Assessment of added amino acids to foods and food supplements

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Assessment of added amino acids to foods and food supplements

There is an increasing interest from the food industry to add high amounts of amino acids to foods, including soft drinks, food supplements and gels intended for exercise. In Denmark, companies need approval to add "certain other substances to food", which include a risk assessment. Performing risk assessments of the amino acids is challenging, as knowledge of the health consequences of consuming single amino acids in high amounts is currently insufficient to make a comprehensive risk assessment. The branched chain amino acids L-leucine, L-isoleucine and L-valine are currently the most commonly used and in addition added in the largest amounts to food supplements and food. However, only a few toxicological studies have been performed on the branched chain amino acids and even less for most other amino acids.

In 2011, the Norwegian authorities (VKM) carried out an assessment of 30 amino acids and amino acid compounds. The accompanying report has contributed to a certain overview, but cannot constitute a complete basis for risk assessment.

Specification of the task

The aim of the present report is to review experiments and studies performed with the 13[1] (out of 20 proteinogenic) amino acids, which it is permitted to add to food, cf. Appendix 2 in Executive Order no. 1342 of 28/11/2018 on the addition of certain substances other than vitamins and minerals to food. The present report aims to uncover data gaps to identify where future research should focus in order to perform a comprehensive risk assessment of the individual amino acids. The present report will also provide an updated basis for the future risk assessment of amino acids.

Method

The project is based on literature studies, and no toxicological studies has been carried out.

The report will cover the following

- Based on VKM's Risk Grouping of amino acids from 2011 and the subsequent reports on individual amino acids, 2015-2017, a literature search will be carried out until now in relevant databases to uncover any new toxicological experiments or human studies that refer to negative physiological effects after ingestion of amino acids
- Updated safe intake levels for the single amino acids based on previous assessments and new literature

[1] Glycine, Arginine, Cysteine, Glutamine, Histidine, Isoleucine, Leucine, Valine, Lysin, Methionine, Phenylalanine, Tryptophan, Tyrosine, Forgrenede aminosyrer

- This will also include studies that look at whether a high intake of single amino acids can result in a skewed balance of amino acids, which can have negative physiological effects
- Calculate the intake and composition of amino acids in an average Danish diet for different age groups (15-17 years, 18-24 years, 25-75 years)
- Generate data on the content and composition of the various amino acids in a variety of supplements and in individual specific foods. This is proposed to be handled by DTU students who from February 2019 have a 20-week internship period in FVST
- Clarify differences in the amino acid composition of a customary diet and fortified foods / supplements for e.g. athletes
- Assess whether there may be a changed bioavailability of amino acids in dietary supplements / fortified foods compared to amino acids in normal non-fortified foods.

End product(s) / delivery(ies): Memorandum, data basis for risk assessment and possibly a scientific article.

Participants in the project: Karin Nørby and Morten Poulsen, DTU National Food Institute.

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Content

Current basis for risk assessment of amino acids in DK

There is an increasing interest from the food industry to add high amounts of amino acids to foods, including soft drinks, supplements and gels intended for exercise. In Denmark, it requires approval to add certain other substances to food, which includes a risk assessment thereof. Based on these risk assessments, general authorizations have so far been granted and limit values have been set for 13 of the 20 proteinogenic amino acids associated with applied supplements, flavoured beverages, sports gels, biscuits, cookies, chocolate, bars and the like (see Appendix 1). The general authorizations apply to the total content of the amino acid in question in the product, regardless of the source, and the quantity limit is determined on the basis of the quantity applied for and the corresponding risk assessment. If a company request to add an additional amount of amino acids to a product, a new risk assessment must be performed.

Results of the literature search

The literature search was conducted in PubMed in the period 2011 to December 2020 with focus on human and animal studies with the amino acids mentioned in the table below. In Table 1 is shown the number of publications (hits) for each amino acid

Table 1

PubMed search January 2011 to end 2020 – relevant hits						
	Human studies			Animal studies		
Amino acid	Hits	Selected 1	Selected 2	Hits	Selected 1	Selected 2
Glycine	113	12	3	295	10	7
Arginine	498	78	8	437	71	66
Cysteine	309	10	2	405	17	6
Glutamine	315	36	8	265	58	1
Histidine	110	5	3	98	5	1
Isoleucine	9	1	1	7	1	1
Leucine	163	36	7	198	15	6
Valine	17	2	0	13	3	3
Lysine	164	8	1	169	17	13
Methionine	192	16	1	225	28	26
Phenylalanine	77	1	0	65	9	8
Tryptophan	319	14	3	210	17	17
Tyrosine	324	7	1	469	20	9
BCAA	12	3	2	5	3	3

PubMed search January 2011 to end 2020 – relevant hits						
	Human studies			Animal studies		
Amino acid	Hits	Selected 1	Selected 2	Hits	Selected 1	Selected 2
Branched-chain amino acids	462	110	29	Human and animal studies in the same file		
Branched-chain amino acids supplementation	65	30	3	Human and animal studies in the same file		

All hits identified in PubMed have been through a double screening check. The first check (shown in the above table as: Selected 1) was a selection of possible relevant papers fulfilling the search criteria – the second check: Selected 2) was a more closely and thoroughly examination of the possible relevant papers ending up with the ones to be considered in the present report. Selection 1 and 2 was not performed by the same individual.

Results of relevant papers to be covered in the project

Glycine

(Tell, Frøyland, Haugen, Henjum, et al., 2018) "Glycine is a non-essential amino acid which is synthesized from 3-phosphoglycerate via serine, or derived from threonine, choline and hydroxyproline via inter-organ metabolism involving primarily the liver and kidneys. Endogenous synthesis is estimated to be in the magnitude of 8 g per day in adults. Glycine is a constituent of all proteins in the human body. It also functions as a neurotransmitter, and can play both stimulatory and depressant roles in the brain. Foods rich in glycine are generally protein rich foods such as meat, fish, dairy products and legumes".

Animal studies

Three groups of six male rats were treated with glycine solution once daily by oral gavage at doses of 500, 1000, or 2000 mg/kg per day in a volume of 10 mL/kg for 28 consecutive days. No animals died, and no glycine-related changes were observed in body weight, food consumption, water consumption, hematology, organ weight, gross pathological examination or histopathological examination. In urinalysis, daily urinary volume and urinary Cl excretion were significantly higher in the 2000 mg/kg per day dose group, and urine pH and urinary protein showed lower trends in the glycine-treated groups. There were no histopathological changes in the kidneys or urinary bladder and no changes in other urinary parameters. As regards blood chemistry, phospholipids were significantly higher in the 2000 mg/kg per day dose group. The no-observed-adverse-effect level of glycine was estimated to be at least 2000 mg/kg per day under the conditions of this study (Shibui, Miwa, Yamashita, Chin, & Kodama, 2013).

Ten male rats exposed by glycine (2000 mg/kg bw/day) in drinking water for 7 days after the induction of diabetes for up to 16 weeks, gained less than the control group (Gholami, Kamali, & Rostamzad, 2019).

Glycine was administered at doses of 100, 300 and 500 mg/kg body weight per day by oral gavage once in a day for two weeks with 25 animals in each group. Histopathological investigation of brain tissues showed cellular clumps at cortical junctions at higher doses of glycine as compared to control. Authors conclude that this is a beneficial effect (Imtiaz, Ikram, Ayaz, Qadir, & Muhammad, 2018).

Human studies

Glycine is utilized to synthesize serine, sarcosine, purines, creatine, heme group, glutathione, and collagen. Glycine is a major quantitative component of collagen. In addition, the role of glycine maintaining collagen structure is critical, as glycine residues are required to stabilize the triple helix of the collagen molecule. This quality of glycine likely contributes to explain the occurrence of medial arterial calcification and the elevated cardiovascular risk associated with diabetes and chronic kidney disease, as emerging evidence links normal collagen content with the initiation and progression of vascular calcification in humans. No quantitative data was given in this paper (Adeva-Andany et al., 2018).

In a randomised, double-blind clinical trial performed in 60 patients diagnosed with metabolic syndrome (MetS), 30 patients received 5 g of glycine three times per day (15 g of glycine per day) for three months - control group received placebo. MetS was defined by at least 3 of the following parameters are necessary for MetS to be diagnosed: fasting glucose ≥110 mg/dL; blood pressure (BP) ≥130/85 mmHg (1 mmHg = 133.322 Pa); triacylglycerols (TG) ≥150 mg/dL; high density lipoprotein (HDL) <50 mg/dL (women) and <40 mg/dL (men); and waist circumference >88 cm (women) and >102 cm (men). Glycine supplementation showed significant effects and the results were clearly associated with gender. Males had a significant reduction in systolic blood pressure (−7.66mm Hg) and glycated hemoglobin (−1.39%), an increase in high-density lipoprotein (4.66 mg/dL) and total cholesterol (26.08 mg/dL), and an increase in waist/hip ratio (0.02). By comparison, females showed significant decreases in low-density lipoprotein (−14.80 mg/dL) and significant increases in high-density lipoprotein (4.33 mg/dL) and glucose levels (11.75 mg/dL). After glycine treatment, our data showed a 58% (7/12 males) reduction of at least one of the risk parameters established metabolic syndrome diagnosis (Díaz-Flores et al., 2013).

In a Japanese population-based cohort study, the association between glutamic acid and glycine intakes and the risk of mortality from stroke was investigated. A high intake of glutamic acid in terms of a percentage of total protein was significantly associated with a decreased risk of mortality from total stroke in women after controlling for covariates. Glycine intake was significantly associated with an increased risk of mortality from total and ischemic stroke in men without history of hypertension at baseline. The authors concluded that glutamic acid and glycine intakes may be associated with an increased risk of stroke mortality (Nagata et al., 2015).

Glycine: conclusion on the safety evaluation

No subchronic or chronic animal studies were identified. Different clinical and cohort studies demonstrated conflicting results. In the Díaz-Flores et al. study they concluded that increasing percentage of glycine compared to total protein intake was significantly associeted with increased risk of stroke mortality whereas Nagata et al. found that doses of 15 g of glycine per

day for three months showed a potential beneficial significant increases in HDL in both gender and reduction in systolic blood pressure. As the human data are inconclusive, it was decided to use the 28-day study in rats by Shibui et al. (2013). The NOAEL in this study was 2000 mg/kg per day (highest dose tested), and when applying an assessment factor of 100 this corresponds to a safe intake level of 20 mg/kg per day, or 1.4 g per day for a person weighing 70 kg. VKM used the same study for their safety assessment.

Arginine

(Tell, Frøyland, Haugen, Holvik, et al., 2018) "Arginine is a conditionally essential amino acid, meaning that under most circumstances endogenous synthesis by the human body is sufficient. However, the biosynthetic pathway may under certain conditions produce insufficient amounts. In such cases a dietary supply is needed. Individuals with poor nutrition or certain physical conditions are examples of vulnerable groups. Under normal conditions, endogenous production of arginine is 15-20 g/day. The requirements for L-arginine in adults are 117 mg/kg body weight (bw) per day (WHO, 2007), i.e. for a 70 kg adult person, the requirement is 8.2 g per day. Arginine is a constituent of all food proteins. Dairy products, beef, pork, poultry, wild game and seafood, as well as plant sources such as wheat germ and flour, oatmeal and nuts are good sources of arginine".

Animal studies

A metabolism study was performed using pigs and rats. In Experiment 1, male and female pigs were for 120 days fed a corn- and soybean meal-based diet supplemented with L-arginine-HCl, corresponding to 315 and 630 mg/kg bw per day. In Experiment 2, male and female rats were fed a casein-based diet for 90 days, while receiving drinking water containing supplemental L-arginine-HCl to provide 1800 and 3600 mg L-arginine/kg bw per day. In Experiment 3, pigs and rats received a single oral dose of 1 or 10 mg L-homoarginine/kg body-weight, respectively. 95-96 % of orally administered L-homoarginine was recovered in urine and L-homoarginine was quantitatively a minor product of L-arginine catabolism in the body. Dietary L-arginine supplementation dose-dependently increased whole-body L-homoarginine synthesis. No information on possible adverse effects due to the arginine supplementation was included in the study paper (Hou et al., 2016).

L-arginine was tested in a 13 weeks study in rats. Male and female Sprague-Dawley rats (12 of each sec in each dose group) were fed with a standard diet with l-arginine supplementation. The doses for male rats: 3300, 1750 and 875 mg l-arginine/kg bw per day, for female rats: 3900, 1950 and 975 mg/kg bw per day. All diets were administered ad libitum for 13 continuous weeks. To examine recoverability of any potential effects, the administration period was followed by a 5-week-long recovery, during which only a standard diet was provided. In male and female rats in each concentration group, treatment-related changes were not observed for clinical signs, body weights, diet consumption, ophthalmology, gross pathology, organ weight, or histopathology. An elevated level of plasma glucose was detected in some male rats in the highest dose during the analysis conducted in the fifth week of administration. However, no changes were observed at the end of the administration period. In the same group, an increase in hemoglobin, together with a tendency toward an increase in the red blood cell counts, was found. At the end of the recovery period, a significant decline in red blood cell

count and reticulocyte ratio was seen in females in the highest dose group. The authors state that the observed effects are in the physiological range and not of toxicological relevance and they claim that NOAEL values for arginine was 3300 mg/kg bw per day for males and for females 3900 mg/kg bw per day. There is no reporting of data in tables/figures, and data are only described in text. Therefore, the proposed NOAEL cannot be substantiated (Tsubuku, Hatayama, Mawatari, Smriga, & Kimura, 2004a).

Lin et al., (2020) examined the effect of a dietary supplement of arginine in Duroc boars. Fifteen male piglets (Duroc boars) in the control group were fed the basic diet, and 15 piglets in the treatment group were fed a diet containing 0.8% more arginine than the control group. They were weaned at 28 days old and fed when 33 days old and for 117 days. That corresponds to approximately 112 g arginine/kg bodyweight and 196 g arginine/kg body weight for the control group and arginine fed group, respectively at the start of the experiment. At the end of the experiment the arginine dose was approximately 20 g/kg body weight and 34 g/kg body weight in the control group and arginine fed group, respectively. Dietary supplementation with arginine significantly increased testicular weight, the number of spermatogonia, and the height of the seminiferous epithelium. The serum levels of luteinizing hormone (LH) and c (FSH) were also significantly increased in the arginine fed group (Lin et al., 2020).

Hines et al. (2019) investigated arginine supplementation on the reproductive parameters in gilts. Control animals (n = 143; 0% supplemental arginine) or 1 of 3 supplemental arginine (1% as fed) treatments: from 15 to 45 d of gestation (n = 138; Early-arginine); from 15 day of gestation until farrowing (n = 139; Full-arginine); or from 85 day of gestation until farrowing (n = 128; Late-arginine). The feeding corresponds to about 1,5 g/kg body weight/day. The results show that gestational arginine supplementation had no effect on reproductive performance in first parity sows (Hines et al., 2019).

Male mice, aged 6–8 weeks, with ad libitum access to water and a 60% high fat diet for 8 weeks were orally exposed to arginine through water corresponding to 420 mg/kg bw per day. The results show that L-arginine reduced food intake and stimulated gut hormone release in obese mice (Alamshah et al., 2016).

Voloshin et al. (2014) investigated the L-arginine effects on non-alcoholic fatty liver disease. Five-week-old C57BL/6 J male mice were treated for 10 weeks with a high fat diet (HFD) ad libitum to induce hepatic fat accumulation. Thereafter, the HFD-fed mice were divided into two groups, with or without CDCArg 0.75 mg/kg bw per day. In this experiment, mice were pair-fed (15.5 kcal per day per mouse) to compensate the hyperphagic effect of CDCArg. The low fat diet (LFD) control group consumed on average the same amount of calories per day per mouse. The duration of treatment with CDCArg was four weeks. In comparison to HFD treated mice, mice treated with HFD supplemented with CDCArg, showed reduced liver steatosis, reduced body weight and decreased testicular fat and liver tissue mass. Blood glucose, cholesterol, insulin and leptin levels were also lower in this group. No evidence of toxicity of CDCArg was observed (Voloshin, Hahn-Obercyger, Anavi, & Tirosh, 2014).

Arginine is in research papers used to induce acute pancreatitis in animal models to investigate protective effects of proteins etc. and to investigate mechanisms of behind the protective effects. In these studies intraperitoneal injection of arginine has been applied in doses ranging from 0.4 g/kg to 400 g/kg bw ((Al-Hashem et al., 2020)¸ (Abdel-Aziz, Rifaai, & Abdel-Gaber,

2020); (Ren et al., 2018); (Shao & Hathcock, 2008); (Siriviriyakul, Chingchit, Klaikeaw, Chayanupatkul, & Werawatganon, 2019); (Stojanović et al., 2019); (Ohkawara, Takeda, & Nishihira, 2017); (N. Wang et al., 2017); (Cheng, Qiao, Xu, & Shen, 2017); (Hu et al., 2017); (Uçmak et al., 2016); (Ateyya, Wagih, & El-Sherbeeny, 2016); (Zhu et al., 2015); (Guo, Zheng, Gao, Zhang, & Liu, 2015); (Ning et al., 2013); (Shen et al., 2012); (Khan et al., 2012); (Chen et al., 2012); (Shen et al., 2012)).

Human studies

In a systematic review and meta-analysis of clinical trials the authors of the review reported that results of subgroup analysis showed that 1) supplementation with L-arginine could significantly decrease serum insulin levels when the dosage of L-arginine is > 6.5 g/day, 2) when the duration of supplementation is ≤ 12.8 weeks , 3) when the participants are not diabetic patients and 4) when the baseline serum level of insulin was > 20 µIU/mL. Although the results of this study confirmed that supplementation with L-arginine could have significant effects on some glycemic profile indices of participants in clinical trials, the clinical importance of this reduction may not be meaningful (Yousefi Rad, Nazarian, Saboori, Falahi, & Hekmatdoost, 2020).

In a randomized, placebo-controlled, clinical trial the aim was to evaluate the safety and tolerability of oral arginine in overweight or obese but otherwise healthy adults with a body mass index of ≥ 25 kg/m2. A total of 142 subjects completed a 7-day wash-in period using a 12 g arginine per day dose. All the remaining eligible 101 subjects who tolerated the wash-in dose (45 men and 56 women) were assigned randomly to ingest 0, 15 or 30 g arginine (as pharmaceutical grade Arg-HCl) per day for 90 days. Arginine was taken daily in at least two divided doses by mixing with a flavoured beverage. At Days 0 and 90, blood pressures of study subjects were recorded, their physical examinations were performed, and their blood and 24-h urine samples were obtained to measure: (1) serum concentrations of amino acids, glucose, fatty acids, and related metabolites; and (2) renal, hepatic, endocrine and metabolic parameters. The results indicate that the serum concentration of arginine in men or women increased ($P < 0.05$) progressively with increasing oral arginine doses from 0 to 30 g/day. Dietary supplementation with 30 g arginine per day reduced ($P < 0.05$) systolic blood pressure and serum glucose concentration in females, as well as serum concentrations of free fatty acids in both males and females. Based on physiological and biochemical variables, study subjects tolerated oral administration of 15 and 30 g arginine/day without adverse events (McNeal, Meininger, Wilborn, Tekwe, & Wu, 2018).

In a single-blind randomized controlled trial ninety obese patients were included. Patients were randomized to receive either L-arginine (3 or 6 g three times per day) or placebo for 8 weeks. Anthropometric and biochemical indices, dietary intake, and blood pressure values were measured at the baseline and after the 8-week intervention. Results showed significant decreases in anthropometric parameters, blood pressure (SBP, DBP), FBS, HbA1c, LDL, MDA ($P < 0.001$), TG ($P = 0.02$), and TC ($P = 0.002$) and a significant increase in HDL ($P < 0.001$) were observed in the intervention group, compared to the control group. In the control group, no significant differences were found between the baseline and end-of-intervention measurements. In conclusion, oral L-arginine supplementation appears to improve anthropometric parameters, blood pressure values, and some blood biochemical indices

associated with cardiovascular disease prevention (Dashtabi, Mazloom, Fararouei, & Hejazi, 2016).

In a double-blind randomized controlled trial, 56 healthy men selected from sport clubs at the Isfahan University of Medical Science between November 2013 and December 2013 received L-arginine supplementation (2000 mg daily) in the intervention group or placebo (2000 mg maltodextrin daily) in the control group for 45 days. The primary outcome measures were the levels of fasting blood sugar, blood pressure and lipid profile including triglyceride (TG), cholesterol, LDL and HDL in healthy subjects. In this trial, there were complete data for 52 healthy participants with mean age of 20.85±4.29 years. At the end of study, fasting blood sugar (P=0.001) and lipid profile (triglyceride TG (P<0.001), cholesterol (P<0.001), LDL (P=0.04), HDL (P=0.015)) were decreased in the L-arginine group but there was no significant change in the placebo group. In addition, the reduction of fasting blood sugar and lipid profile in L-arginine was significant compared with placebo group. No significant changes were found about systolic (P=0.81) and diastolic blood pressure either in L-arginine or placebo group (P=0.532)(Pahlavani et al., 2017).

Several trials with arginine supplementation have been performed during the years both in healthy individuals or in persons with various cardiovascular conditions. Doses up to 15 g arginine per day has been reported. Some of the trials reports confirms a significant improvement in exercise duration time by oral L-arginine as compared with placebo in individuals with stable heart failure - in contrast - other clinical studies showed no effects. 15 g per day of L-arginine supplementation or placebo for 2 weeks had no significant effect on endothelial function, blood flow and markers of oxidative stress or exercise performance (Böger, 2014).

Brown et al. (2018) showed in a small pilot study that exercise training (walking 45 minutes six days a week for 12 weeks) along with arginine supplementation (6000 mg per day) is beneficial in pulmonary arterial hypertension (PAH). No control group was included. No adverse events occurred, and right ventricular function and brain natriuretic peptide levels remained stable, suggesting safety of the intervention. Further investigation in a randomized controlled trial is warranted (Brown et al., 2018).

Shao & Hathcock (2008) established a so-called "Observed Safe Level" (OSL) at 20 g per person per day based on data from several clinical trials with doses up to 42 g per day (Shao & Hathcock, 2008). An OSL means that a daily intake of up to the OSL-level during a longer period do not result in health related complications. An OSL is intended to provide an upper limit (UL) for substances with limit or no toxic effect. However, most of the human studies used to provide the OSL do not include parameters to assess the safety.

As also cited in the VKM report (Tel et al., 2018) it seems that the two most relevant randomised placebo-controlled trials for the current risk assessment are those published by Monti et al. (2012) and Lucotti et al. (2009). Both provided a daily dose of 6.4 g arginine, for a duration of 6 and 18 months, respectively. In both studies, adverse events did not differ between arginine and placebo groups (VKM).

Arginine: conclusion on the safety evaluation

Although many animal studies are available, a NOAEL could not be derived from any of them. There has also been published several human pilot and clinical trials with arginine supplementation. One of the most relevant from the updated literature search is a randomized, placebo-controlled, clinical trial with doses of 0, 15 or 30 arginine per day for 90 days (McNeal, Meininger, Wilborn, Tekwe, & Wu, 2018). These doses were well tolerated, although the dose of 30 g arginine per day reduced ($P < 0.05$) systolic blood pressure and serum glucose concentration in females, as well as serum concentrations of free fatty acids in both males and females. None of these effects were seen at the medium dose level.

To establish a safe dose level, the randomised placebo-controlled trials by (Monti et al., 2012) and (Lucotti et al., 2009) will be used due to the length of the studies. Both studies showed that a daily dose of 6.4 g arginine for up to 18 months caused no adverse effects, which therefore is considered as the safe intake level.

Cysteine

(Strand et al., 2018) "L-cysteine is a central compound in sulphur metabolism in the human body. L-cysteine is a conditionally essential sulphur-containing amino acid, obtained from L-methionine and from serine. Sulphur-containing amino acids are mainly found in cereal proteins and animal proteins, and less abundantly in pulses. Cysteine may occur in proteins either as cysteine itself or as cystine. Cystine is the disulphide dimer of cysteine, and is a more stable compound than cysteine".

Animal studies

Six to nine adult male Wistar rats were orally gavaged three times (at 1900, 2300 and 0300 hours) with 484 mg/kg bw per day L-cysteine for 14 weeks. Eight to ten obese male C57BL/6 mice aged 6 weeks were also orally gavaged three times (at 1900, 2300 and 0300 hours) with 484 mg L-cysteine/kg bw per day for 14 weeks. Following the repeated l-cysteine administration, the only significant effect observed was a decreased food intake in rats and obese mice. Information on possible adverse effects due to the cysteine supplementation was not included in the study paper (Mcgavigan et al., 2015).

In a review by McPherson & Hardy (2011), it is stated that cysteine itself readily oxidizes to the insoluble cystine dimer. Both free cysteine and cystine are toxic at high levels in the diet when given to rats and chicks. (Harper, Benevenga, & Wohlhueter, 1970) observed 50% mortality in rats fed a casein diet (10 g/100 g) supplemented with 2.4 g/100 g L-Cysteine (free base). Dietary cysteine in protein form as well as cysteine derivatives, largely lack this toxicity when included in animal diets and can effectively substitute for free cysteine to boost antioxidant defences. (Baker, 2006) studied graded levels of supplemental sulphur containing amino acids (SAA) (0, 1, 2, 3 or 4 g/100 g) added to a standard corn–soybean meal diet (23 g/100 g protein) for young chicks. Thus, 0, 1, 2, 3, or 4 g/100 g of L-methionine, L-cysteine or L-cystine as well as levels of N-acetyl-L-cystine equimolar to the L-cysteine addition were supplemented. Because the basal diet contained methionine and cyst(e)ine at their required concentrations for maximal chick growth (i.e., 0.50 g/100 g of each), the supplemental SAA concentrations represented 0, 3, 5, 7 and 9 times the required level of either methionine or cysteine. The 1 g/100 g additions, regardless of SAA source, had no effect on growth rate. Likewise, 2 g/100 g of supplemental

cystine or N-acetyl-L-cysteine were without effect on chick growth. This same level of supplemental methionine, however, depressed weight gain by 34%, and 2 g/100 g of added L-cysteine reduced weight gain by 10%. At 3 g/100 g of L-cysteine, half of the chicks died by day 5 of the 9-day bioassay; and 92% mortality occurred by day 5 when 4 g/100 g of L-cysteine was supplemented. No mortality occurred at any level of supplemental methionine, cystine, or N-acetyl-cysteine, although the growth depression at 4 g/100 g of methionine was severe (94%). With 4 g/100 g of supplemental L-cystine or an isosulfurous level of N-acetyl-L-cystine, growth depressions were 20% and 34%, respectively. That excess dietary L-cystine and N-acetyl-L-cysteine were far less noxious than L-cysteine suggests these SAA compounds may have been absorbed from the gut more slowly than cysteine and with N-acetyl-L-cysteine, it may have been deacetylated more slowly such that tissue concentrations of cysteine could not reach the same levels as those caused by cysteine itself (McPherson & Hardy, 2011).

Human studies

In a review (McPherson & Hardy, 2011), it is stated that cysteine itself readily oxidizes to the insoluble cystine dimer. Both free cysteine and cystine are toxic at high levels in the diet when given to rats and chicks. L-cysteine has a critical role in methionine, taurine and GSH metabolism. Oral or enteral supplementation with diets enriched with cysteine can lead to increased cysteine utilization and improved antioxidant status in various inflammatory conditions, but the simple amino acid and its derivatives, such as N-acetylcysteine, have limited practical applications in clinical nutrition because of stability issues and potential adverse reactions. Cysteine-rich proteins, such as keratin, are abundant. Clearly, something is different in how the body handles oral L-cysteine, L-cystine, and *N*-acetyl-L-cysteine, resulting in a different toxicity profile for each compound. Pharmacokinetic studies with *N*-acetyl-L-Cys have demonstrated that <10% of orally administered *N*-acetyl-L-cysteine is absorbed into portal blood as N-acetyl-L-Cysteine per se (Olsson, Johansson, Gabrielsson, & Bolme, 1988). Thus, first-pass metabolism in the gut wall causes formation of cysteine, GSH, and inorganic sulphate. (McPherson & Hardy, 2011).

In a population based prospective cohort study, a Swedish research group examined 34.250 Swedish women in 1997. The incidence of stroke cases in the group of women was evaluated in 2008. The aim was to evaluate the hypothesis that cysteine intake is inversely associated with stroke incidence. Dietary intake of cysteine was obtained via a comprehensive questionnaires on food intake. The lowest quintile had an intake below 559 mg cysteine per day while the women in the highest quintile group had an average intake above 703 mg cysteine per day. The group observed an inverse association between dietary cysteine intake and risk of stroke. Their findings further suggest that cysteine, at least partly, may account for the reported inverse relation between animal protein (contrary to cysteine sources from vegetable or fish/shellfish origin) intake and stroke risk (Larsson, Håkansson, & Wolk, 2015).

Cysteine: conclusion on the safety evaluation

No data in the identified animal studies could be used for establishment of a NOAEL. It seems that the free cysteine base is quite toxic especially in chicks and to a lesser degree in rats. In contrast it seems that cysteine does not show these toxic effects in the animal studies when the amino acids is incorporated into e.g. a protein. According to the amino acid intake in FRIDA,

cysteine (expressed as cystine, the dimer of cysteine) the amino acids which is present in the lowest amount among all the amino acids in food proteins. Due to lack of human intervention studies with L-cysteine, studies with N-acetylcysteine (or N-acetyl-L-cysteine, NAC), which is readily converted to cysteine, is included instead. Most of the included studies with various study groups have tested NAC in doses of about 600-1200 mg/day. In the randomised controlled trials there have been no differences in severe adverse events between the placebo and NAC-groups. The adverse effects reported are generally limited to mild gastrointestinal symptoms (Strand et al., 2018).
The dose 1200 mg of NAC yields maximum 900 mg of L-cysteine (corresponding to 450 mg of L-cystine). Therefore, the safe dose level of cysteine will be 900 mg per person per day.

Glutamine

(Løvik et al., 2020) "L-glutamine is considered a non-essential amino acid in humans. In addition to its role in protein synthesis and the handling by the body of ammonia (via urea cycle), L-glutamine participates in other complex metabolic pathways e.g. in the central nervous system, immune system, and insulin secretion. L-glutamine is deaminated by glutaminase to form glutamic acid. L-glutamine is available from all protein-containing foods. High-protein foods contain the most (e.g. meat, fish, eggs and dairy products" ("L-glutamine is considered a non-essential amino acid in humans. In addition to its role in protein synthesis and the handling by the body of ammonia (via urea cycle), L-glutamine participates in other complex metabolic pathways e.g. in the central nervous system, immune system, and insulin secretion. L-glutamine is deaminated by glutaminase to form glutamic acid. L-glutamine is available from all protein-containing foods. High-protein foods contain the most (e.g. meat, fish, eggs and dairy products".

Animal studies

No relevant animal studies identified

Human studies

Durante (2019) has examined the role of L-glutamine in cardiovascular disease. Many studies report that glutamine supplementation protects against cardiometabolic disease, ischemia-reperfusion injury, sickle cell disease, cardiac injury by inimical stimuli and may be beneficial in patients with heart failure. However, excessive shunting of glutamine to the Krebs cycle can precipitate aberrant angiogenic responses and the development of pulmonary arterial hypertension (Durante, 2019).

Kim & Kim (2017) has reviewed the literature regarding the roles of glutamine in intestinal physiology and management of multiple intestinal diseases. Glutamine is the most abundant amino acid in human blood, skeletal muscle and the free amino acid pool. In gut physiology, glutamine promotes enterocyte proliferation, regulated tight junction proteins, suppresses pro-inflammatory signalling pathways and protects cells against apoptosis and cellular stresses during normal and pathologic conditions. Studies in healthy adults have demonstrated that 75% of enteral provided glutamine is absorbed into the splanchnic tissues and most of the absorbed glutamine is metabolized within the intestine. Garcia-de Lorenzo et al. describes that

glutamine-enriched diets were shown to improve immunologic aspects in trauma patients and to ameliorate mucositis in post-chemotherapy patients. The authors determined how much glutamine is required to observe improved clinical outcomes: 21 g glutamine per day for 28 days for Crohn's disease and 42 g per day for 21 days for short bowel syndrome. In a randomized controlled trial. (Benjamin et al.) reported that glutamine supplementation (0.5 g/kg bw per day for two months) in patients with Crohn's disease in remission phase reduced the intestinal permeability and morphology (Kim & Kim, 2017).

McRae has performed a review of meta-analyses of the therapeutic benefits of glutamine. Glutamine may be a conditionally essential amino acid in patients with catabolic disease as it has been demonstrated that circulating plasma glutamine concentrations drop during critical illness and following major surgery. Clinical studies have suggested that glutamine supplementation in this group of patients may improve nitrogen balance, constitutive protein levels and improve immune functions together with decreasing infection rates, the length of hospitalisation and mortality rates. In these clinical trials doses of 0.3 and up to 0.45 g/kg bw per day of glutamine was administered (no information on treatment days). The American society for parenteral and enteral nutrition suggests that glutamine supplementation should probably be given early and in doses between 0.2 and 0.5 g/kg bw per day (McRAE, 2017).

Laviano et al. conducted a pilot study designed as a cross-over trial whether the supplementation of glutamine to overweight/obese patients may favour weight loss and improve glucose metabolism, independently of calorie restriction. Patients were given a daily dose of 0.5 g/kg bw for four weeks either of glutamine or an isonitrogenous standard protein supplement. Only one patient reported dizziness early during glutamine supplementation, which disappeared when the daily dose was reduced to 0.25 g/kg bw per day for one week and the investigational dose was reinstated without further appearance of side effects (Laviano et al., 2014).

Shao & Hathcock (2008) established a so-called "Observed Safe Level" (OSL) at 14 g glutamine per person per day based on data from several clinical trials with doses up to 45 g per day (Shao & Hathcock, 2008). An OSL means that a daily intake of up to the OSL-level during a longer period do not result in health related complications. An OSL is intended to provide an upper limit (UL) for substances with limit or no toxic effect. However, most of the human studies used to provide the OSL do not include parameters to assess the safety.

Glutamine: conclusion on the safety evaluation

No new studies were identified that could establish a NOAEL. In a clinical trial a dose of 0.5 g/kg bw for four weeks was well tolerated (Laviano et al., 2014). Due to the lack of more recently human intervention studies it is suggested to use the before mentioned OSL-level of 14 g per person per day as the safe level to be used in risk assessments. Studies have indicated that even higher levels could be acceptable but the uncertainty is high and 14 g per person per day is chosen safe intake level.

Histidine

(Holvik, Iversen, et al., 2020) "L-histidine is a conditionally essential amino acid which is a normal constituent of most body proteins. L-histidine is also a part of many plasma proteins. It has

anti-oxidant and anti-inflammatory properties. Moreover, L-histidine is also a precursor of histamine and is necessary for the regulation and metabolism of trace elements such as metal ions. The human body has a large pool of L-histidine in plasma proteins, but also as carnosine in skeletal muscles and in haemoglobin. Foods rich in histidine are generally protein rich foods such as meat, dairy products, legumes, fish, nuts, seeds, eggs and whole grains".

Animal studies

Many studies have shown that histidine acts on feeding behaviour in rats where it is reported that food intake is decreased with increased dietary histidine. A research group showed a decrease in food intake with a daily dose of 20% casein plus 2.5% or 5% histidine (25 g/kg or 50 g/kg of diet) for eight days, compared with a group that received 20% casein without supplemental histidine. In a study in which the level of histidine was >2 g/kg bw per day, rats from 7 to 46 weeks showed growth retardation, hepatomegaly and hypercholesterolemia; when histidine was administered in doses >4 g/kg bw per day , hypercholesterolemia was observed after 46 days of treatment. In a 13-week feeding study diets containing 0, 3.1, 6.2, 12.5, 25 and 50 g/kg of histidine in male and female F344 rats demonstrated that with the higher dose of histidine, body weight and food intake decreased in males and an increase in hemoglobin volume and hematocrit was seen. This effect seemed due to the high dose of histidine, because it is a major component of hemoglobin. The level of blood urea nitrogen and creatinine increased in females and the level of blood urea nitrogen also increased in females fed 12.5 g/kg of histidine in the diet. Concerning body composition, there was an increase in the weight of some organs, including the kidney for males of the 25 g/kg and 50 g/kg groups, testis for males of the 50 g/kg group and kidney for females of the 50 g/kg group. Owing to the different adverse effects observed for 50 g/kg fed rats, the authors concluded that the maximum tolerable dose of histidine is 25 g/kg of histidine in the diet corresponding to 2232 mg/kg bw per day (Moro, Tomé, Schmidely, Demersay, & Azzout-Marniche, 2020).

Human studies

Gheller et al. (2020) evaluated the clinical safety of histidine intake above the average dietary intake (1.52-5.20 g per day) of graded doses of histidine supplement given to healthy adult persons (aged 21-50 years). The doses given were: (4, 8 and 12 g per day, Study 1) (20 men and 20 women) and/or a 16-g per day dosage of histidine (Study 2, 21 men and 19 women); 27 participants (12 men and 15 women) completed both studies. Participants consumed encapsulated histidine for 4 weeks followed by a 3-wk recovery period. No changes were observed in vitals or body composition occurred with histidine supplementation in either study. Plasma histidine (measured in subjects who completed all dosages for Studies 1 and 2) was elevated at the 12- and 16-g per day dosages (compared with 0-8 g per day, ($P < 0.05$) and blood urea nitrogen increased with dosage ($P = 0.013$) and time ($P < 0.001$) in Study 1 and with time in Study 2 ($P < 0.001$). In Study 1, mean ferritin concentrations were lower in 12 g per day (46.0 ng/mL; 95% CI: 34.8, 60.9 ng/mL) than in 4 g per day (51.6 ng/mL; 95% CI: 39.0, 68.4 ng/mL; $P = 0.038$). In Study 2, 16 g per day increased mean aspartate aminotransferase from baseline (19 U/L; 95% CI: 17, 22 U/L) to week 4 (24 U/L; 95% CI: 21, 27 U/L; $P < 0.001$) and mean serum zinc decreased from baseline (0.75 µg/dL; 95% CI: 0.71, 0.80 µg/dL) to week 4 (0.70 µg/dL; 95% CI: 0.66, 0.74 µg/dL; $P = 0.011$). Although values in this study remained within the normal reference ranges for all analytes measured, in all dosages tested, the human no-

observed adverse effect level was determined to be 8 g per day owing to changes in blood parameters at the 12-g per day dosage (Gheller et al., 2020).

In order to investigate the possible efficacy of oral histidine as anorectic therapy in men, a study group fed eight healthy men (ages 32 to 38) with 4 g of histidine supplement per day for four weeks. Results showed that this amount had no significant effect on appetite, taste or smell perception, food intake, or body weight. In addition, total serum zinc, albumin-bound zinc, macroglobulin-bound zinc concentrations, and urinary histidine excretion had not significantly changed. Based on these studies and a risk assessment, the Norwegian Scientific Committee for Food Safety concluded that supplementation with 4 to 4.5 g/day, corresponding to 57 mg/kg of body weight per day for a 70 kg adult, does not have adverse effects in humans. The scientific committee concluded that the specified doses of 0.55 and 0.6 g/day of histidine in food supplements are unlikely to cause adverse health effects in all age groups from 10 years and up (Holvik, Iversen, et al., 2020). Geliebter et al., studied the effect of daily histidine doses from 24 to 64 g on healthy subjects for four weeks. They reported ensuing headaches, weakness, drowsiness and nausea. For subjects receiving 64 g per day, two reported painful sensations in their eyes and difficult focusing, one showed mental confusion, poor memory and depression with episodes of crying. In contrast, Moro et al. reported that daily histidine intake (1.65 g/day) actually decreased feelings of fatigue, increased efficiency while performing memory tasks and promoted clear thinking and concentrations in subjects with high fatigue and sliip disruptions scores. The large difference in histidine doses likely explains the contradictory effects between the two study groups. In comparison according to the FAO, the daily requirement for histidine is 8 to 12 mg/kg of body weight per day in adults. The average intake of histidine in typical, adult diets in Europe, the USA and Japan was reported to be between 2.12 and 2.40 g per day (i.e., about 30 to 35 mg/kg bw per day), where men of 50-70 years of age at 5.20 g per day represented the 99th percentile intake (Moro et al., 2020).

Histidine: conclusion on the safety evaluation

Histidine was investigated in a 90-day study in rats where a NOAEL of 2232 mg/kg bw per day was established. Applying an assessment factor of 100 this corresponds to 22 mg/kg per day in humans, or 1540 mg per day for a person weighing 70 kg (Moro et al., 2020). In the Gheller et al. study, the human no-observed adverse effect level was determined to be 8 g per day owing to changes in blood parameters at the 12-g per day dosage. (Holvik, Iversen, et al., 2020) concluded that supplementation with 4 to 4.5 g per day, corresponding to 57 mg/kg of body weight per day for a 70 kg adult, does not have adverse effects in humans. The two human studies are only indicative for the safety due to the limited number of participant, population groups and parameters that are safety-related. Data from the animal study will therefore be used to set the safe intake level at 1540 mg per person per day.

Lysine

(Henjum et al., 2020) "L-Lysine, an indispensable amino acid, is present in all proteins in the human body. Its catabolisation takes place mainly in the liver. The two nitrogen groups are transferred to alpha-ketoglutarate to form glutamate. The remaining carbon skeleton is broken down to acetyl-CoA. Lysine is exclusively ketogenic i.e. does not enter gluconeogenesis for the

production of glucose. Foods rich in L-lysine are generally protein rich foods such as meat, dairy products, eggs, legumes, and some fish".

Animal studies

Sprague-Dawley rats were fed with transgenic rice and non-transgenic rice with 17.5%, 35% and 70% rice in the diet for 90 days. There were 10 male and females in each group. In one elite transgenic line, HFL1, the free lysine content in mature seeds is 437.8 mg/g dry seed weight, increased 25- fold compared with the non-transgenic wild type (17.3 mg/g dry seed weight). In non-transgenic rice the feeding with rice corresponds to 0.5, 1 and 2 mg lysine/kg bw/day under the assumption that in wild type rice the lysine content is 0.3% dry matter in paddy rice. In transgenic rice the feeding corresponds to 12.5, 50 and 100 mg lysine/kg bw/day. Some effects (such as body weight and hematological parameters) were observed in both male and female rats with both transgenic and non-transgenic rice. There was no dose response relationship and the transgenic mice with up to 100 mg lysine/kg bw per day did not induce more pronounced effects compared to the wild type rice with low level of lysine (Yang et al., 2017).

Tsubuku et al. (2004b) evaluated toxicological and behavioural effects of Lysine in a 90-day study with male and female Sprague-Dawley rats. The amino acid (lysine hydrochloride) was incorporated into a standard diet at doses equal to 1.25%, 2.5% and 5.0% (w/w). A control group received a standard diet. To examine stability of any potential effects, the administration period was followed by a 5 weeks recovery period, during which only the standard diet was provided to all animals. No treatment-related changes were observed in any of the treatment groups, neither in clinical observations, body weights, diet consumption, water intake, ophthalmology, gross pathology, organ weights or histopathology. A Lysine-related drop in serum concentration and an increase in urine excretion of chlorides was a compensatory reaction to the ingested hydrochloride. No functional, biochemical, or histological changes in renal function were found. The NOAEL for Lysine was estimated at 5.0% for both genders corresponding to 3357 and 3986 mg/kg bw per day for males and females respectively (Tsubuku, Mochizuki, Mawatari, Smriga, & Kimura, 2004).

In a transgenic animal model, Gcdh-/- mice were fed from 30 days of age up to 60 days with a diet containing normal or increased amounts of lysine (5600 mg/kg bw/day). Increased amount of lysine in the diet caused myelin damage that seems to be mediated by a long-term increased levels of GA-I metabolites having deleterious effects in myelinating oligodendrocytes over neurons (Olivera-Bravo et al., 2019).

Human studies

Unni et al. (2012) examined the effect of an eight weeks lysine supplementation diet on muscle mass and function. Healthy, under- (n=20) and well-nourished (n=20) men were studied before and after 8 weeks, during which low (n=20) and high (n=20) lysine diets were consumed. The low and high lysine diets (~25 and ~40 mg/kg bw per day) for under and well-nourished men, respectively, were based on the subjects' habitual lysine intake, while the high lysine diet supplied 80 mg/kg bw per day. Anthropometry, muscle function, insulin sensitivity and leucine kinetics were measured before and after the experimental period. The high lysine diet had a small positive effect (about +7.5%) on muscle strength, but no effect on other parameters. In

conclusion: Over an 8 weeks controlled feeding period, an intake of 80 mg lysine/kg bw per day had a small positive effect on muscle strength, but only a very limited number of safety parameters were included (Unni et al., 2012).

Lysine: conclusion on the safety evaluation

No new animal or human study was identified. (Tsubuku, Mochizuki, Mawatari, Smriga, & Kimura, 2004b) investigated Lysine (lysine hydrochloride) in a 90-day study in rats where a NOAEL of approximately 4000 mg lysin /kg bw per day was established. Applying an assessment factor of 100 this corresponds to 40 mg/kg per day in humans, or 2800 mg per day for a person weighing 70 kg, which will be considered the safe dose in the assessment.

Methionine

Animal studies

Wallis et al. (2020) investigated the sex-specific effects of dietary methionine restriction on the intestinal microbiome. Each group contained 5 mice except for the males on the methionine-restricted diet with 4 animals. Diets were changed at the beginning of the experiment to a methionine-restricted diet corresponding to 300 mg/kg bw/day, or an otherwise identical control diet corresponding to 1300 mg/kg bw/day and diets were maintained continuously for 30 days. In summary, feeding a methionine-restricted diet for one month was associated with significant and sex-specific changes in the intestinal microbiome (Wallis, Melnyk, & Miousse, 2020).

After 4 weeks of a methionine-enriched diet at a dose of 2 g/kg of bw per day, adult male Wistar rats underwent 4-vessel occlusion lasting for 15 min, followed by a reperfusion period varying from 3 to 7 days. Histo-morphological analyses showed that the subsequent ischemia-reperfusion insult (IRI) aggravates the extent of the sole hHcy-induced degeneration of the hippocampal neurons. Decreased volume in the grey matter, extensive changes in the metabolic ratio, deeper alterations in the number and morphology of neurons, astrocytes and their processes were demonstrated in the hippocampus 7 days post-ischemia in the hHcy animals. In summary, there were effects of rat hippocampal neurodegenerative effects at a dose of 2 g/kg (diet) of animal weight per day (Kovalska et al., 2020).

Diets supplemented with methionine (corresponding to 1000 mg/kg bw/day) to mice dams that were fed test diets from parturition through d 17 of lactation. Supplementation with methionine independently increased litter weight gain 10%. Supplementation with methionine increased phosphorylation of mTOR by 47% (mTOR is a protein kinase regulating cell growth, survival, metabolism, and immunity) (Liu et al., 2017).

One month old CBS +/- heterozygous mice were fed (six mice) with a high methionine diet (1540 mg/kg bw/day) for two months. A High methionine diet poses a cardiac threat by increasing oxidative stress, inflammatory manifestations, matrix/vascular remodelling, and decreased cardiac function (Chaturvedi, Kamat, Kalani, Familtseva, & Tyagi, 2016).

Human studies

Recently, VKM made a safety assessment of methionine (Strand et al., 2020). In this assessment the following is stated "In 2005, Institute of Medicine, US (IOM) concluded that there was

insufficient data to establish a tolerable upper intake level (UL) for methionine. One relevant new animal and four human studies with methionine were identified after 2002. Two of the new studies in humans reported on methionine-loading tests. One study in infants showed serious adverse health effects in infants given a protein hydrolysate with L-methionine equivalent to 8800 mg/L. There are indications that intake of methionine during the so called acute methionine-loading test is associated with adverse health effects such as dizziness, nausea, sleepiness and decreased or increased blood pressure. In the loading test, 100 mg methionine per kg body weight is given after a 12-hour fast. This intake (100 mg/kg body weight) of L-methionine may be regarded as the lowest observed adverse effect level (LOAEL). Although IOM has concluded that no UL could be established for methionine it has been reported that use of methionine as a single amino acid may have adverse health effects. An intake at 100 mg/kg body weight of L-methionine may be regarded as a LOAEL. With a conservative approach and the use of an assessment factor of 10 for between people variations and a factor of 3 for the uncertainty of LOAEL, a tentative guidance level (GL) of 100/30 ~ 3 mg of L-methionine per kg body weight can be suggested. In a 70 kg man this is equivalent to an intake of 210 mg per daily dosage" (Strand et al., 2020)

Methionine: conclusion on the safety evaluation

A few new animal studies have been found showing adverse effects following high administration of methionine. These studies are perform in specialized animal models, and include few animal fed for a short duration. Therefore, they cannot be used to provide a NOAEL. In addition, no new human studies have been identified that can be used to set a NOAEL. To provide a safe intake level, Strand et al., 2020 made use of an assessment from the Institute of Medicine US, where, an intake of 100 mg/kg body weight of L-methionine is regarded as a LOAEL. With the use of an assessment factor of 10 for between people variations and a factor of 3 for the uncertainty of LOAEL compared with an NOAEL, a tentative guidance level of 100/30 ~ 3 mg of L-methionine per kg body weight can be suggested. In a 70 kg person this is equivalent to an intake of 210 mg per day. The intake of 210 mg per day will be considered the safe intake level.

Phenylalanine

(Frøyland, Haugen, Holvik, et al., 2020) "L-phenylalanine is an essential amino acid which means it has to be obtained from the diet. Amino acids are building blocks for proteins and present in protein rich food such as milk, meat, fish, eggs and cheese".

Animal studies

Mice (females 15/group, males 5/group) were dosed via oral gavage with 150 and 300 mg/kg bw. Pregnant mice received phenylalanine from 8-12 days of pregnancy. Minor significant effects on body weight, uterus weight, liver, kidney, heart and lung weight at both doses. Minor significant increased number of implants and alive fetuses, and decreased number of dead fetuses (decrease) and decreased fetal weight. In summary, minor effects on body weight and reproductive parameters were induced by 150 and 300 mg phenylalanine/kg bw (Oliveira et al., 2014).

Human studies

EFSA re-evaluated aspartame as a food additive in 2013 ("Scientific Opinion on the re-evaluation of aspartame (E 951) as a food additive," 2013). EFSA in this opinion points out that aspartame is rapidly and completely hydrolysed in the gastrointestinal tract to phenylalanine, aspartic acid and methanol. Phenylalanine at high plasma levels is known to cause developmental toxicity in humans. The EFSA Panel concluded that human data on developmental toxicity were more appropriate for the risk assessment. Concentration-response modelling was used to determine the effects of aspartame administration on plasma phenylalanine using human data after phenylalanine administration to normal, PKU heterozygote or PKU homozygote individuals. In healthy and PKU heterozygotes, aspartame intakes up to the ADI of 40 mg/kg bw per day (equivalent to 22.4 mg/kg bw per day phenylalanine) in addition to dietary phenylalanine, would not lead to peak plasma phenylalanine concentrations above the current clinical guideline (360 µM) for the prevention of adverse effects in fetuses (EFSA, 2013). This ADI is not applicable to PKU patients.

Phenylalanine: conclusion on the safety evaluation

No new data were identified in the literature search that could provide the basis for a safety assessment of phenylalanine. In their risk assessment of phenylalanine, Frøyland et al. (2020) used the EFSA opinion on the re-evaluation of aspartame (E 951) as a food additive. They used the aspartame ADI of 40 mg/kg bw per day, which is equivalent to 22.4 mg/kg bw per day of phenylalanine. This correspond to 1568 mg per day for a person weighing 70 kg) will be considered the safe intake level dose. This safe dose is not applicable to PKU patients.

Tryptophan

(Holvik, Frøyland, et al., 2020) "L-tryptophan is an indispensable amino acid in humans, which in addition to its role in protein synthesis, also participates in complex metabolic pathways where it acts as a precursor to the potent neurotransmitter serotonin, the hormone melatonin, and the vitamin niacin (vitamin B3). L-tryptophan is available from a wide variety of protein-rich foods in the normal diet, including meat, fish, milk and dairy products, egg, beans, lentils and also bread and grains, pasta, rice, fruit and vegetables.".

Animal studies

L-tryptophan was investigated in a 90-day study. L-tryptophan at doses of 0, 1.25%, 2.5% and 5.0% were administered in the diet to Sprague–Dawley rats for 13 weeks. Twelve males and 12 females were used in each group. Body weight gain and food consumption significantly decreased throughout the administration period in males in the 2.5% group and in both sexes in the 5% group. At the end of the dosing period, decreases in water intake in males in the 5% group and in serum glucose in females in the 5% group were observed. The NOAEL of l-tryptophan in the present study was 948 mg/kg bw/day for males and 1956 mg/kg bw/day for females (Shibui et al., 2018).

Male Wistar rats were dosed with a tryptophan-enriched diet at a dose of 600 mg/kg bw/day for 30 days via the diet. The study authors suggest that the physiological and behavioral alterations that follow the administration of tryptophan are associated with the activation of brain regions that regulate cognition and mood/anxiety-related responses (SILVA et al., 2017).

Five pregnant rats were fed via the diet with 1200 mg/kg bw/day from gestational day 1. At postnatal day 20, surviving pups of the experimental group and control group were sacrificed. The high-tryptophan diet in pregnant rats induces hyperserotonemia in the fetus. Hyperserotonemia results in an excess of serotonin in the brain where it has a negative influence on development of serotonergic neurons and consequently on growth hormone production (Musumeci et al., 2014).

Human studies

Lieberman et al. (2016) examined the intake of tryptophan and its associations with biochemical, behavioural, sleep, and health and safety outcomes in adults in a secondary analysis of a large, publicly available database of the US population. The usual tryptophan intake by US adults was 826 mg/d, several fold higher than the Estimated Average Requirement for adults of 280 mg/d for a 70-kg adult. Most health- and safety-related biochemical markers of liver function, kidney function, and carbohydrate metabolism were not significantly (P-trend > 0.05) associated with deciles of tryptophan intake and were well within normal ranges, even for individuals in the 99th percentile of intake. Usual intake deciles of tryptophan were inversely associated with self-reported depression measured by the Patient Health Questionnaire raw score (0-27; P-trend < 0.01) and calculated level (1 = no depression, 5 = severe depression; P-trend < 0.01) and were positively associated with self-reported sleep duration (P-trend = 0.02) (Lieberman, Agarwal, & Fulgoni, 2016).

Tryptophan: conclusion on the safety evaluation

L-tryptophan was investigated in a 90 day study in rats where a NOAEL of 948 mg/kg bw per day was established (Shibui et al., 2018). Applying an assessment factor of 100 this corresponds to 10 mg/kg per day in humans, or 700 mg per day for a person weighing 70 kg, which is considered as the safe intake level.

Tyrosine

(Frøyland, Haugen, Henjum, et al., 2020) "L-tyrosine, an aromatic amino acid, is considered a conditionally indispensable amino acid because it can be synthesised from L-phenylalanine in the liver. The magnitude of endogenous synthesis of L-tyrosine is not known, but is related to the intake of phenylalanine. L-tyrosine is a precursor of several biologically active substances, including catecholamine neurotransmitters, thyroid hormones and melanin skin pigments. L-tyrosine is available from all protein-containing foods such as meat, eggs, fish, dairy products, grains and pulses".

Animal studies

Rats of both sexes were administered l-tyrosine by oral gavage once daily for 13 weeks at doses of 0 (vehicle), 200, 600 or 2000 mg/kg bw/day. Ten males and ten females were used in each dose group. Effects were observed in forestomach, liver, kidney and in blood chemistry. The no-observed-adverse-effect level (NOAEL) of l-tyrosine was 600 mg/kg bw/day for males and females (Shibui, Manabe, Kodama, & Gonsho, 2016).

Human studies

No relevant human studies were identified.

Tyrosine: conclusion on the safety evaluation

To provide a safe intake level for tyrosine, the 90 day study in rats by (Shibui et al., 2016) are used. The NOAEL derived was 600 mg/kg bw per day. Applying an assessment factor of 100 this corresponds to 6 mg/kg per day in humans, or 420 mg per day for a person weighing 70 kg, which is considered as the safe intake level.

Isoleucine

Animal studies

No relevant animal studies identified

Human studies

A research group investigated the effects of the intragastric administration of leucine and isoleucine on the gastric emptying of, and blood glucose responses to a physiologic mixed-macronutrient drink and subsequent energy intake. In two separate study, 12 healthy, lean subjects received on 3 separate occasions an intragastric infusion of 5 g of leucine or isoleucine, or 10 g of leucine or isoleucine - a control group was included. 15 minutes later subjects consumed a mixed-nutrient drink and gastric emptying, blood glucose, plasma insulin, C-peptide, glucagon, glucagon-like peptide-1, glucose-dependent insulinotropic polypeptide and cholecystokinin were measured for 60 minutes. Immediately afterward energy intake from a cold meal was assessed. Compared with the control, leucine 10 g decreased the blood glucose area under the curve ($P<0.05$) and tended to reduce peak blood glucose ($O=0.07$), whereas effects of leucine 5 g were NS. Leucine 10 g but not leucine 5 g increased plasma insulin and C-peptide ($P<0.01$ for both), but neither dose affected glucagon, FLP-1, GIP, cholecystokinin, gastric emptying or energy intake. Compared to controls isoleucine 10 g reduced the blood glucose and peak blood glucose ($P<0.01$), whereas effects of isoleucine 5 g were NS. Neither dose levels affected insulin, C-peptide, glucagon, GLLP-1 or GIP. Isoleucine 10 g but not isoleucine 5 g slowed gastric emptying ($P<0.05$), but gastric emptying was not correlated with the blood glucose AUC. Isoleucine did not affect energy intake. In conclusion, in healthy subjects, both leucine and isoleucine reduced blood glucose in response to a mixed-nutrient drink but did not affect subsequent energy intake. The mechanisms underlying glucose lowering appear to differ: leucine stimulated insulin, whereas isoleucine acted independently on insulin (Ullrich et al., 2016).

Isoleucine: conclusion on the safety evaluation

Very few animal and human studies have been identified. From the human study by Ullrich et al. (2016), no effects were seen following administration of 5 g of isoleucine or leucine. See assessment of BCAA.

Leucine

Animal studies

A toxicokinetic study of BCAAs in dogs following oral administration of 0.25 g/kg and 0.50 g/kg. All these BCAAs (including leucine) were well absorbed with a substantial increase in the plasma concentration (T. Wang, Xie, Chen, Jiang, & Wang, 2015).

Male C57BL/6 mice were exposed for 14 weeks to semi-synthetic diets containing either 20% (adequate protein content, AP) or 50% whey protein (high-protein content, HP). A third group was fed the AP diet supplemented with L-leucine (AP + L; 4,5 g Leucine/100 g) corresponding to 9000 mg L-leucin/kg bw per day. There were 10-11 mice in each diet group. Leucine supplementation did not affect glucose tolerance. There was no effect on liver and skeletal muscle triglyceride and glycogen concentrations. There was a decrease in post-absorptive plasma concentrations of branched-chain amino acids. An exposure of mice to a leucine supplementation has no significant effect on energy homeostasis and UCP expression compared with AP diets when feeding a low-fat diet (Noatsch, Petzke, Millrose, & Klaus, 2011).

35-day-old piglets of both sexes were for 35 days fed diets containing two fold (L2)- (19.7 g/kg corresponding to about 700 mg leucine/kg bw/day) and four-fold (L4: 37.5 g/kg corresponding to about 1400 mg leucine/kg bw/day) higher leucine contents than the recommended amount (control). There were 36 piglets in the control dose group and the two dose groups. L4 diet led to a pronounced increase in BCKDH activity in the brain, liver and cardiac muscle, whereas there were no changes in enzyme activity in the pancreas, skeletal muscle, adipose tissue and intestinal mucosa. The L2 diet had only weak effects on BCKDH activity. L2 and L4 diets reduced the concentrations of free valine and isoleucine in nearly all tissues. Compared to the controls, pigs treated with the L2 and L4 diets consumed less food, showed increased plasma concentrations of 3-hydroxybutyrate and reduced levels of circulating serotonin (Wessels et al. 2016).

Human studies

Leucine, a branched-chain amino acid, has been shown to stimulate muscle protein synthesis and has been suggested to play a role in the prevention of age-related muscle atrophy (sarcopenia).

Two studies were performed to determine the UL for leucine in young and elderly men (Elango, Chapman, Rafii, Ball, & Pencharz, 2012); (Rasmussen, Gilbert, Turki, Madden, & Elango, 2016). Initially, in young men the conceptual model of determining the maximum oxidative capacity of an amino acid was found to be an ideal marker for identifying the UL. Leucine oxidation, measured with the use of l-[1-13C]leucine, increased with increasing leucine intakes and reached a plateau at higher intakes. Two-phase linear regression analysis identified a breakpoint of 550 mg/kg per day (95% CI: 454, 646 mg/kg per day), with a simultaneous increase in blood ammonia concentrations above normal values (35 µmol/L). The other study was conducted in elderly men (~72 y old). A breakpoint in leucine oxidation was observed at 431 mg/kg bw per day (95% CI: 351, 511 mg/kg bw per day), with blood ammonia concentrations above normal (35 µmol/L) at leucine intakes >550 mg/kg bw per day. Taking the data together, the UL for leucine intake in healthy elderly men could be set at a value similar to young men, at 500 mg/kg bw per day or ~35 g/d for an individual weighing 70 kg; or, as a cautious estimate, the leucine UL could also be considered as 351 mg/kg bw per day (the lower 95% CI), which would be ~24.5 g/d for an elderly individual weighing 70 kg. These studies to determine the UL for leucine in humans are acute diet studies, and future studies with

additional biomarkers and long-term supplementation of leucine will be necessary (Elango et al., 2012); (Rasmussen et al., 2016).

Ullrich et al. (2016) investigated the effects of the intragastric administration of leucine and isoleucine on the gastric emptying of, and blood glucose responses to a physiologic mixed-macronutrient drink and subsequent energy intake. In two separate studies 12 healthy, lean subjects received on 3 separate occasions an intragastric infusion of 5 g of leucine and isoleucine or 10 g of leucine and isoleucine - a control group was included. 15 minutes later subjects consumed a mixed-nutrient drink and gastric emptying, blood glucose, plasma insulin, C-peptide, glucagon, glucagon-like peptide-1, glucose-dependent insulinotropic polypeptide and cholecystokinin were measured for 60 minutes. Immediately afterward energy intake from a cold meal was assessed. Compared with the control, leucine 10 g decreased the blood glucose area under the curve ($P<0.05$) and tended to reduce peak blood glucose ($O=0.07$), whereas effects of leucine 5 g were NS. Leucine 10 g but not leucine 5 g increased plasma insulin and C-peptide ($P<0.01$ for both), but neither dose affected glucagon, FLP-1, GIP, cholecystokinin, gastric emptying or energy intake. Compared to controls isoleucine 10 g reduced the blood glucose and peak blood glucose ($P<0.01$), whereas effects of isoleucine 5 g were NS. Neither dose affected insulin, C-peptide, glucagon, GLLP-1 or GIP. Isoleucine 10 g but not isoleucine 5 g slowed gastric emptying ($P<0.05$), but gastric emptying was not correlated with the blood glucose AUC. Isoleucine did not affect energy intake. In conclusion in healthy subjects, both leucine and isoleucine reduced blood glucose in response to a mixed-nutrient drink but did not affect subsequent energy intake. The mechanisms underlying glucose lowering appear to differ: leucine stimulated insulin, whereas isoleucine acted insulin independently (Ullrich et al., 2016).

Cocate et al. (2015) assessed the potential association of BCAA consumption with central obesity and cardiometabolic risk factors in a cross-sectional study with 296 Brazilian middle-aged men. Anthropometry, lifestyle features, blood biochemical parameters were assessed and dietary intake was estimated by a food frequency questionnaire. Participants were classified by the occurrence of central obesity (Cobesity), hypertriglyceridemia (HTG), hypertriglyceridemic waist phenotype (HWP) and metabolic syndrome (MetS). Participants in the BCAA highest tertile (≥0.17 g/kg/d) presented lower occurrence of Cobesity (36.0% vs 72.4%, $P<0.01$), HTG (17.0% vs 30.6% $P<0.032$, $z=-2.32$), HWP (23.0% vs 46.9%, $P<0.01$) and MetS (19.0% vs 34.7%, $P<0.01$). They also exhibited lower values for Castelli index (total cholesterol:HDL-c) and triglycerides: HDL-c ratio than those in the first tertile, regardless of interfering factors (i.e. habitual physical activity, work position, smoking habit, and energy intake). Interestingly, leucine consumption showed similar associations with cardiometabolic risk factors, as compared to BCAA consumption ($P<0.05$) (Cocate, Natali, de Oliveira, Alfenas, & Hermsdorff, 2015).

Leucine: conclusion on the safety evaluation

Only few animal and human studies have been identified and most of them are merely investigating endpoint to reveal beneficial effects. From the human study by Ullrich et al. (2016), no effects were seen following administration of 5 g of isoleucine or leucine. See assessment of BCAA.

Valine

Animal studies

A toxicokinetics study of BCAAs in dogs following oral administration of 0.25 g/kg and 0.50 g/kg, corresponding to 60 and 120 mg valine/kg body weight. All these BCAAs (including valine) were well absorbed with a substantial increase in the plasma concentration (T. Wang et al., 2015).

Six barrows [16.5 kg body weight (BW)] were exposed to an intravenous infusions of either 150 mM valine (1.5 mmol/kg BW, corresponding to 180 mg/kg bodyweight) or physiological saline. Blood samples were taken 10 min prior to infusion, at the end of infusion, at 10-min intervals for 60 min post-infusion, and at 90 and 120 min post-infusion. The administered dose of valine did not cause a substantial change in the metabolic status of growing pigs (Libao-Mercado, Columbus, & de Lange, 2015).

Human studies

No relevant human studies identified

Valine: conclusion on the safety evaluation

Only a few animal and not of any safety relevance have been identified. Therefore, no safe intake level has been derived for valine. See assessment of BCAA.

Branched-chain amino acids

(Skålhegg et al., 2018) "L-leucine, L-isoleucine and L-valine are essential amino acids. L-leucine, L-isoleucine and L-valine are commonly known as Branched Chain Amino Acids (BCAAs), and are found in food items containing proteins and in particular, in protein-rich foods such as dairy products, meats, eggs, nuts, whole grains, seeds, avocadoes and edible seaweed. It has been shown that BCAAs are not metabolized in the liver as is common for most other amino acids but taken up by most peripheral tissues (in particular muscle) where they are either used in protein synthesis or as precursors for nitrogen and/or a number of carbon containing molecules".

Branched-chain amino acids (BCAAs), are proteinogenic essential amino acids with aliphatic-branched side chains. In addition to their role as key building blocks for peptide synthesis, BCAAs are also important sources for the biosynthesis of sterol, ketone bodies and glucose. They account for about 21% of the total body protein content and 35% of the dietary essential amino acids in muscle proteins. BCAAs enter the blood circulation by absorption, largely escape the first-pass hepatic metabolism and appear directly in the systemic circulation. About 95–99% of all circulating BCAAs are reabsorbed in the kidney nephrons, largely through the proximal convoluted tubules (Bifari & Nisoli, 2017).

Animal studies

See also Leucine and Valine in the sections above.

In a 9-week animal study in rats, supplementation with a higher level of BCAA (150% compared with controls) in either high-sucrose or high-fat in the diets of the rats lead to a decrease in brain tryptophan levels, which could lead to neurobehavioral impairment Coppola et al., 2012).

Results from a 12-week mice study suggested that treatment of mice with BCAA induced myocardial injury by triggering excessive ROS production and by enhancing AMPK-ULK1 pathway-dependent autophagy. The findings suggested that inhibition of either ROS production or autophagy may alleviate myocardial injury induced by BCAA. The dose of BCAA added was 2% in the drinking water but no information of water intake was reported (Jiang et al., 2021).

Human studies

Most of the human studies reported are focusing on the beneficial effects of BCAA supplementation. In these study doses up to 30 g per person has been tested, with no adverse effects reported. However, in these studies very few if any safety parameters were included.

In a review of human intervention studies, it was reported that elevations in plasma levels of BCAA is associated with obesity and diabetes and strongly associated with insulin resistance. Increased levels were most likely the result of inhibition of BCAA catabolism in specific tissues (adipose and liver). Some epi studies indicate that a diet high in BCAA can be associated with circulating BCCA, but others have not found this association (Arany and Neinast, 2018).

A meta-analysis was performed to assess whether oral branched-chain amino acids (BCAA) supplementation exerts influence on circulating BCAA and the significance of dietary BCAA in type 2 diabetes and obesity risk. The outcome of this meta-analysis showed that oral BCAA supplementation modestly elevates circulating leucine and that dietary BCAA intake were positively related to Type-2-diabetes. The difference in BCAA intake between low and high users, and thereby contributing to the Type-2-diabetes, were 5-8 g BCAA per person per day (Okekunle et al., 2019).

A review systematically investigated observational studies in humans that evaluated the dietary intake of branched-chain amino acids (BCAA) and its association with insulin resistance.

The outcome revealed that the associations between BCAA and insulin resistance are inconsistent, potentially due to other longitudinal outcomes (Vieira et al., 2020).

This paper assessed associations from 3 prospective cohorts. The data obtained suggest that high consumption of BCAA is associated with an increased risk of type-2-diabetes (Zheng et al., 2016).

In a study from 2011 and therefore not included in the updated literature search, five young healthy men received a single dose of 5 g BCAA (1:2.3:1.2 of isoleucine, leucine and valine, respectively). After 3 hours, reduced plasma levels of methionine, tyrosine, tryptophan and phenylalanine was measured. In plasma, increased insulin concentration and level of free fatty acids were observed (Hang et al., 2011). The study is performed with a low number of participants and no follow-up of the parameters investigated. Despite limitations, the study is valuable in the safety assessment, especially when considering the lack of other relevant human intervention studies (Hu et al., 2017).

BCAA: conclusion on the safety evaluation

Considering the popularity of adding BCAA to different types of foods and food supplements, the lack of thorough and relevant safety studies in both animals and humans are striking.

Many of the human studies carried out with BCAA of doses up to 30 g per day are focusing on beneficial effects like increased physical performance. The studies has therefore not included parameters that could be used to evaluate the safety.

In the majority of these studies, the ratio of isoleucine, leucine and valine is approximately 1:2:1.

The study by Hang et al. (2011) showed that intake of 5 g BCAA can lead to physiological effects, but whether these in the long-term can lead to adverse effects are not known.

In the updated literature search, we have found few relevant animal studies. Information about dose levels in these studies are not very accurate, but findings indicated that high intake of BCAA can lead to lower brain tryptophan levels (rats) and potential myocardial injury (mice).

In human intervention studies, it was reported that elevated plasma levels of BCAA is associated with obesity and diabetes and strongly associated with insulin resistance. As the relation between BCAA intake and plasma levels are not clear such information can be only indicative.

However, a meta-analysis of oral BCAA supplementation showed that dietary BCAA intake were positively related to Type-2-diabetes. In these studies was the difference in BCAA intake around 5-8 g BCAA per person per day. A number of epidemiological studies came to the same positive association between high BCAA intake and increased risk of type-2-diabetes.

Even though, there seems to be strong indication for adverse effects, it is difficult based on these studies to come up with an exact safety level of the BCAA.

From the studies cited above, it seems that a moderate to high additional intake of BCAA can lead to adverse effects. As adverse effects are reported from above 5 g BCAA per person per day, this will also be the safe intake level.

In table 2 an overview of the conclusions of the safety evaluation is presented, and in Appendix 1 a comparison of existing UL in DK and the present DTU assessment of safe doses.

Table 2

Conclusion of safety evaluation based on the literature search and former VKM assessments		
	Maximum tolerated dose (mg/person/day)	Comments
Arginine	6400	Monti et al., 2012; Lucotti et al., 2009
Cysteine	900	Strand et al., 2018
Glutamic acid	14000	Shao & Hathcock (2008)
Glycine	1400	Shibui et al., 2013
Histidine	1540	Moro et al., 2020
Isoleucine	5000 (provided that Leucine and Valine is not simultaneously consumed from other supplements)	Ullrich et al., 2016
Leucine	5000 (provided that Isoleucine and Valine is not simultaneously consumed from other supplements)	Ullrich et al., 2016
Lysine	2800	Tsubuku et al., 2004b
Methionine	210	Strand et al., 2020
Phenylalanine	1568	Strand et al., 2020
Tryptophan	700	Shibui et al., 2018
Tyrosine	420	Shibui et al., 2016
Valine	No safe level could be derived	No relevant data identified that could establish a safe level
BCAA	5000	Several human studies

Daily intake of amino acids from an average Danish diet

From the DTU Foods public food database – FRIDA the average daily intake of amino acids in three age groups of Danish consumers has been calculated. The data extracted from FRIDA are based on the Danish National Survey of Dietary Habits and Physical Activity (DANSDA) in 2011-2013 with respect to food intake data and on analytical data which were latest updated in FRIDA version 4 with data published in 2019. This last update now provide the possibility to cover 98% of the protein intake in the Danish population distributed on the single amino acids.

Intake figures have been calculated for the age groups 14-17 years, 18-24 years and 25-75 years for males and females. The protein intake is 52% higher for 14-17 years males compared to females, this figure drops to 47% for the 18-24 years group, and to 32% for the 25-75 year group. The distribution of the intake of the single amino acids is comparable across all age groups and gender. In table 3 the intake of the 13 amino acids covered in this report is shown and a detailed intake distribution for all amino acids is given in Appendix 2 "Distribution of mean daily intake of amino acids in three age groups (14-17, 18-24 and 25-75) of the Danish population".

Table 3

Intake of amino acids in different age groups (mg/person per day)			
	15-17 year	18-24 year	25-75 year
Isoleucine	3737	3911	4118
Leucine	6177	6449	6779
Lysine	5432	5694	6053
Methionine	1769	1859	1978
Cystine (dimer of cysteine)	799	830	858
Phenylalanine	3525	3696	3874
Tyrosine	2705	2854	3042
Tryptophan	928	975	1021
Valine	4674	4894	5216
Arginine	3940	4293	4517
Histidine	2183	2310	2422
Glutamic acid	15028	15473	16081
Glycine	3210	3445	3619

Norwegian report on risk grouping of amino acids

The Norwegian Scientific Committee for Food Safety (VKM) has on request from the Norwegian Food Safety Authority conducted a risk categorisation of about 30 amino acids and amino acid compounds based on potential health risks related to high intakes of the amino acids.

High risk	Direct effect on organs or effect on the central nervous system or increased risk of disease development
Moderate risk	Changes in biomarkers with known negative health effects
Low risk	Changes in biomarkers without known negative health effects or no known adverse effects

Histidine, methionine and tryptophan were grouped at high risk, arginine, asparagine, leucine, isoleucine, glutamine and lysine were grouped at moderate risk, while for valine, cysteine, phenylalanine, glycine, proline, serine, threonine and tyrosine were not found scientifically documentation that one could conclude something about. risk but which is considered to be a moderate risk based on a bioactive potential. Alanine is not included in the Norwegian assessment. Subsequently, VKM has carried out an actual risk assessment of the individual amino acids in the period 2015-2017.

The risk assessment is based on literature studies and is performed for three age groups (children 10-13 years, teenagers 14 to 17 years and adults 18 years and over) and for different specific doses of the individual amino acids.

Below is given a short summary of the VKM conclusions for the 13 amino acids which will be considered in the present report.

Glycine

The Norwegian Food Safety Authority (NFSA) requested the Norwegian Scientific Committee for Food Safety (VKM) to assess the safety of glycine in food supplements at the doses 20, 50, 100, 300, 500 and 650 mg/day for the general population, ages 10 years and above. VKM concluded: No particular vulnerable groups was identified for glycine in supplements up to 650 mg/day ((Tell, Frøyland, Haugen, Henjum, et al., 2018).

Arginine

NSFA requested a risk assessment of L-arginine, which according to the information provided by NFSA is found in food supplements in the doses 3000, 3500, 4000, 4500, 5000, 5500, 6000 and 6800 mg/day. VKM concluded that for adults the high dose of 6800 mg/day may represent a risk of adverse health effects and for teenagers this would apply from the dose of 6000 mg/day and for children this would apply from the dose of 4000 mg/day.

Cysteine (VKM, 2015)

NFSA requested a risk assessment of the following doses of cysteine and cystine in food supplements: L-cysteine 10 mg/day and L-cystine 250, 500, 750 and 1000 mg/day. VKM concluded that for adults and teenagers only the dose of 1000 mg L-cystine per day would represent a risk of adverse health effects and for children this would apply for doses from 750 mg per day

Glutamine (VKM, 2016)

The NFSA requested a risk assessment of the following doses of L-glutamine in food supplements: 3500, 5000, 8000, 10000, 12000, 15000 or 16500 mg/day. VKM concluded that for all age groups doses up to 16500 mg/day L-glutamine in food supplements are considered unlikely to cause adverse health effects.

Histidine (VKM, 2016)

NSFA requested a risk assessment of 550 and 600 mg/day of L-histidine from food supplements. VKM concludes that for all age groups doses up to 600 mg/day L-histidine in food supplements are considered unlikely to cause adverse health effects.

Isoleucine, Leucine and Valine – BCAA (VKM, 2016)

NFSA requested a risk assessment of the following doses of L-leucine, L-isoleucine and L-valine in food supplements for adults, teenagers and children 10 years and above: L-leucine: 2500, 3000, 4000, 5000 or 5250 mg/day, L-isoleucine: 1500, 1750, 2000 or 2500 mg/day and L-valine: 1500, 1750, 2000, 2250 and 2500 mg/day. VKM concluded that due to lack of studies addressing adverse effects for the specified doses of the three amino acids in food

supplements, no conclusions could be made for any of the age groups (adults, teenagers or children).

Lysine (VKM, 2016)

NSFA requested a risk assessment of 1000, 2000, 2500, 2750 or 3000 mg/day of L-lysine from food supplements. VKM concluded that for all age groups doses up to 3000 mg/day L-lysine in food supplements were considered unlikely to cause adverse health effects.

Methionine (VKM, 2013)

See previous section on methionine

Phenylalanine (VKM, 2016)

NFSA requested a risk assessment of the following doses of L-phenylalanine in food supplements: 100, 250, 500, 750 or 1000 mg/day. VKM concluded that for all age groups doses up to 1000 mg/day of L-phenylalanine in food supplements are considered unlikely to cause adverse health effects – this does not apply to persons with phenylketonuria.

Tryptophan (VKM, 2016)

NFSA requested a risk assessment of the following doses of L-tryptophan in food supplements: 250, 300 or 450 mg/day for adults, teenagers and children. VKM concluded that for all age groups doses of 250 mg/day and above of L-tryptophan in food supplements may represent a risk of adverse health effects.

Tyrosine (VKM, 2016)

The NFSA requested a risk assessment of the following doses of L-tyrosine food supplements: 1250, 1500, 1750 or 2000 mg/day. VKM concluded that for all age groups doses of 1250 mg/day and above of L-tyrosine in food supplements may represent a risk of adverse health effects.

In figure 1 the VKM conclusions are presented in graphic form and in Appendix 3 an overview of the data on which the VKM conclusions are based for the individual amino acids.

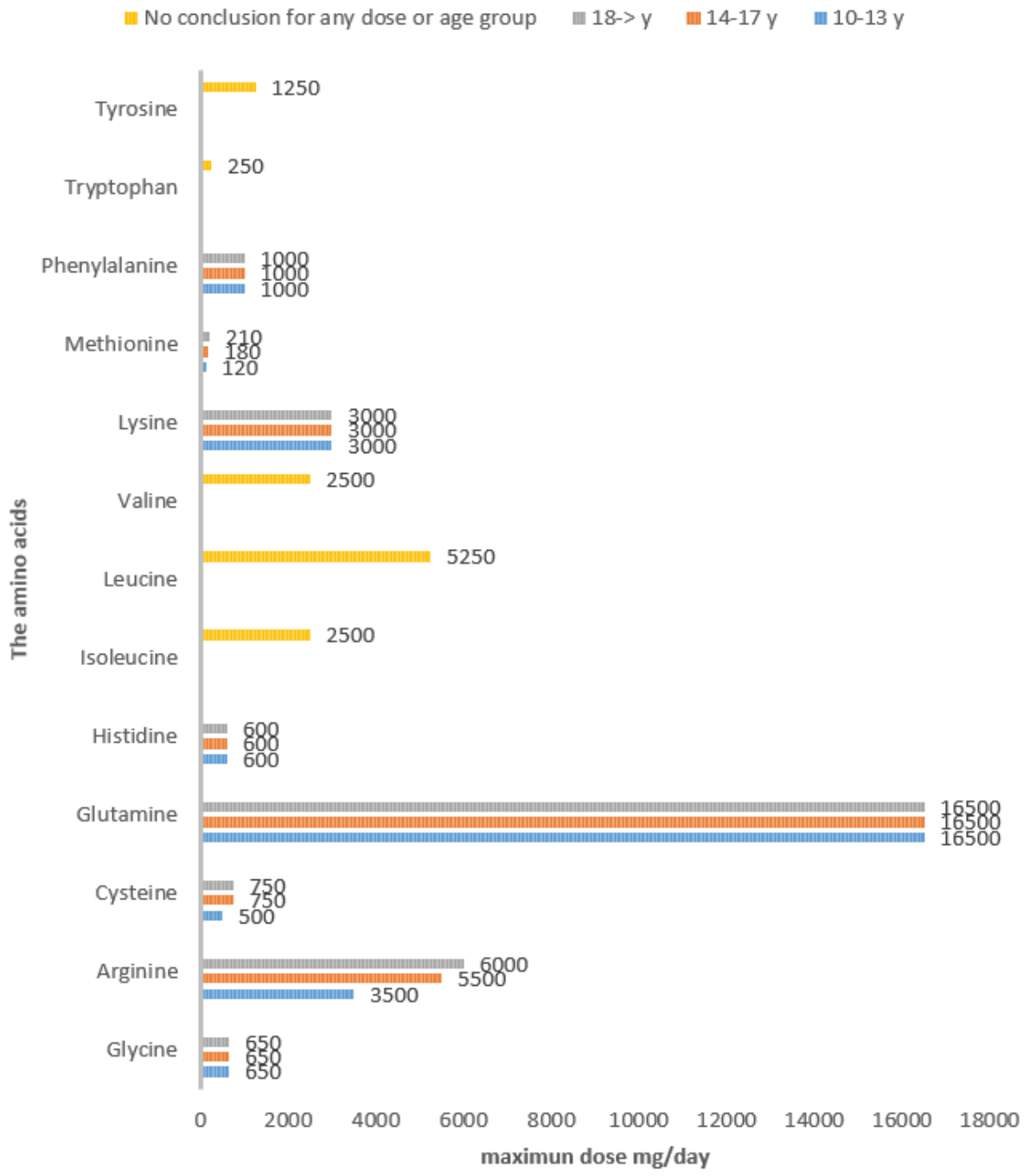
In table 4 the conclusions for the single amino acids based on the present report is compared to the conclusion given by the VKM.

Table 4

Comparison of DTU and VKM conclusions on adults for the single amino acids (mg/person per day)		
Amino acids	DTU	VKM*
Arginine	6400	6000
Cysteine	900	750
Glutamine	14000	16500
Glycine	1400	650
Histidine	1540	600
Isoleucine	5000 (provided that Leucine and Valine is not simultaneously consumed from other supplements)	No conclusion
Leucine	5000 (provided that Isoleucine and Valine is not simultaneously consumed from other supplements)	No conclusion
Lysine	2800	3000
Methionine	210	210
Phenylalanine	1568	1000
Tryptophan	700	No conclusion
Tyrosine	420	No conclusion
Valine	No safe level could be derived	No conclusion

* In the VKM conclusions on the single amino acids they have only considered the doses requested by the NFSA – this means that in cases where no concern arises from the highest dose requested – the safe dose might be higher

Figure 1 VKM conclusion of 13 amino acids for three age groups



The amino acid composition of ordinary diets and safe amounts to be added to supplements

Table 5 shows the amount of each amino acid that can be added to e.g. food supplements without giving adverse effects, and are thereby summarising the safety assessment performed in this report. In the last columns the average daily dietary intake of the different amino acids for different age groups are given. For most of the amino acids, the assessed safe level is slightly below or comparable to the average daily dietary intake. One exception is methionine where average daily intake is almost a factor 10 higher that the level assessed as safe.

Table 5

	DTU conclusion[1]	Average daily dietary intake[2]		
	mg/person per day	mg/person per day		
	Adults	15-17 year	18-24 Years	25-75 years
Leucine	5000[3]	6.177	6.449	6779
Isoleucine	5000[4]	3.737	3.911	4118
Valine	No conclusion	4.674	4.894	5216
Arginine	6400	3.940	4.293	4517
Cysteine and cystine	900	799 (cystine)	830 (cystine)	858 (cystine)
Histidine	1540	2.183	2.310	2422
Lysine	2800	5.432	5.694	6053
Methionine	210	1.769	1.859	1978
Phenylalanine	1568	3.525	3.696	3874
Tryptophan	700	928	975	1021

1) Safe amount of the amino acid to be consumed via addition to e.g. food supplements for an adult

2) The average daily intake of the amino acid via a normal diet for different age groups (analytical composition of food from FRIDA - DTU Foods public food database (up to 2019))

3) Provided that Leucine and Valine is not simultaneously consumed from other supplements

4) Provided that Isoleucine and Valine is not simultaneously consumed from other supplements

Consequence of different amino acid composition in ordinary diet and fortified foods

One emerging question is whether administration of high amounts of single amino acid in e.g. food supplements will have a different health impact than the same amount of the specific amino acid ingested via a variety of foods during the day. Information that could help clarify this are only addressed in a few number of publication found in the updated literature search, and often in animal studies performed with different study design. It is therefore not possible to provide an answer that could clarify this issue about bioavailability of amino acids in different dosing regimen. From a theoretically point of view a high load of amino acids in a single product could both lead to more and less adverse health effects. As a default in future risk assessments, we will therefore assume that a daily high amount of a single amino acid in e.g. food supplements will have the same health impact than the same amount of the specific amino acid ingested via a variety of foods during the day.

Conclusion

Based on literature search, updated safe intake levels could be established for 12 of the 13 amino acids evaluated in the present report. The estimated safe levels are presented in Table 2. Only for valine a safe level could not be established. A safe level of the sum of the three branched chained amino acids valine, leucine and isoleucine was estimated to 5000 mg/person per day. When comparing the present UL for adding amino acids per daily dose in food supplements and others in DK (Appendix 1 Executive Order no. 1342 of 28/11/2018) a new safe dose could be established for all the amino acids in question except for cysteine which is at the same level (see Appendix 1). A comparison between the DTU conclusions and the conclusions from the Norwegian VKM reports is presented in Table 4. Table 5 gives an overview of the new safe doses of amino acids established in this report along with the average intake via the food in DK of the same amino acids for different age groups.

Uncertainty evaluation

Regarding animal data and safety of amino acids there were no chronic studies available and only a few sub-chronic studies. This can lead to higher uncertainty when assessing the safety, as the uncertainty in risk assessments is generally lower if data from an equal quality chronic study is used, compared to data from a sub-chronic (90 days) or subacute (28 days) study. Moreover, most of the studies does not have focus on toxicological effects, but more on the beneficial, protective and feeding effects of amino acids.

The main source of uncertainty regarding the human studies is that almost none of the human studies are performed with the purpose to assess the safety for longer-term use. Instead, the human studies mainly focus on the beneficial aspect and efficacy of the administered doses of amino acids. In some of the studies, a few or medium number of parameters are included that could be of relevance in the safety assessment. The uncertainty arise, when we based on a limited number of parameters should determine whether it represent an overall long-term safe intake level. Also, responsible for the uncertainty in the assessment is the fact that many of the

reported studies are performed in a low number of subjects, is of short duration and sometimes include patient groups.

The uncertainty on the safe levels for the different amino acids proposed in this report cannot be quantified according to the methodology proposed by EFSA due to the limited number of studies and data (Benford et al., 2018). Overall, the uncertainty can only be described qualitatively and is in this report estimated to be medium to high.

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Appendix 1

Comparison of existing UL in DK and the DTU assessment of safe doses		
	Generally accepted UL for adding amino acids per daily dose in DK (Appendix 1 Executive Order no. 1342 of 28/11/2018)	DTU assessment of safe dose of the single amino acids (January 2021)
Amino Acid	Supplements per recommended daily dose (mg/person/day)	Maximum tolerated dose as supplement (mg/person/day)
Glycine	Total amount of maximum 30	1400
L-Arginine	Total amount of maximum 5000	6400
L-Cystine	Total amount of maximum 500	900 (cysteine)
L-Glutamine	Total amount of maximum 3500	14000
L-Histidine	Total amount of maximum 56	1540
L-Isoleucine	Total amount of 1500	5000 (provided that Leucine and Valine is not simultaneously consumed from other supplements)
L-Leucine	Total amount of maximum 1300	5000 (provided that Isoleucine and Valine is not simultaneously consumed from other supplements)
L-Lysine	Total amount of maximum 500	2800
L-Methionine	Total amount of maximum 125	210
L-Phenylalanine	Total amount of maximum 50	1568
L-Tryptophan	Total amount of maximum 500	700
L-Tyrosine	Total amount of maximum 50	140
L-Valine	Total amount of maximum 1500	No safe level could be derived

Appendix 2 Distribution of mean daily intake of amino acids in three age groups (14-17, 18-24 and 25-75) of the Danish population

Distribution of mean daily intake of amino acids of the Danish population (mg/person/day)*			
	15-17 year	18-24 year	25-75 year
Isoleucine	3.737	3.911	4.118
Leucine	6.177	6.449	6.779
Lysine	5.432	5.694	6.053
Methionine	1.769	1.859	1.978
Cystine	799	830	858
Phenylalanine	3.525	3.696	3.874
Tyrosine	2.705	2.854	3.042
Threonine	2.974	3.124	3.294
Tryptophan	928	975	1.021
Valine	4.674	4.894	5.216
Arginine	3.940	4.293	4.517
Histidine	2.183	2.310	2.422
Alanine	3.625	3.879	4.101
Asparagic acid	6.498	6.922	7.365
Glutamic acid	15.028	15.473	16.081
Glycine	3.210	3.445	3.619
Proline	5.649	5.785	6.032
Serine	3.776	3.964	4.159
sum AA	76.629	80.355	84.530

* Data based on the “Den danske kostundersøgelse 2011-2013” and “analytical composition of food from FRIDA - DTU Foods public food database (up to 2019)”

Distribution of mean daily intake of amino acids for males and females of the Danish population (mg/person/day)*						
	Men 15-17	Women 15-17	Men 18-24	Women 18-24	Men 25-75	Women 25-75
Isoleucine	4.572	2.995	4.686	3.162	4.707	3.564
Leucine	7.544	4.960	7.713	5.228	7.741	5.874
Lysine	6.697	4.306	6.902	4.527	6.962	5.198
Methionine	2.170	1.412	2.232	1.498	2.266	1.707
Cystine	975	643	986	680	982	742
Phenylalanine	4.291	2.843	4.398	3.018	4.414	3.367
Tyrosine	3.299	2.176	3.402	2.324	3.452	2.656
Threonine	3.649	2.372	3.763	2.506	3.784	2.834
Tryptophan	1.129	749	1.163	792	1.168	883
Valine	5.645	3.810	5.807	4.011	5.924	4.551
Arginine	4.834	3.143	5.163	3.451	5.160	3.913
Histidine	2.690	1.732	2.794	1.843	2.787	2.078

Distribution of mean daily intake of amino acids for males and females of the Danish population (mg/person/day)*						
	Men 15-17	Women 15-17	Men 18-24	Women 18-24	Men 25-75	Women 25-75
Alanine	4.472	2.871	4.695	3.090	4.737	3.502
Aspartic acid	7.935	5.219	8.297	5.595	8.432	6.361
Glutamic acid	18.201	12.204	18.291	12.751	18.294	14.001
Glycine	3.980	2.525	4.202	2.714	4.205	3.067
Proline	6.823	4.604	6.832	4.775	6.842	5.271
Serine	4.596	3.045	4.710	3.243	4.729	3.624
Sum AA	93.502	61.610	96.037	65.208	96.586	73.194

* Data based on “Danskernes kostvaner 2011-2013” and “analytical composition of food from FRIDA - DTU Foods public food database (up to 2019)”

Appendix 3

VKM Risk assessment of amino acids, 2015-2017					
	Doses mg/day	Children 10-13	Teenagers 14-17	Adults ≥18	reference
Glycine	20, 50, 100, 300, 500, 650	unlikely to cause adverse health effects	unlikely to cause adverse health effects	unlikely to cause adverse health effects	Shibui Y., Miwa T., Yamashita M., Chin K., Kodama T. (2013) A 4-week Repeated Dose Toxicity Study of Glycine in Rats by Gavage Administration. J Toxicol Pathol 26:405-12. DOI: 10.1293/tox.2013-0026
Alanine	3500, 3750, 4000, 4250, 4500	unlikely to cause adverse health effects	unlikely to cause adverse health effects	unlikely to cause adverse health effects	Chow F.C., Dysart M.I., Hamar D.W., Lewis L.D., Udall R.H. (1976) Alanine: a toxicity study. Toxicol Appl Pharmacol 37:491-7
Arginine	3000, 3500, 4000, 4500, 5000, 5500, 6000, 6800	the doses 4000, 4500, 5000, 5500, 6000 and 6800 mg/day may represent a risk of adverse health effects	the doses 6000 and 6800 mg/day may represent a risk of adverse health effects	the dose 6800 mg/day may represent a risk of adverse health effects	Monti et al., 2012. Effect of a long-term oral l-arginine supplementation on glucose metabolism: a randomized, double-blind, placebo-controlled trial. Diabetes Obes Metab 14:893-900. DOI: 10.1111/j.1463-1326.2012.01615.x Lucotti et al., 2009. Oral L-arginine supplementation improves endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass. Metabolism 58:1270-6. DOI: 10.1016/j.metabol.2009.03.029

VKM Risk assessment of amino acids, 2015-2017					
	Doses mg/day	Children 10-13	Teenagers 14-17	Adults ≥18	reference
Aspartic acid	3000, 3500, 4000, 4500, 5000, 5700	All doses may represent a risk of adverse health effects	All doses may represent a risk of adverse health effects	All doses may represent a risk of adverse health effects	Tada et al., 2008. Toxic effects 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 Chem Toxicol 46:2789-95. DOI: 10.1016/j.fct.2008.05.013
Cysteine	10	unlikely to cause adverse health effects	unlikely to cause adverse health effects	unlikely to cause adverse health effects	Several studies with different population groups
Glutamine	3500, 5000, 8000, 10000, 12000, 15000, 16500	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	Wong et al., 2011 Oral subchronic and genotoxicity studies conducted with the amino acid, L-glutamine. Food Chem Toxicol 49:2096-102. DOI: 10.1016/j.fct.2011.05.023
Glutamic acid	1000, 2000, 3000, 4000, 5000, 5500	doses of 5000 and 5500 mg/day may represent a risk of adverse health effects	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	Harper et al., 2009. N-acetyl-glutamic acid: evaluation of acute and 28-day repeated dose oral toxicity and genotoxicity. Food Chem Toxicol 47:2723-9. DOI: 10.1016/j.fct.2009.07.036. Test substance: N-acetyl-glutamic acid

VKM Risk assessment of amino acids, 2015-2017					
	Doses mg/day	Children 10-13	Teenagers 14-17	Adults ≥18	reference
Histidine	550, 600	Both doses are unlikely to cause adverse health effects	Both doses are unlikely to cause adverse health effects	Both doses are unlikely to cause adverse health effects	Feng et al., 2013. Histidine supplementation improves insulin resistance through suppressed inflammation in obese women with the metabolic syndrome: a randomised controlled trial. Diabetologia 56:985-94. DOI: 10.1007/s00125-013-2839-7. Blumenkrantz et al., 1975. Histidine supplementation for treatment of anaemia of uraemia. Br Med J 2:530-3
Isoleucine	1500, 1750, 2000, 2500	No data available	No data available	No data available	
Leucine	2500, 3000, 4000, 5000, 5250	No data available	No data available	No data available	
Valine	1500, 1750, 2000, 2250, 2500	No data available	No data available	No data available	

VKM Risk assessment of amino acids, 2015-2017					
	Doses mg/day	Children 10-13	Teenagers 14-17	Adults ≥18	reference
Lysine	1000, 2000, 2500, 2750, 3000	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	Wass et al., 2011. L-lysine as adjunctive treatment in patients with schizophrenia: a single-blinded, randomized, cross-over pilot study. BMC Medicine 9:40 Zeinoddini et al., 2014. L-lysine as an adjunct to risperidone in patients with chronic schizophrenia: A double-blind, placebo-controlled, randomized trial. Journal of Psychiatric Research 59:125-131
Methionine	200, 300, 500, 600, 700	No new data available	No new data available	No new data available	VKM maintains the guidance level from 2013 at 210 mg methione per day
Phenylalanine	100, 250, 500, 750, 1000	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	EFSA (2013) opinion for aspartame
Proline	50, 500, 1000, 1500, 1800	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	Tada et al., 2010. Toxicological evaluation of L-proline in a 90-day feeding study with Fischer 344 rats. Regul Toxicol Pharmacol 58:114-20. DOI: 10.1016/j.yrtph.2010.04.011

VKM Risk assessment of amino acids, 2015-2017					
	Doses mg/day	Children 10-13	Teenagers 14-17	Adults ≥18	reference
Serine	50, 500, 1000, 1250, 1500, 1750	All doses considered unlikely to cause adverse health effects NB doses of 50, 500, 1000, 1500, 1800 mg/day in the conclusion	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	Kaneko et al., 2009. A 13-week subchronic oral toxicity study of L-serine in rats. Food Chem Toxicol 47:2356-60. DOI: 10.1016/j.fct.2009.06.030
Threonine	1000, 1200, 1500, 2000, 2400 mg/day	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	All doses considered unlikely to cause adverse health effects	
Tryptophan	250, 300 or 450 mg/day	All doses may represent a risk of adverse health effects	All doses may represent a risk of adverse health effects	All doses may represent a risk of adverse health effects	Food, Consumer Products and the Environment (COT) in 2004 – dose of up to 2228 mg/day as antidepressive drug
Tyrosine	1250, 1500, 1750 or 2000 mg/day	All doses may represent a risk of adverse health effects	All doses may represent a risk of adverse health effects	All doses may represent a risk of adverse health effects	Shibui Y., Manabe Y., Kodama T., Gonsho A. (2016) 13-week repeated dose toxicity study of l-tyrosine in rats by daily oral administration. Food Chem Toxicol 87:55-64. DOI: 10.1016/j.fct.2015.11.017.