demonstrate that N-acetyl-L-methionine is a good source of L-methionine.

From a nutritional point of view, the estimated required daily intake of methionine+cysteine for children aged from 7 months to 18 years is to the order of 22 mg/kg b.w. and for adults it is to the order of 15-19 mg/kg b.w., equivalent to approximately 1 g/day for adults with a body weight of 60 kg (IOM, 2005). The potential toxic effects of N-acetyl-L-methionine lie in an excess intake of methionine mainly manifested in a reduction in the intake of food and weight gain together with tissue injury.

L-methionine is an essential amino acid whose metabolism implies conversion to S-adenosyl L-methionine by the enzyme methionine-adenosyltransferase mainly found in the liver. This reaction is catalysed by the ATP which donates its adenosyl group to the methionine and forms inorganic phosphate and pyrophosphate. The compound formed, in turn donates its methyl group and forms S-adenosyl L-homocysteine and its hydrolysis generates homocysteine and adenosine. Next the L-homocysteine in combination with serine is transformed to L-cystathionine which in turn is transformed to α-ketobutyrate and L-cysteine (Figure 14):

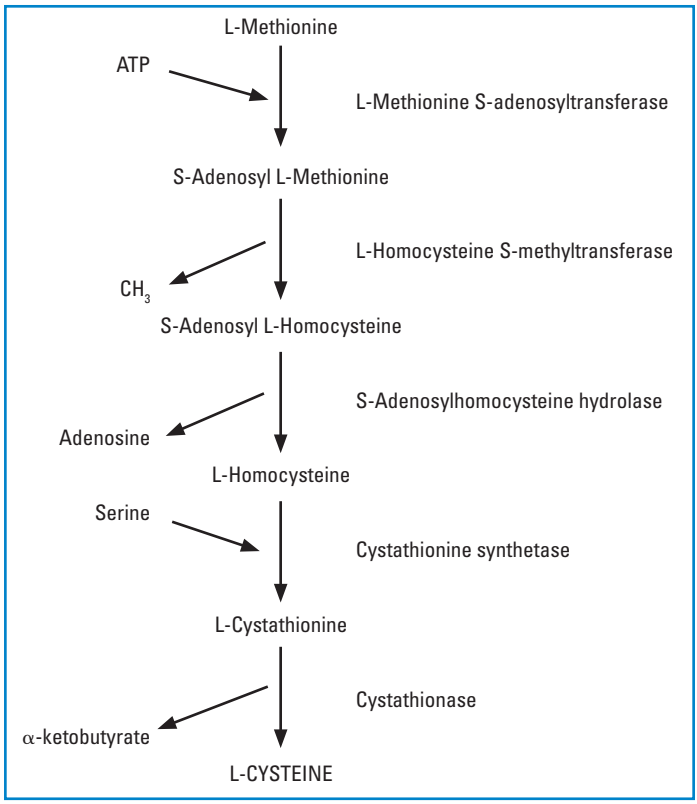

Figure 14. Metabolism of L-methionine. **Adapted from:** (Laster et al., 1965).

4.11.4 Safety

There is no available data for characterising the dose-response relation for L-methionine. Data on the adverse effects of L-methionine from food supplements are considered insufficient for a dose-response assessment and to be able to derive an UL (maximum tolerable limit) for humans.

Since 1960, reports have been received of children with homocystinuria disorders who frequently suffer arterial and venous thrombosis (McCully, 1969). In 1985, discussions were already underway as to whether intolerance to methionine constituted a potential risk of arterial coronary disease (Murphy-Chutorian et al., 1985) and it was suggested that patients under the age of 30 years with hyperhomocysteinemia have a higher probability (around 50 %) of vascular accident (Mudd, 1985).

An important question in relation to the possible toxicity of methionine is whether it arises as a direct response to methionine or whether it is derived from an excess methionine load generated by an increase in the levels of homocysteine and consequently a vascular disorder.

The safety of a load or excess dose of methionine has been examined in humans in epidemiological studies. Although a limited number of studies have been published, the conclusion maintained is that, although this may generate transitory complications in the alteration of

perception and control, no adverse vascular effects have been observed (Krupkova-Meixnerova et al., 2002). Single doses of methionine of 7 g produce lethargy, doses of 10.5 g cause nausea and vomiting (IOM, 2005). Only high doses, repeatedly administered, may pose a risk of coronary disease (IOM, 2005).

Examinations have also been made to determine whether a high intake of methionine in children could be linked to toxicity, concluding that hypermethioninemia is the result of the intake of a formula with a very high methionine content, resulting in a methionine intake in the range of 125-507 mg/kg/day, compared to an average intake of 62-97 mg/kg/day (Harvey et al., 2003). Children with methionine intakes two to five times higher than the normal intake show effects on growth and extremely high methionine plasma levels, but without long-term adverse effects (Garlick, 2006). As methionine is an essential amino acid and can be supplemented in the diet, it is appropriate to ask whether a dietary intake of methionine results in an increase in the concentration of homocysteine and the consequent cardiovascular risk. A number of studies indicate that an increase of plasma homocysteine is only observed with intakes of up to five times the normal intake of methionine. A moderate intake of methionine does not lead to hyperhomocysteinemia and the consequent cardiovascular risk (Chambers et al., 1999).

Although methionine is considered to be the most toxic amino acid with relation to growth in animals (Benevenga and Steele, 1984), there is no evidence to indicate toxicity in humans, except at very high methionine levels of intake. A single dose of 100 mg/kg b.w. has been shown to be safe and is equivalent to seven times the daily requirement for the sulfur amino acids (methionine+cysteine) (WHO, 2006). However if this dose of 100 mg/kg b.w. is administered repeatedly for 7 days, it results in an increase in the plasma levels of homocysteine and has been used as an index of susceptibility to cardiovascular disease (McAuley et al., 1999). Supplements of vitamins B_6, B_{12}, C and folic acid protect against the effects of methionine on the levels of homocysteine and the vascular function (Garlick, 2006).

Although methionine is a precursor of homocysteine and of the role of homocysteine in cardiovascular disease, there is no evidence to suggest that the dietary intake of methionine (L-methionine or N-acetyl-L-methionine) within reasonable limits may cause cardiovascular lesion.

There is no available data for characterising the dose-response relation for L-methionine. Data on the adverse effects of L-methionine from dietary supplements are considered insufficient for a dose-response assessment and to be able to derive an UL (maximum tolerable limit) for humans.

For the purposes of risk assessment, in the absence of a NOAEL, or of a confidence level lower than the benchmark dose lower bound (BMDL), the assessment could be made with the LOAEL (Lowest Observed Adverse Effect Level).

The Norwegian Scientific Committee for Food Safety (VKM) recommends the intake of 100 mg/kg b.w. of L-methionine as the lowest observed adverse effect level (LOAEL) based on the fact that in humans an intake of 100 mg/kg b.w. of L-methionine is associated with somnolence, vertigo and a decrease or increase in blood pressure (VKM, 2013).

Given that the LOAEL recommended by the Norwegian Scientific Committee for Food Safety has not been justified by a dose-response study, nor do we know the exact degree of the adverse

effects observed, an uncertainty factor of 10 should be applied as the inter-individual human variation factor and an uncertainty factor of 3 for the extrapolation of a LOAEL to a NOAEL, plus a factor of 1 as the LOAEL has not been established in dose-response studies. This gives:

LOAEL/40 = 100/40 = 2.5 mg/kg b.w.

Taking the human bodyweight as 70 kg we have a maximum recommended intake of 175 mg/day.

4.11.5 Conclusion

Taking the studies which establish a LOAEL of 100 mg/kg as the reference, the Scientific Committee considers a maximum quantity of 2.5 mg/kg/day (175 mg/day for a person weighing 70 kg) of N-acetyl-L-methionine in adults is acceptable from the safety point of view for use as a food supplement.

References

Baxter, J., Kim, Y. and Snowden, M. (2002). Hydrolysis of N-acetyl-L-glutamine by acylase I. *Journal of Food Science*, 66, pp: 1428-1433.

Benevenga, N.J. and Steele, R.D. (1984). Adverse effects of excessive consumption of amino acids. *Annual Review of Nutrition*, 4, pp: 157-181.

Chambers, J.C., McGregor, A., Jean-Marie, J., Obeid, O.A. and Kooner, J.S. (1999). Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia; an effect reversible with vitamin C therapy. *Circulation,* 99, pp: 1156-1160.

Francis, K.T. and Smith, R.C. (1975). *In vitro* metabolism of ethionine, N-acetyl-ethionine and Nacetyl-methionine by rat liver microsomes. *Experientia,* 31, pp: 890-891.

Garlick, P.J. (2006). Toxicity of methionine in humans. *Journal of Nutrition,* 136 (6), pp: 1722S-1725S.

Giardina, T., Biagini, A., Dalle Ore, F., Ferre, E., Reynier, M. and Puigserver, A. (1997). The hog intestinal mucosa acylase I: subcellular localization, isolation, kinetic studies and biological function. *Biochimie,* 79, pp: 265-273.

Harvey, M.S., Braverman, N., Pomper, M., Tezcan, K., Kronick, J., Jayakar, P. et al. (2003). Infantile hypermethioninemia and hyperhomocysteinemia due to high methionine intake: a diagnostic trap. *Molecular Genetics and Metabolism,* 79 (1), pp: 6-16.

IOM (2005). Institute of Medicine. Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein and Amino Acids. National Academic Press, 2005, Washington DC.

Krupkova-Meixnerova, L., Vesela, K., Vitova, A., Janosikova, B., Andel, M. and Kozich, V. (2002). Methionine-loading test: evaluation of adverse effects and safety in an epidemiological study. *Clinical Nutrition*, 21 (2), pp: 151-156.

Laster, L., Mudd, S.H., Finkelstein, J.D. and Irreverre, F. (1965). Homocystinuria due to cystathionine synthase deficiency: The metabolism of L-methionine. *Journal of Clinical Investigation*, 44 (10), pp: 1708-1719.

McCully, K.S. (1969). Vascular pathology of homosysteinemia: implications for the pathogenesis of arteriosclerosis. *American Journal of Pathololgy*, 56, pp: 111-128.

McAuley, D.F., Hanratty, C.G., McGurk, C., Nugent, A.G. and Johnston, G.D. (1999). Effect of methionine supplementation on endothelial function, plasma homocysteine, and lipid peroxidation. *Journal of Toxicology: Clinical Toxicology*, 37, pp: 435-440.

Mudd, S.H. (1985). Vascular disease and homocysteine metabolism. *New England Journal of Medicine*, 313, pp: 751-753.

Murphy-Chutorian, D.R., Wexman, M.P., Grieco, A.J., Heininger, J.A., Glassman, E., Gaull, G.E., Ng, S.K., Feit, F., Wexman, K. and Fox, A.C. (1985). Methionine intolerance: a possible risk factor for coronary artery disease. *Journal of American College of Cardiology,* 6, pp: 725-730.

Stegink, L.D., Filer, Jr.L.J. and Baker, L. (1980). Plasma methionine levels in normal adult subjects after oral loading with L-methionine and N-acetyl-L-methionine. *Journal of Nutrition*, 110, pp: 42-49.

VKM (2013). Norwegian Scientific Committee for Food Safety. Doc. No 12-704 final 11. March 2013.

WHO (2006). World Health Organization. Protein and Amino Acid Requirements in Human Nutrition. Report of a Joint WHO/FAO/UNU Expert Consultation. WHO Technical Report Series No. 935, Geneva.

5. Hydroxymethylbutyrate

5.1 Proposal

The AECOSAN has recommended a maximum daily quantity for hydroxymethylbutyrate (HMB) in food supplements of 3 g. In Italy it is authorised in food supplements without the establishment of a maximum daily quantity (Italy, 2015). It is also authorised as a food supplement in Belgium.

5.2 Characteristics and sources

HMB can be supplemented in the form of a monohydrate calcium salt (commonly referred to as HMB calcium) or as a free acid (HMB). When the free acid is compared to the calcium salt (at equivalent levels of free acid of 0.8 g compared to 1 g of HMB calcium), a higher maximum plasma concentration (Cmax) is reached with free acid (in 76-97 %) in a shorter time (Tmax) (30 minutes); the area under the curve (AUC) also increases by 91-97 % (Fuller et al., 2011). To conclude, HMB in free acid form improves the bioavailability of the HMB.

5.3 Nutrition and metabolism

HMB is a naturally found metabolite of the amino acid leucine: leucine is converted into its analogous keto (keto isocaproate or KIC) and then to HMB (through the cytosolic KIC dioxygenase enzyme) (Sabourin and Bieber, 1983) (Van Koevering and Nissen, 1992). It should be noted that the mitochondrial version of KIC dioxigenase converts KIC into the CoA derivative of isovaleric acid (β-hydroxyisovalerate) (Sabourin and Bieber, 1983). All the endogenous HMB derives from leucine (Van Koevering and Nissen, 1992), and the production of HMB is correlated with the dietary intake of leucine (following a first order kinetics by the cytosolic KIC dioxygenase) (Sabourin and Bieber, 1983) (Nissen et al., 1996) with approximately 5 % of the total leucine oxidised *in vivo* being converted to HMB (Van Koevering and Nissen, 1992). Although the HMB plasma concentration is 1-4 μM, it may increase 5-10 times after a leucine-rich meal (Nissen et al., 1996).

5.4 Safety

Toxicological evidence indicates that the no observed adverse effect level (NOAEL, the highest dose not associated with toxic symptoms) for HMB in orally administered experiments on rats is 3 490 mg/kg for male rats and 4 160 mg/kg for female rats (Baxter et al., 2005). This implies an estimated equivalent in humans of 558 mg/kg and 665 mg/kg, respectively (CDER, 2005). Assuming a bodyweight of 70 kg, this is equivalent to 39 g (men) and 46 g (women). Other toxicological tests

conducted using pigs shows that doses of approximately 5 g/kg in pigs more than 4 days old did not change any biochemical parameters or the weight of the organs (Wilson et al., 2013).

Toxicological studies in humans indicate that approximately 6 g of HMB per day (76 mg/kg/day) over 8 weeks in young untrained men subjected to exercise did not reveal any adverse effects on the haematological parameters, hepatic enzymes and lipid profile or the kidney function (Gallagher et al., 2000). In addition, nor did 3 g of HMB per day for a maximum of 8 weeks in young people and the elderly alter the serum haematological parameters (Nissen et al., 2000). This dose was also safe for 1 year of administration (Baier et al., 2009). In general, this standard dose of HMB seems to be well-tolerated for long periods of time (according to meta-analysis studies) (Rathmacher et al., 2004).

5.5 Conclusion

The free acid form is absorbed better than the calcium form, and reaches a maximum level in serum more quickly than the hydroxymethylbutyrate calcium salt.

It should be noted that hydroxymethylbutyrate is a metabolite of the dietary leucine in our body and is a mediator in a variety of effects of the leucine. Approximately 5 % of dietary leucine is converted to hydroxymethylbutyrate in the body.

Supplementation with hydroxymethylbutyrate with up to 3 g per day has been shown to be very well-tolerated. Moreover, it is thought that higher doses are equally as safe (although there have been fewer tests in humans).

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

References

Baier, S., Johannsen, D., Abumrad, N., Rathmacher, J.A., Nissen, S. and Flakoll, P. (2009). Year-long changes in protein metabolism in elderly men and women supplemented with a nutrition cocktail of beta-hydroxy-beta-methylbutyrate (HMB), L-arginine, and L-lysine. *Journal of Parenteral and Enteral Nutrition*, 33 (1), pp: 71-82.

Baxter, J.H., Carlos, J.L., Thurmond, J., Rehani, R.N., Bultman, J. and Frost, D. (2005). Dietary toxicity of calcium beta-hydroxy-beta-methyl butyrate (CaHMB). *Food and Chemical Toxicology*, 43 (12), pp: 1731-1741.

CDER 2005). Center for Drug Evaluation and Research. U.S. Department of Health and Human Services. Food and Drug Administration. Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers. Available at: http://www.fda.gov/downloads/Drugs/Guidances/UCM078932.pdf [accessed: 26-03-15].

Fuller, J.C., Rathmacher, J.A., Baier, S.M., Abumrad, N.N., Angus, H.F. and Shar, R.L. (2011). Free acid gel form of β-hydroxy-β-methylbutyrate (HMB) improves HMB clearance from plasma in human subjects compared with the calcium HMB salt. *British Journal of Nutrition*, 105 (3), pp: 367-372.

Gallagher, P.M., Carrithers, J.A., Godard, M.P., Schulze, K.E. and Trappe, S.W. (2000). Beta-hydroxy-beta-methylbutyrate ingestion, part II: effects on hematology, hepatic and renal function. *Medicine & Science in Sports & Exercise*, 32 (12), pp: 2116-2119.

Italia (2015). Elenco sostanze ad effetto nutritivo o fisiológico impiegabili negli intregatori alimentari. Available

at: http://www.salute.gov.it/imgs/C_17_pagineAree_1268_listaFile_itemName_4_file.pdf [accessed: 27-09-15].

Nissen, S., Sharp, R., Ray, M., Rathmacher, J.A., Rice, D., Fuller, J.C.Jr., Connelly, A.S. and Abumrad, N. (1996). Effect of leucine metabolite beta-hydroxy-beta-methylbutyrate on muscle metabolism during resistance-exercise training. *The Journal of Applied Physiology*, 81 (5), pp: 2095-2104.

Nissen, S., Sharp, R.L., Panton, L., Vukovich, M., Trappe, S. and Fuller, J.C. (2000). Beta-hydroxy-beta-methylbutyrate (HMB) supplementation in humans is safe and may decrease cardiovascular risk factors. *Journal of Nutrition,* 130 (8), pp: 1937-1945.

Rathmacher, J.A., Nissen, S., Panton, L., Clark, R.H., Eubanks May, P., Barber, A.E., D'Olimpio, J. and Abumrad, N.N. (2004) Supplementation with a combination of beta-hydroxy-beta-methylbutyrate (HMB), arginine, and glutamine is safe and could improve hematological parameters. *Journal of Parenteral and Enteral Nutrition,* 28 (2), pp: 65-75.

Sabourin, P.J. and Bieber, L.L. (1983). Formation of beta-hydroxyisovalerate by an alpha-ketoisocaproate oxygenase in human liver. *Metabolism,* 32 (2), pp: 160-164.

Van Koevering, M. and Nissen, S. (1992). Oxidation of leucine and alpha-ketoisocaproate to beta-hydroxy-beta-methylbutyrate in vivo. *The American Journal of Physiology*, 262 (1), pp: E27-31.

Wilson, J.M., Fitschen, P.J., Campbell, B., Wilson, G.J., Zanchi, N., Taylor, L., Wilborn, C., Kalman, D.S., Stout, J.R., Hoffman, J.R., Ziegenfuss, T.N., López, H.L., Kreider, R.B., Smith-Ryan, A.E. and Antonio, J. (2013). International Society of Sports Nutrition Position Stand: beta-hydroxy-beta-methylbutyrate (HMB). *Journal of the International Society of Sports Nutrition*, 10, pp: 6.

6. Lipoic acid

6.1 Proposal

The AECOSAN has proposed the inclusion of lipoic acid as a food supplement in a future expansion of the Royal Decree 1487/2009 without specifying a maximum daily allowance. Specifically, lipoic acid is authorised in Italy without a maximum daily allowance and with the warning that when taking glucose-lowering drugs is mandatory to visit a doctor before its eventual use (Italy, 2015). In Belgium it is also authorised for use in food supplements without the establishment of a maximum daily allowance.

6.2 Characteristics and sources

Lipoic acid is a natural compound, present in the human body and in certain food.

Chemically, alpha-lipoic acid (1,2-dithiolane-3-pentanoic acid), also known as thioctic acid, is a non-essential fatty acid with a well-defined structure, containing two atoms of sulphur and a chiral centre, resulting in the enantiomeric forms R and S, as shown in figure 15:

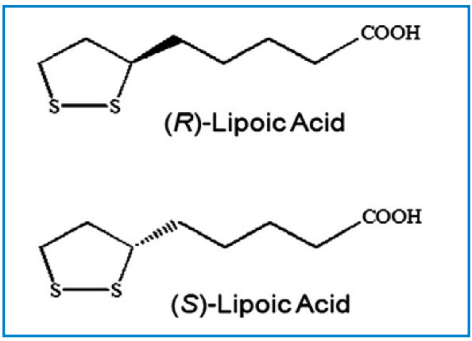

Figure 15. Chemical structure of lipoic acid with its two enantiomeric forms. **Source:** (Shay et al., 2009).

In foods, it appears bonded to proteins (lipolysine) in low concentrations of 0.3 mg/100 g in the richest natural plant sources (spinach) or animal sources (kidneys). Other sources include: broccoli, green cabbage, lettuce and chard, and entrails such as the heart and liver. Lipolysine is also found in tomatoes, peas and Brussel sprouts.

The natural compound is the R form whereas in food supplements the racemic R/S combination is more common. It is found in redox equilibria, the oxidised form is a closed cycle which has a disulfide bond and the reduced form is an open ring containing two thiol groups (dihydrolipoic acid).

It should be noted that trivalent arsenic is able to react with lipoic acid. In the decarboxylation of pyruvic acid, trivalent arsenic, which would act as toxic, is able to inhibit the lipoic acid responsible for the transformation of pyruvate to AcetylCoA. Lipoic acid is a thiol and this chemical characteristic is exploited by the trivalent arsenic to react with the same (Figure 16).

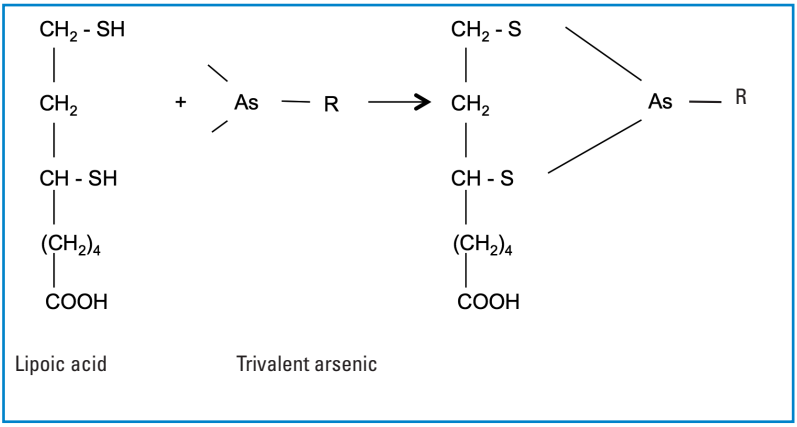

Figure 16. Reaction between lipoic acid and arsenic.

6.3 Nutrition and metabolism

Lipoic acid is synthesised in the liver, heart and kidneys (Ghibu et al., 2008), in particular in the mitochondria via enzymatic synthesis from octanoic acid playing a significant role in the energy metabolism.

Although *de novo* synthesis could cover the lipoic acid demands for energy metabolism, it can be absorbed from dietary sources. In fact, lipoic acid is absorbed from plant and animal foods and is accumulated temporarily in different tissues (liver, heart and skeletal muscle). Several studies have shown that lipoic acid bioavailability depends on a number of factors, whether it is taken in the form of acid or of salt, with food or alone, etc. (Shay et al., 2009).

Moreover, there are cellular systems to transport and excrete free lipoic acid (not bonded to proteins). Cellular transport of lipoic acid depends on pH, being accelerated when is acidic. During biliar transport, lipoic acid is transform in dihydrolipoic with a high oxidant activity (Takaishi et al., 2007), that could also be excreted.

The common metabolic pathway for *in vivo* lipoic acid is β-oxidation, being the principal metabolites: bisnorlipoate, tetranorlipoate, β-hydroxy-bisnorlipoate, and bis-methylates mercapto derivatives (Figure 17).

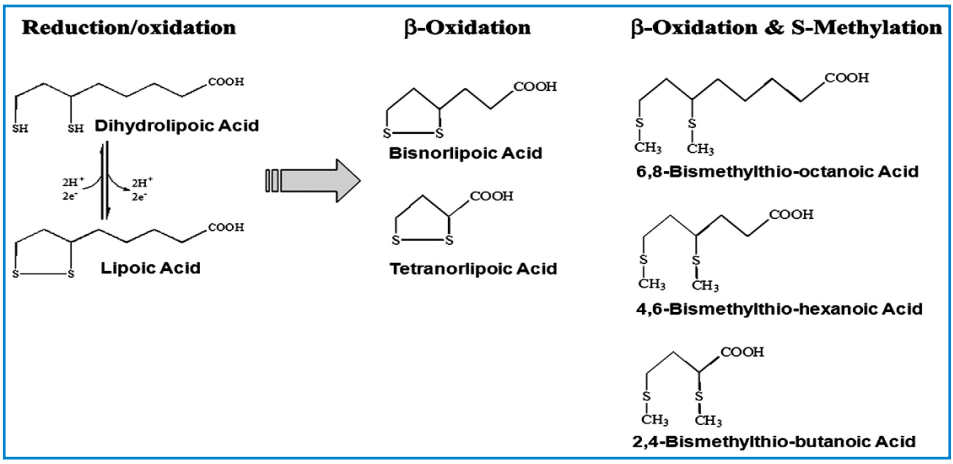

Figure 17. Metabolism of lipoic acid. **Source:** (Shay et al., 2009).

Lipoic acid acts mainly as cofactor for proteins involved in energy metabolism. Only the R form of lipoic acid is conjugated in order to conserve the lysine residues in an amide bond, thereby making this isoform as an essential cofactor for cellular systems, in particular for dehydrogenase enzymes, principally the pyruvate dehydrogenase and the branched-chain of ketoacid dehydrogenase (subunit E2 of PDH and of KADH), and also the 2-oxoglutarate dehydrogenase complex (Shay et al., 2009).

Both lipoic acid and dihydrolipoic acid have antioxidant properties. Moreover, dihydrolipoic acid has pro-oxidant properties in systems in which hydroxyl radicals are generated (Ghibu et al., 2008, 2009).

6.4 Safety

Subchronic toxicity studies in rats establish a NOAEL of 60 mg/kg/day (Cremer et al., 2006). If we consider a mean NOAEL of 60 mg/kg/day and we use an uncertainty factor of 100, the ADI is 0.6 mg/kg/day. For an individual weighing 70 kg, this would permit a daily intake of 42 mg of lipoic acid.

The most frequently used dose is 600 mg/day and this is the dose approved in Japan and Germany for the diabetic neuropathy. In that country it has been indicated for some pathologies for more than 50 years. The maximum dose used is 2 400 mg/day in clinical trials and no adverse effects have been detected. None of them were observed with a dose of 1 800 mg/day for 6 months (Costantino et al., 2014).

Nevertheless, there are insufficient studies to confirm its safety in children, pregnant or lactating women, or in individuals with diabetes, kidney or liver pathologies, and therefore for safety reasons, its use is not recommended in these situations.

6.5 Conclusion

The Scientific Committee concludes that the toxicological information for lipoic acid is limited. Clinical trials have been conducted in healthy adult population (excluding pregnant and lactating women) and no adverse effects have been observed with a range of doses between 1 800 and 2 400 mg/day in than 6 month time period. Moreover, taking as the reference the subchronic toxicity studies in rats with an mean NOAEL of 60 mg/kg/day, the Scientific Committee considers that, given the information available to date, and the general considerations listed in this report, a maximum quantity of 0.6 mg/kg/day (42 mg/day for a person weighing 70 kg) of lipoic acid in adults is acceptable from a safety point of view for use as a food supplement.

References

Cremer, D.R., Rabeler, R., Roberts, A. and Lynch, B. (2006). Long-term safety of alpha-lipoic acid (ALA) consumption: A 2-year study. *Regulatory toxicology and pharmacology,* 46 (3), pp: 193-201.

Costantino, M., Guaraldi, C., Costantino, D., De Grazia, S. and Unfer, V. (2014). Peripheral neuropathy in obstetrics: efficacy and safety of α-lipoic acid supplementation. *European review for medical and pharmacological sciences,* 18 (18), pp: 2766-2771.

Guibu, S., Richard, C., Delemasure, S., Vergely, C., Mogosan, C. and Muresan, A. (2008). An endogenous dithiol with antioxidant properties: alpha-lipoic acid, potential uses in cardiovascular diseases. *Annales de Cardiologie et de Angéiologie,* 57 (3), pp: 161-165.

Ghibu, S., Richard, C., Vergely, C., Zeller, M., Cottin, Y. and Rochette, L. (2009). Antioxidant properties of an endogenous thiol: Alpha-lipoic acid, useful in the prevention of cardiovascular diseases. *Journal of Cardiovascular Pharmacology,* 54 (5), pp: 391-398.

Italia (2015). Elenco sostanze ad effetto nutritivo o fisiológico impiegabili negli intregatori alimentari. Available at: http://www.salute.gov.it/imgs/C_17_pagineAree_1268_listaFile_itemName_4_file.pdf [accessed: 27-09-15].

Shay, K.P., Moreau, R.F., Smith, E.J., Smith, A.R. and Hagen, T.M. (2009). Alpha-lipoic acid as a dietary supplement: Molecular mechanisms and therapeutic potential. *Biochimica et Biophysica* Acta, 1790 (10), pp: 1149-1160.

Takaishi, N., Yoshida, K., Satsu, H. and Shimizu, M. (2007). Transepithelial transport of alpha-lipoic acid across human intestinal Caco-2 cell monolayers. *Journal of Agricultural and Food Chemistry,* 55, pp: 5253-5259.

7. *Monascus purpureus*

7.1 Proposal

The AECOSAN have recommended a maximum daily quantity for *Monascus purpureus* conditioned to a maximum input of 10 mg of monacolin expressed as monacolin K.

This proposal is based on the existence of a health claim approved by the EFSA stating that "monacolin K from red yeast rice contributes to maintaining normal blood cholesterol levels". This claim can only be used with respect to foods which provide a daily intake of 10 mg of monacolin K from red yeast rice.

In order for a product to carry this claim, the consumer will be informed that the beneficial effect is obtained with a daily intake of 10 mg of monacolin K, from fermented preparations of red yeast rice (EFSA, 2011) (EU, 2012).

7.2 Characteristics and sources

The commercial red yeast used as a supplement in phytotherapy is obtained by fermenting rice with a mixture of fungi of the genus *Monascus*, such as *M. purpureus* Went (Monascaceae) and others, which are used to obtain rice wine. The principal active compound of red yeast is monacolin K, an active ingredient which has been marketed with the generic name of lovastatin. It is also produced by other fungi such as *Aspergillus terreus* or *Pleurotus ostreatus*.

7.3 Nutrition and metabolism

Monacolin K inhibits 3-hydroxy-methyl-glutaril (HMG)-CoA reductase, a limiting enzyme in the endogenous biosynthesis of cholesterol. Monacolin K is a prodrug derived from hexahydronaphthalene β-hydroxy-δ-lactone, which is hydrolysed to mevinolinic acid, with a similar structure to that of HMG-CoA. Monacolin K combines with the HMG reductase, blocking the conversion of HMG-CoA to mevalonic acid and the subsequent synthesis of cholesterol (Castillo and Martínez, 2007).

The inactive compound is administered orally and is absorbed irregularly (31 %), becoming converted to active hydroxy-acids in the intestinal mucosa and liver. It is metabolised by the isozyme of cytochrome P-450 CYP3A4, which may lead to important interactions (Flórez et al., 2014).

It has a very low bioavailability (<5 %) due to poor absorption and a first-pass hepatic metabolism. The bonding to plasma proteins is high (95 %), and passes the blood-brain and placental barriers. The maximum plasma concentration is reached after approximately 2 hours, and the half-life in plasma is 2.5 to 3 hours. It is eliminated through faeces and urine (Flórez et al., 2014).

The recommended dose for red yeast is two capsules twice a day. As the capsules usually contain 600 mg of a standardised mixture of yeast and rice, with a content of 2.4 mg of monacolin K, the recommended dose is 4.8 mg × 2 doses, that is, 9.6 mg/day in two intakes (Bratman and Girman, 2003).

7.4 Safety

Pregnant and lactating women must not take red yeast, nor should children under the age of 12 years. Patients with liver or kidney failure may only take it with precaution (SEFIT, 2015).

The tolerability of monacolin K, as for the other statins, is high. A significant percentage (7-29 %) of patients treated with statins present muscular disorders (myalgia, contractions and loss of strength), with normal or high serum concentrations of muscular enzymes (for example, creatine phosphokinase). Lower percentages present fatigue, an increase in hepatic enzymes, peripheral neuropathy, insomnia, neurocognitive alterations and even diabetes mellitus (Stroes et al., 2015). A reduced percentage of patients may present a severe immune-mediated inflammatory myopathy. The incidence of these effects is related to the dose of statin (usually more than 20 mg of lovastatin or the equivalent), the type of statin, association with other hypolipemiant drugs (for example, gemfibrozil), age and gender, and other conditioning factors (Osakidetza, 2008) (Stroes et al., 2015).

The European Atherosclerosis Society consensus document considers red yeast as effective and well-tolerated (Stroes et al., 2015). A meta-analysis which included 13 clinical trials concluded that red yeast rice is a relatively safe product for use in the treatment of dyslipidemias (Li et al., 2014). No differences were observed in the figures for serum creatine phosphokinase, or in those for glycemia among the treatment groups and those treated with the placebo. Although the intervention time for these studies was relatively long (between 4 weeks and 6 months), the equivalent doses of lovastatin were low, less than 10 mg in all the studies, except one (Huang et al., 2007). Moreover, it has been observed that it may be an effective and well-tolerated treatment in patients who are intolerant of statins (mainly myalgia), as only 5 % of the patients treated suffered myalgia (Osakidetza, 2008) (Halbert et al., 2010).

Therefore, although at the established doses the undesirable effects do not appear to be relevant, it is necessary to conduct clinical trials with doses equivalent to 10 mg/day of lovastatin for a prolonged period of time (1-2 years). Until these studies have been conducted, monitoring is advised in the event of muscular pain. The intake of this product with grapes should also be avoided as its serum level could be increased 5-20 times. It may interact with immunosuppressants, antifungals and antibiotics (Harkness and Bratman, 2003). As the inhibitors of HMG-CoA reductase also reduce the synthesis of coenzyme Q10, a supplement of CoQ10 is recommended if intake is long-term.

7.5 Conclusion

There is no direct correlation between the administration of monacolin K at doses of 10 mg and possible undesirable effects. Therefore, the Scientific Committee concludes that the AECOSAN recommendation of a maximum daily quantity for *Monascus purpureus* conditioned to a maximum input of 10 mg of monacolin expressed as monacolin K is acceptable from the safety point of view for use as a food supplement.

As monacolin K is a medicine used at a standard dose of 20 and 40 mg, and in some cases an initial dose of 10 mg is recommended (Naci et al., 2013) (IQB, 2015), pregnant and lactating women

or children under the age of 12 years should not take red yeast (Osakidetza, 2008). Patients with liver or kidney failure may only take it with precaution and subject to medical consultation.

References

Bratman, S. and Girman, A.M. (2003). In book: *Mosby's Handbook of Herbs and Supplements, and their Therapeutic Uses*. Mosby Health Gate: St. Louis. pp: 848-851.

Castillo, E. and Martínez, I. (2007). In book: *Manual de Fitoterapia*. Elsevier-Masson. Barcelona. pp: 152.

EFSA (2011). European Food Safety Authority. Scientific Opinion on the substantiation of health claims related to monacolin K from red yeast rice and maintenance of normal blood LDL cholesterol concentrations (ID 1648, 1700) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *The EFSA Journal*, 9 (7), pp: 2304.

EU (2012). Commission Regulation (EU) No 432/2012 of 16 May 2012 establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children's development and health. OJ L 136 of 25 May 2012, pp: 1-40.

Flórez, J., Armijo, J.A. and Mediavilla, A. (2014). In book: *Farmacología Humana*, 6ª Ed., Elsevier-Masson, Barcelona. pp: 864-878.

Halbert, S.C., French, B., Gordon, R.Y., Farrar, J.T., Schmitz, K., Morris, P.B., Thompson, P.D., Rader, D.J. and Becker, D.J. (2010). Tolerability of red yeast rice (2,400 mg twice daily) versus pravastatin (20 mg twice daily) in patients with previous statin intolerance. *American Journal of Cardiology*, 105, pp: 198-204.

Harkness, R. and Bratman, S. (2003). In book: *Mosby's Handbook of Drug-Herb and Drug-Supplement Interactions*. Mosby Health Gate: St. Louis. pp: 237-238.

Huang, C.F., Li, T.C., Lin, C.C., Shih, H.C. and Lai, M.M. (2007). Efficacy of *Monascus purpureus* Went rice on lowering lipid ratios in hypercholesterolemic patients. *European Journal of Cardiovascular Prevention and Rehabilitation,* 14, pp: 438-440.

IQB (2015). Instituto Químico Biológico. Medciclopedia. Monografías: Lovastatina. Available at: http://www.iqb.es/cbasicas/farma/farma04/l032.htm. [accessed: 11-06-15].

Li, Y., Jiang, L., Jia, Z., Xin, W., Yang, S., Yang, Q. and Wang, L. (2014). A meta-analysis of red yeast rice: an effective and relatively safe alternative approach for dyslipidemia. *PLoS One*, 9, pp: e98611.

Naci, H., Brugts, J.J., Fleurence, R. and Ades, A.E. (2013). Dose-comparative effects of different statins on serum lipid levels: a network meta-analysis of 256,827 individuals in 181 randomized controlled trials. *The European Journal of Preventive Cardiology*, 20, pp: 658-670.

Osakidetza (2008). Guía de práctica clínica sobre el manejo de los lípidos como factor de riesgo cardiovascular. Osakidetza y Departamento de Sanidad: Vitoria. pp: 124-126, 184-252.

SEFIT (2015). Sociedad Española de Fitoterapia. Available at: http://www.fitoterapia.net/ [accessed: 11-06-15].

Stroes, E.S., Thompson, P.D., Corsini, A., Vladutiu, G.D., Raal, F.J., Ray, K.K., Roden, M., Stein, E., Tokgözoglu, L., Nordestgaard, B.G., Bruckert, E., De Backer, G., Krauss, R.M., Laufs, U., Santos, R.D., Hegele, R.A., Hovingh, G.K., Leiter, L.A., Mach, F., März, W., Newman, C.B., Wiklund, O., Jacobson, T.A., Catapano, A.L., Chapman, M.J. and Ginsberg, H.N. (2015). Statin-associated muscle symptoms: impact on statin therapy-European Atherosclerosis Society Consensus Panel Statement on Assessment, Aetiology and Management. *European Heart Journal,* 36, pp: 1012-1022.

8. Active carbon

8.1 Proposal

The AECOSAN has recommended a maximum daily quantity of 2 g of active carbon.

This proposal is based on the authorisation of a health claim that active carbon contributes to reducing excessive flatulence after eating. The claim can only be used with foods that contain 1 g of active carbon per quantified portion. For a product to carry this claim, the consumer will be informed of the beneficial effects of taking 1 g of active carbon at least 30 minutes before eating and another gram shortly after the meal (EU, 2012).

8.2 Characteristics and sources

Active carbon is an inert material made up of carbon organised in microcrystalline form which has undergone an activation process resulting in a dense network with pores of diameters ranging between 10 and 2 000 Å (Marín, 2003). Thanks to this layout the carbon has a high absorption surface of 300 to 2 000 m^2/g approximately (TAP, 2002), used to absorb gases and vapours from a mixture of gases and to dissolve and disperse substances present in liquids.

There are numerous sources for preparing active carbon, and activation may take place through different mechanisms. Sources of active carbon include animal bones, meat, blood, hard and soft wood, fruit peel, cereal, vegetable fibres, lignin, refinery and carbon waste.

The carbon sources are treated to obtain the active carbon using a wide variety of methods, and differentiating between two stages: carbonisation followed by oxidation. Activation may include the use of synthetic acids, bases or other substances in a stream of gases such as nitrogen or carbon dioxide. The quality and performance can be improved by eliminating the humidity (FAO, 1985). Microwaves or ion exchange resins may also be used (FCC, 1996).

Once the active carbon has been manufactured, it is classified according to its surface area and pore distribution. In 1996, the Food Chemical Codex established food grade specifications for active carbon (FCC, 1996).

In 2013, the EFSA published an opinion in relation to the use of active carbon for its use in contact with foods, concluding that active carbon should comply with the same purity requirements as those established for the food additive Vegetable Carbon (E-153) established by Directive 95/45/EC, with the exception of the ash content, which may be up to 10 % (p/p) (EFSA, 2013).

Four types of action are identified: absorption, mechanical filtration, ion exchange and surface oxidation.

8.3 Nutrition and metabolism

Active carbon is considered inert from the nutritional point of view, and is therefore not expected to be absorbed by the body.

In addition, the huge absorbent potential of active carbon must be considered, hence its two best-known applications: its use in overcoming aerophagia, meteorism and flatulence, and in treating intoxications, thanks to its capacity for absorbing toxic substances. This same

characteristic means that prolonged use is not to be advised due to possible interference in the absorption of certain nutrients.

8.4 Safety

From the safety point of view regarding the use of active carbon, no toxicological studies of the product have been found that permit a strict scientific assessment of this aspect.

Nevertheless, its use as a medicine to treat the same symptoms as those for which it is considered efficient when used as a food supplement is known. When used as a medicine, the technical specification sheet shall indicate that it may damage the intestinal motility. The sheet shall also indicate that "preclinical research has not revealed any toxic effects of the active ingredient", nevertheless this preclinical research has not been published.

In addition, from a safety point of view, it is necessary to know the size of the particle of the food supplement, paying special attention to the possible presence of nanoparticles.

8.5 Conclusion

The Scientific Committee considers that the available toxicological information is insufficient to determine the maximum daily quantity of active carbon that might be considered safe in its use as a food supplement.

References

FAO (1985). Food and Agriculture Organization of the United Nations. Industrial charcoal making. FAO forestry paper 63. Mechanical Wood Products Branch. Forest Industries Division. FAO Forestry Department. Food and Agriculture Organization of the United Nations. Rome, 1985.

FCC (1996). Food Chemicals Codex Committee. In book: *Food Chemicals Codex (4th ed.)*. Washington: National Academy.

EFSA (2013). European Food Safety Authority. Scientific Opinion on the safety evaluation of the active substances iron, sodium chloride, water, silica gel, activated carbon, monosodium glutamate, potassium acid tartrate, powdered cellulose, malic acid, chabazite, hydroxypropyl cellulose, potassium carbonate, sodium thiosulfate, propylene glycol, glycerin, polyethyleneglycol sorbitan monooleate, sodium propionate and clinoptilolite for use in food contact materials. *The EFSA Journal,* 11 (4), pp: 3155.

EU (2012). Commission Regulation (EU) No 432/2012 of 16 May 2012 establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children's development and health. OJ L 136 of 25 May 2012, pp: 1-40.

Marín, R. (2003). In book: *Físicoquímica y microbiología de los medios acuáticos. Tratamiento y control de calidad de aguas*. Díaz de Santos S.A. Madrid.

TAP (2002). Technical Advisory Panel. Activated carbon processing. National Organic Standards Board Technical Advisory Panel Review. Available at: http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELPRDC5066960 [accessed: 8-02-15].

9. Lactulose

9.1 Proposal

The AECOSAN has recommended a maximum daily amount of lactulose of 10 g.

This proposal is based on the authorisation of a health claim that lactulose contributes to the acceleration of bowel transit. This claim may only be used for food which contains at least 10 g of lactulose per quantified portion. In order for a product to carry this claim, the consumer will be informed that the beneficial effect is obtained after an intake of 10 g of lactulose in one single dose (EFSA, 2010) (EU, 2012).

With respect to its use as a food supplement, lactulose is considered as such in Belgium and Italy.

In other countries of the European Union including Slovenia, the United Kingdom, Sweden and the Czech Republic, it has a medicinal use.

At present in Spain it is considered as a medicine according to the Spanish Agency of Medicines and Health Products. The technical specification sheets for the different medicines, in all cases, recommend daily doses of between 10 and 20 g in one or two intakes, reaching 30 g/day in the case of hepatic encephalopathy.

9.2 Characteristics and sources

Lactulose is a disaccharide with the formula 4-O-β-D-Galactopyranosyl-β-D-fructofuranose (EFSA, 2010).

This non-digestible carbohydrate is not found as such in nature, although it can be obtained through the alkaline isomerisation of lactose or by enzyme-catalysed synthesis (Illanes, 2011).

With regard to its uses, as it is sweeter and more soluble than lactose, it is used in confectionery. Traditionally, it has been used as a laxative, in chronic and acute cases, and in the treatment of hyperammonemia and chronic hepatic encephalopathy. Studies have also been published showing that its administration increases the proportion of bacteria that promote the health of the gastrointestinal tract, including bifidobacteria and lactobacilli, and in studies with animals it reduces the presence of pathogenic bacteria including *Salmonella* (Schuster-Wolff-Bühring et al., 2010) (Bouhnik et al., 2014).

9.3 Nutrition and metabolism

The absorption of lactulose in the gastrointestinal tract is very poor and in humans it is not digestible due to the lack of enzymes that are able to hydrolyse it. Consequently, oral doses of lactulose solution reach the colon almost unchanged. There, they are decomposed by the action of the bacteria in the colon, mainly to lactic acid and to a lesser extent to formic acid and acetic acid, resulting in an increase in osmotic pressure and a slight acidification of the contents of the colon. This in turn leads to an increase in the water content of the faeces and their softening.

9.4 Safety

The safety sheet from the Spanish Agency of Medicines and Health Products states that "Data from the preclinical studies do not reveal any special risks for humans, in accordance with single dose and repeat dose toxicity studies".

Studies on laboratory animals (mouse and rabbit) show that lactulose does not have a teratogenic effect, or an adverse effect on reproduction (Baglioni and Dubini, 1976). It is not well-absorbed in the adult intestine (0.2-2.8 %) (Hallmann, 2000), in infants (less than 1.7 %) (Dmitriev, 1997) and children (less than 1 %) (Miki et al., 1996). It cannot be hydrolysed by human enzymes and is converted into different short-chain organic acids, mainly by intestinal bacteria.

Lactulose is included among the so-called osmotic laxatives (Hallmann, 2000). No serious adverse effects have been described in healthy individuals, only minor gastrointestinal symptoms, including swelling, gas, stomach pain, diarrhoea, nausea or headaches (Gordon et al., 2013), a result of an intolerance that is not dose-dependent (Als-Nielsen et al., 2004).

Its administration worsens diarrhoea and specialists recommend it is avoided in the case of chronic intestinal disease given its osmotic effect and the reduction of the transit time. However, given its minor effect it continues to be used in permeability studies on these patients (Van Citters and Lin, 2005), even the administration of 25 g of lactulose has been proposed for the unequivocal diagnosis of irritable colon (Le Neve et al., 2013).

The higher quantities administered described include two meta-analyses in which its effect on hepatic encephalopathy was studied. In these studies, doses of 30 to 120 and 20 to 80 g per day, respectively, were administered. None of these studies found serious adverse effects associated with its intake, only certain gastrointestinal reactions and one case of allergy (Als-Nielsen et al., 2004) (Luo et al., 2011).

These figures suggest that the daily intake of up to 10 g of lactulose would only pose a minor risk.

9.5 Conclusion

The toxicology studies carried out indicate that the daily intake of up to 10 g of lactulose would only pose a slight risk to certain particularly sensitive individuals.

Therefore, the Scientific Committee concludes that, based on the information available to date and taking into account the considerations reflected in this report, the AECOSAN proposal of a maximum daily quantity of 10 g of lactulose is acceptable from the safety point of view for use as a food supplement.

References

Als-Nielsen, B., Gluud, L.L. and Gluud, C. (2004). Non-absorbable disaccharides for hepatic encephalopathy: systematic review of randomised trials. *British Medical Journal*, 328, pp: 1046.

Baglioni, A. and Dubini, F. (1976). Lattulosio: valutazione tossicologica. *Bollettino chimico farmaceutico*, 115 (8), pp: 596-606.

Bouhnik, Y., Attar, A., Joly, F.A., Riottot, M., Dyard, F. and Flourie, B. (2004). Lactulose ingestion increases faecal bifidobacterial counts: A randomised double-blind study in healthy humans. *European Journal of Clinical Nutrition,* 58, pp: 462-466.

Dmitriev, A., Gmoshinski, I., Mazo, V., Dmitrieva, N., Uzebekova, D., Chugunova, L. and Mirgorodskaya, L. (1997). Intestinal permeability to alpha-lactalbumine of breast milk and lactulose in infants. *Gut,* 41, pp: 1.

EFSA (2010). European Food Safety Authority. Scientific Opinion on the substantiation of health claims related to lactulose and decreasing potentially pathogenic gastro-intestinal microorganisms (ID 806) and reduction in intestinal transit time (ID 807) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *The EFSA Journal*, 8 (10), pp: 1806.

EU (2012). Commission Regulation (EU) No 432/2012 of 16 May 2012 establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children's development and health. OJ L 136 of 25 May 2012, pp: 1-40.

Gordon, M., Naidoo, K., Akobeng, A.K. and Thomas, A.G. (2013). Cochrane Review: Osmotic and stimulant laxatives for the management of childhood constipation (Review). *Evidence-Based Child Health: A Cochrane Review Journal,* 8, pp: 57-109.

Hallmann, F. (2000). Toxicity of commonly used laxatives. *Medical Science Monitor*, 6, pp: 618-628.

Illanes, A. (2011). Whey upgrading by enzyme biocatalysis. *Electronic Journal of Biotechnology*, 14 (6).

Le Neve, B., Posserud, I., Bohn, L., Guyonnet, D., Rondeau, P., Tillisch, K., Naliboff, B., Mayer, E.A. and Simren, M. (2013). A Combined Nutrient and Lactulose Challenge Test allows symptom-based clustering of patients with irritable bowel syndrome. *American Journal of Gastroenterology,* 108, pp: 786-795.

Luo, M., Li, L., Lu, C.Z. and Cao, W.K. (2011). Clinical efficacy and safety of lactulose for minimal hepatic encephalopathy: a meta-analysis. *European Journal of Gastroenterolology & Hepatology*, 23, pp:1250-1257.

Miki, K., Butler, R., Moore, D. and Davidson, G. (1996). Rapid and simultaneous quantification of rhamnose, mannitol, and lactulose in urine by HPLC for estimating intestinal permeability in pediatric practice. *Clinical Chemistry*, 42, pp: 71-75.

Schuster-Wolff-Buhring, R., Fischer, L. and Hinrichs, J. (2010). Production and physiological action of the disaccharide lactulose. *International Dairy Journal*, 20, pp: 731-741.

Van Citters, G. and Lin, H. (2005). Nutrition in inflammatory bowel disease. In book: Inflammatory Bowel Disease: From Bench to Bedside. Targan, S., Shanahan, F. and Karp, L. (Ed.), *Springer US*. pp: 587-604.