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Risk assessment of "other substances" – L-Citrulline

Opinion of the Panel Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics of the Norwegian Scientific Committee for Food Safety

Report from the Norwegian Scientific Committee for Food Safety (VKM) 2016: 66
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(Panel members in alphabetical order after chair of the panel)

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Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

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Summary

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has, at the request of the Norwegian Food Safety Authority (Mattilsynet; NFSA), assessed the risk of "other substances" in food supplements and energy drinks sold in Norway. VKM has assessed the risk of doses in food supplements and concentrations in energy drinks given by NFSA. These risk assessments will provide NFSA with the scientific basis while regulating the addition of "other substances" to food supplements and other foods.

"Other substances" are described in the food supplement directive 2002/46/EC *as substances other than vitamins or minerals that have a nutritional and/or physiological effect*. It is added mainly to food supplements, but also to energy drinks and other foods. VKM has not in this series of risk assessments of "other substances" evaluated any claimed beneficial effects from these substances, only possible adverse effects.

The present report is a risk assessment of L-citrulline, and it is based on a previous risk assessment and articles retrieved from a literature search.

According to information from NFSA, L-citrulline is an ingredient in food supplements sold in Norway. NFSA has requested a risk assessment of 1000, 1500 and 2000 mg/day of L-citrulline in food supplements. The intake of L-citrulline was estimated for the age groups children (10 to <14 years), adolescents (14 to <18 years) and adults (≥ 18 years).

Other sources of L-citrulline, such as foods and cosmetics, have not been included in the present risk assessment.

The natural isoform of citrulline is the L-form. In mammals, it is found in all organisms and tissues. L-Citrulline is not part of the amino acids that are incorporated into proteins by the standard genetic code; therefore it is classified as a non-protein amino acid. Thus, its presence in a protein always results from a post-translational modification of the protein.

L-citrulline is found in high levels in certain Cucurbitacea, including watermelon, cucumber, pumpkin and courgette, and in certain algae such as *Grateloupia vulgaris*. It is also present in fish, meat, pulses and milk, and in vegetables such as onions and garlic.

Following oral intake of L-citrulline, plasma L-citrulline concentration increases rapidly but returns to baseline values within 5-8 hours post-exposure. There are three interconnected metabolic pathways for L-citrulline: 1) arginine biosynthesis, 2) nitric oxide (NO) cycle, and 3) the complete urea cycle. Renal L-citrulline reabsorption appears very efficient because urinary loss is very low even at high (up to 15 g) L-citrulline intake.

No adverse health effects of L-citrulline were observed in six human studies covering the ages 12 months to 56 years, with L-citrulline exposure lengths varying from less than one day (acute doses) to 2 years. The doses varied from 2.1-179 mg/kg bw per day for children

(<14 years), 1.5-175 mg/kg bw per day for adolescents (14 to <18 years) and 21-214 mg/kg bw per day in adults. The human studies available had low number of participants and, with exception of one study, included non-healthy populations. In a 2-year study by Rajantie et al. (1980), 19 patients with lysinuric protein intolerance, ages 1.9-32.7 years, were included. No adverse effects were reported from daily intakes of 65 mg/kg bw in children (10 to <14 years), 46 mg/kg bw in adolescents (14 to <18 years) and 40 mg/kg bw in adults (highest doses applied). These age-specific reference points were used for comparisons with the estimated exposures in the risk characterization.

For children, from a daily intake of 1000, 1500 and 2000 mg the estimated exposures are 23.0, 34.6 and 46.1 mg/kg bw per day, respectively. These intake values are below 65 mg/kg bw per day. VKM therefore considers it unlikely that a daily intake of 1000, 1500 or 2000 mg L-citrulline from food supplements causes adverse health effects in children (10 to <14 years).

For adolescents, from a daily intake of 1000, 1500 and 2000 mg the estimated exposures are 16.3, 24.5 and 32.6 mg/kg bw per day, respectively. These intake values are below 46 mg/kg bw per day. VKM therefore considers it unlikely that a daily intake of 1000, 1500 or 2000 mg L-citrulline from food supplements causes adverse health effects adolescents (14 to <18 years).

For adults, from a daily intake of 1000, 1500 and 2000 mg the estimated exposures are 14.3, 21.4 and 28.6 mg/kg bw per day, respectively. These intake values are below 40 mg/kg bw per day. VKM therefore considers it unlikely that a daily intake of 1000, 1500 or 2000 mg L-citrulline from food supplements causes adverse health effects in adults (\geq 18 years).

Since LPI patients have a different intestinal absorption, renal reabsorption and reduced intracellular efflux of cationic amino acids compared to healthy individuals, it is uncertain whether doses given to LPI patients can be directly extrapolated to healthy individuals.

Persons with citrullinemia caused by mutations in enzymes involved in citrulline metabolism are potentially vulnerable to intake of additional L-citrulline from supplements. In addition, humans with chronic renal failure and/or mutations in renal citrulline transporters are potentially vulnerable to supplementation of L-citrulline.

Short summary

According to information from NFSA, L-citrulline is an ingredient in food supplements sold in Norway. NFSA has requested a risk assessment of 1000, 1500 and 2000 mg/day of L-citrulline in food supplements. The values used for comparison with the estimated exposures in the risk characterization are 65 mg/kg bw per day for children (10 to <14 years), 46 mg/kg bw per day for adolescents (14 to <18 years), and 40 mg/kg bw per day for adults (\geq 18 years) based on a human study.

VKM concludes that it is unlikely that daily doses of 1000, 1500 and 2000 mg L-citrulline in food supplements causes adverse health effects in children (10 to <14 years), adolescents (14 to <18 years) and adults (\geq 18 years).

Key words: Adverse health effect, L-citrulline, food supplement, negative health effect, Norwegian Food Safety Authority, Norwegian Scientific Committee for Food Safety, other substances, risk assessment, VKM

Sammendrag på norsk

På oppdrag for Mattilsynet har Vitenskapskomiteen for mattrygghet (VKM) vurdert risiko ved tilsetning av «andre stoffer» i kosttilskudd og energidrikk som selges i Norge. VKM har risikovurdert ulike doser brukt i kosttilskudd. Disse risikovurderingene vil gi Mattilsynet vitenskapelig grunnlag for å regulere andre stoffer.

«Andre stoffer» er beskrevet i kosttilskuddsdirektivet 2002/46/EC som *stoffer som har en ernæringsmessig og/eller fysiologisk effekt, og som ikke er vitaminer og mineraler*. De tilsettes i hovedsak til kosttilskudd, men også til energidrikker og andre næringsmidler. I disse risikovurderingene har VKM ikke sett på påståtte gunstige helseeffekter, men kun vurdert mulige negative helseeffekter.

Denne rapporten er en risikovurdering av L-citrullin, og den er basert på en tidligere risikovurdering og artikler hentet fra et litteratursøk.

I følge informasjon fra Mattilsynet er L-citrullin en ingrediens i kosttilskudd som selges i Norge. Oppdraget fra Mattilsynet var å risikovurdere inntak på 1000, 1500 og 2000 mg/dag av L-citrullin i kosttilskudd. Inntaket av L-citrullin ble beregnet for aldersgruppene barn (10 til <14 år), ungdom (14 til <18 år) og voksne (≥ 18 år).

Andre kilder til L-citrullin, som mat og kosmetikk, er ikke inkludert i denne risikovurderingen.

Den naturlige isoformen av citrullin er L-formen. L-citrullin har flere funksjoner i pattedyr, og finnes i alle organismer og alle vev. L-citrullin er ikke en av aminosyrene som inngår i proteinsyntesen, og den er derfor klassifisert som en ikke-protein aminosyre. Når L-citrullin inngår i et protein er det derfor alltid et resultat av post-translasjonell modifikasjon.

Det er høye nivåer av L-citrullin i Cucurbitacea, inkludert vannmelon, agurk, gresskar og squash, og i noen alger, slik som *Grateloupia vulgaris*. L-citrullin finnes også i fisk, kjøtt, belgfrukter og melk, og i grønnsaker som løk og hvitløk.

Plasmanivået av L-citrullin øker raskt etter oralt inntak, men synker til bakgrunnsnivå innen 5-8 timer etter inntaket. L-citrullin tas opp fra tarmen. L-citrullin metaboliseres via tre veier som er innbyrdes forbundet: 1) argininbiosyntese, 2) nitrogenoksidcyklus og 3) ureasyklus. Utskillelse av L-citrullin med urinen er veldig lav, noe som viser at reabsorpsjonen i nyrene er meget effektiv.

Ingen negative helseeffekter av L-citrullin ble observert i seks humane studier som dekket aldersspennet 12 måneder til 56 år, og med eksponeringslengder som varierte fra under en dag (akutte doser) til 2 år. Dosene var 2.1-179 mg/kg kroppsvekt per dag for barn (<14 år), 1.5-175 mg/kg kroppsvekt per dag for ungdom (14 til <18 år), og 21-214 mg/kg kroppsvekt per dag for voksne. Studiene hadde få deltakere og, med unntak av en studie, inkluderte de pasientgrupper, ikke friske individer. I en studie av Rajantie et al. (1980) (19 pasienter med lysinurisk proteinintoleranse i alderen 1,9 – 32,7 år, 2 års behandling), ble det rapportert at daglig inntak av 65 mg/kg kroppsvekt for barn (10 til <14 år), 46 mg/kg kroppsvekt for

ungdom (14 til <18 år) og 40 mg/kg kroppsvekt for voksne (høyeste doser gitt) ikke resulterte i negative helseeffekter.

Inntaksverdiene brukt til sammenligning med de estimerte daglige inntakene i risikovurderingen er 65 mg/kg kroppsvekt for barn (10 til <14 år), 46 mg/kg kroppsvekt for ungdom (14 til <18 år) og 40 mg/kg kroppsvekt for voksne (≥ 18 år) (høyeste dose gitt). Disse verdiene er basert på studien av Rajantie et al. (1980).

For barn (10 til <14 år) var det estimerte daglige inntaket fra doser på 1000, 1500 og 2000 mg per dag av L-citrullin henholdsvis 23,0, 34,6 og 46,1 mg/kg kroppsvekt per dag. Det estimerte inntaket er lavere enn 65 mg/kg kroppsvekt per dag. VKM konkluderer derfor at det er usannsynlig at et daglig inntak av 1000, 1500 eller 2000 mg L-citrullin vil representere en risiko for negative helseeffekter hos barn (10 til <14 år).

For ungdom (14 til <18 år) var det estimerte daglige inntaket fra doser på 1000, 1500 og 2000 mg per dag av L-citrullin henholdsvis 16,3, 24,5 og 32,6 mg/dag. Det estimerte inntaket er lavere enn 46 mg/kg kroppsvekt per dag. VKM konkluderer derfor at det er usannsynlig at et daglig inntak av 1000, 1500 eller 2000 mg L-citrullin vil representere en risiko for negative helseeffekter hos ungdom (14 til <18 år).

For voksne (≥ 18 år) var det estimerte daglige inntaket fra doser på 1000, 1500 og 2000 mg per dag av L-citrullin henholdsvis 14,3, 21,4 og 28,6 mg/dag. Det estimerte inntaket er lavere enn 40 mg/kg kroppsvekt per dag. VKM konkluderer derfor at det er usannsynlig at et daglig inntak av 1000, 1500 eller 2000 mg L-citrullin vil representere en risiko for negative helseeffekter hos voksne (≥ 18 år).

Siden LPI-pasienter har forskjeller i absorpsjon i tarm, reabsorpsjon i nyrene og redusert intracellulær utskillelse av kationske aminosyrer sammenlignet med friske personer, er det en usikkerhet når det gjelder om dosene gitt til LPI-pasienter kan ekstrapoleres direkte til friske personer.

Personer med forhøyet citrullin-nivå i blodet på grunn av mutasjoner i enzymer som er involverte i citrullin-metabolisme er potensielt sårbare for inntak av ekstra mengder av L-citrullin via kosttilskudd. I tillegg vil personer med kronisk nyresvikt og/eller mutasjoner i transport-proteiner for citrullin i nyrene potensielt være sårbare for inntak av ekstra mengder av L-citrullin via kosttilskudd

Kort sammendrag

I følge informasjon fra Mattilsynet er L-citrullin en ingrediens i kosttilskudd som selges i Norge. Oppdraget fra Mattilsynet var å risikovurdere inntak på 1000, 1500 og 2000 mg/dag av L-citrullin i kosttilskudd. Verdiene som ble sammenlignet med den estimerte eksponeringen i risikokarakteriseringen var 65 mg/kg kroppsvekt for barn (10 til <14 år), 46 mg/kg kroppsvekt for ungdom (14 til <18 år) og 40 mg/kg kroppsvekt for voksne, basert på en human studie.

VKM konkluderer at det er usannsynlig at et daglig inntak av 1000, 1500 eller 2000 mg L-citrullin vil representere en risiko for negative helseeffekter hos barn (10 til <14 år), ungdom (14 til <18 år) eller voksne (≥ 18 år).

Abbreviations and glossary

Abbreviations

AECOSAN	- Spanish Agency for Consumer Affairs, Food Safety and Nutrition
ASL	- arginosuccinate lysate
ASS	- arginosuccinate synthase
bw	- body weight
C _{max}	- maximum concentration
L-NAME	- L-N ^G -nitro-arginine-methyl ester
LOAEL	- lowest observed adverse effect level
LPI	- lysinuric protein intolerance
NFSA	- Norwegian Food Safety Authority [<i>Norw.</i> : Mattilsynet]
NO	- nitric oxide
NOAEL	- no observed adverse effect level
NOS	- nitric oxide synthase
OCT	- ornithine carbamoyltransferase
OSL	- observed safe level
T _{max}	- time of maximum concentration
VKM	- Norwegian Scientific Committee for Food Safety [<i>Norw.</i> : Vitenskapskomiteen for Mattrygghet]
WHO	- World Health Organization

Glossary

"Other substances": a substance other than a vitamin or mineral that has a nutritional or physiological effect (The European Parliament and the Council of the European Union, 2006).

"Negative health effect" and "adverse health effect" are broad terms. VKM uses the definition established by EFSA for "adverse effect": a change in morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences (EFSA, 2006; WHO, 1994).

Background as provided by the Norwegian Food Safety Authority

«Other substances» are substances other than vitamins and minerals, with a nutritional and/or physiological effect on the body. "Other substances" are mainly added to food supplements, but these may also be added to other foods and beverages, such as sports products and energy drinks. Ingestion of these substances in high amounts presents a potential risk for consumers.

In Norway, a former practice of classification of medicines had constituted an effective barrier against the sale of potentially harmful "other substances". Ever since this practice was changed in 2009, it has become challenging to regulate and supervise foods with added "other substances". Meanwhile, in the recent years, the Norwegian market has witnessed a marked growth in the sales of products containing "other substances". In 2011, food supplements containing "other substances" constituted more than 50% of the market share.

While within the European Economic Area, these substances fall under the scope of the European Regulation (EC) No. 1925/2006 on the addition of vitamins, minerals and certain other substances to foods and the European Regulation (EC) No 258/97 concerning novel foods and novel food ingredients, "other substances" remain largely unregulated. In order to ensure safe use of "other substances" many countries have regulated their use at a national level. For example, Denmark regulates these substances in a positive list, i.e. a list of substances with maximal daily doses, permitted for use in food supplements and other foods (FVM, 2014).

The Norwegian Food Safety Authority (NFSA) is working on the establishment of a regulation on the addition of "other substances" to foods at a national level. The regulation will include a list of substances with permitted maximal doses, based on the substances and doses found in products on the Norwegian market. In preparation for a regulation, NFSA has therefore requested the Norwegian Scientific Committee for Food Safety (VKM) to assess the safety of "other substances" found on the Norwegian market. NFSA, in consultation with the industry, has compiled a list of "other substances" found in products marketed in Norway. Only substances with a purity of minimum 50% or concentrated 40 times or more have been included in the list. Substances regulated by other legislations like those for novel foods, food additives, flavourings, foods for special medical purposes etc., have been excluded from the list.

Terms of reference as provided by the Norwegian Food Safety Authority

The Norwegian Food Safety Authority (NFSA) has requested the Norwegian Scientific Committee for Food Safety (VKM) to assess the safety of L-citrulline in food supplements at the following doses: 1000, 1500 and 2000 mg/day.

NFSA requested VKM to assess the safety of "other substances" (in accordance to the guidance document developed in Phase 2) at the doses specified (Phase 3). The safety assessments of "other substances" present in food supplements shall be carried out for the general population, ages 10 years and above.

Assessment

1 Introduction

"Other substances" are described in the food supplement directive 2002/46/EC *as substances other than vitamins or minerals that have a nutritional and/or physiological effect*, and may be added to food supplements or e.g. energy drinks.

This risk assessment regards the substance L-citrulline *per se*, and no specific products.

VKM has in this series of risk assessments of "other substances" not evaluated documentation of any claimed beneficial effects from these substances, but merely possible adverse effects at specified doses used in Norway. Thus, potential high intake consumer groups of the substance may not be identified and therefore not included in this assessment.

According to information from the Norwegian Food Safety Authority (NFSA), L-citrulline is an ingredient in food supplements sold in Norway. NFSA has requested a risk assessment of the intake of 1000, 1500 and 2000 mg L-citrulline per day from food supplements. The total exposure to L-citrulline from other sources than food supplements, such as foods and cosmetic products, is not included in the risk assessment.

The natural isoform of citrulline is the L-form. Its basic function is detoxification of ammonia through conversion via the urea cycle. However, L-citrulline also have other functions such as a key role in the regulation of nitrogen homeostasis, a central role in the cardiovascular system and as regulator of immunity (summarised in Breuillard et al. (2015)). L-citrulline can be found in all organisms and tissues (Curis et al., 2005). L-citrulline is not part of the 22 amino acids that are translated into proteins by the standard genetic code; therefore it is classified as a non-protein amino acid (Pizzarello, 2011). Thus, its presence in a protein is due to a post-translational modification of the protein.

In mammals, L-citrulline has several functions and can be found in all organisms and tissues. Concentrations of L-citrulline in human plasma are 12-39 and 20-50 $\mu\text{mol/l}$ in children and adults, respectively. In humans, the mean levels of L-citrulline \pm standard deviation in muscles, cerebrospinal fluid and urine have been reported to be 170 ± 13 , 1.5 ± 21.6 and 4 ± 4 $\mu\text{mol/l}$, respectively. The level in brain has been reported to be 0.02 ± 0.1 $\mu\text{mol/g}$ (Curis et al., 2005).

Following oral intake of L-citrulline, plasma L-citrulline concentration increases rapidly but returns to baseline values within 5-8 hours post-exposure. There are three interconnected metabolic pathways for L-citrulline: 1) arginine biosynthesis, which involves citrulline exchange at whole body level, 2) nitric oxide (NO) cycle, which can involve local recycling of L-citrulline, and 3) the complete urea cycle, which takes place in the liver. The conversion of

L-citrulline into arginine is important in nitrogen homeostasis in the body in that there is an inter-organ cycle of arginine-citrulline-arginine which can be seen as a mechanism for protecting dietary arginine from excessive liver degradation and thus maintaining protein homeostasis.

No data on dietary intake values of L-citrulline has been found.

2 Hazard identification and characterisation

The present risk assessment is based on one previous risk assessment and articles retrieved from a literature search.

In some studies, the authors have not defined citrulline as being either L-citrulline or D-citrulline, and the term citrulline is then used in the present risk assessment.

2.1 Literature

2.1.1 Previous risk assessments

Report of the Scientific Committee of the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AESAN) on the condition of use of certain substances to be used in food supplements-4 (AECOSAN, 2015)

In 2015, AECOSAN performed a risk assessment of L-citrulline used in food supplements and the conclusion was: *“No data has been found in the scientific bibliography of adverse effects or clinical alterations due to the oral intake of L-citrulline, therefore it is not possible to establish a NOAEL/LOAEL for its oral administration. In 2012, AECOSAN recommended a maximum daily intake of 3 g for L-arginine which was considered acceptable by the Scientific Committee given that there is an Observed Safe Level (OSL) of 20 g/day. Given that L-citrulline is a precursor of this, the same value of 3 g is acceptable from the safety point of view for use as a food supplement, as studies of supplementation exist with higher long-term doses that do not give signs of toxicity.”*

Since both human and animal studies reporting on adverse effects were found in VKM's literature search (presented in section 2.4), VKM did not use the L-arginine-based maximum daily intake value of L-citrulline determined by AECOSAN in the present risk assessment.

2.1.2 Literature search

Literature searches were performed in Medline and Embase in order to retrieve publications on adverse effects caused by L-citrulline. These databases were chosen to ensure comprehensive study retrieval. The literature searches were performed December 2015. The search strategy is included in Appendix 1.

2.1.2.1 Publication selection and data extraction

The literature search identified 124 articles. In the primary screening, titles and abstracts of all publications retrieved were independently screened against the inclusion criteria checklist.

Inclusion criteria checklist:

- Adverse effects in relation to the substance alone are addressed
- Route of exposure for humans is oral
- Route of exposure for animals is oral, in addition, subcutaneous exposure is included if the toxicokinetic is equal to oral exposure
- Human studies are performed in apparently healthy individuals or patient groups assumed to have normal absorption and metabolism of the assessed substance
- Animal model studies that address adverse effects relevant to human health

The inclusion criteria checklist was developed by members of the Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics and the Panel on Nutrition, Dietetic Products, Novel Food and Allergy. Articles that did not appear to meet the inclusion criteria were excluded from further analysis. In situations where it was unclear whether the publication was of relevance to the study, it was retained for further screening. The primary screening was performed independently by two persons.

The full text of articles that passed the primary screening was retrieved for secondary screening. In this screening, the full text articles were reviewed and compared against the inclusion criteria checklist. The secondary screening was performed by one person.

The secondary screening resulted in 25 full text articles. Additionally, the references cited in the included studies were screened. Two studies from the manual search were identified and included. A final total of 8 publications were identified and included in the results in this report (see Figure 2.1.2.1-1).

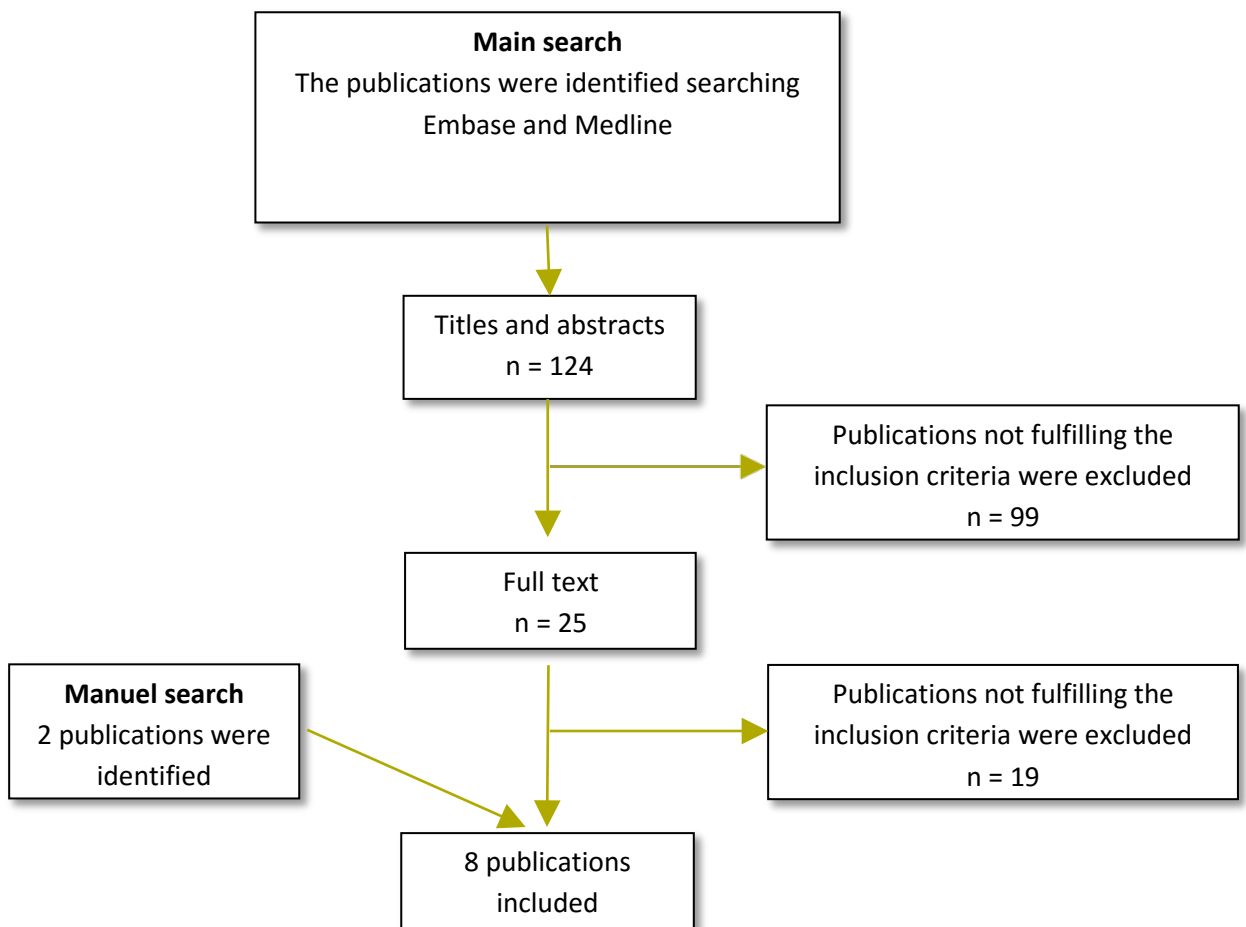


Figure 2.1.2.1-1 Flowchart for the literature search for L-citrulline and the subsequent publication selection.

2.2 General information

2.2.1 Chemistry

The molecular formula of L-citrulline (CAS No. 372-75-8) is $C_6H_{13}N_3O_3$ and the molecular weight is 175.19 g/mol. The IUPAC name is (2S)-2-amino-5-(carbamoylamino) pentanoic acid. It is a colourless solid at ambient temperature and pressure. The natural isoform is the L-form. The structural formula is shown in Figure 2.2.1-1.

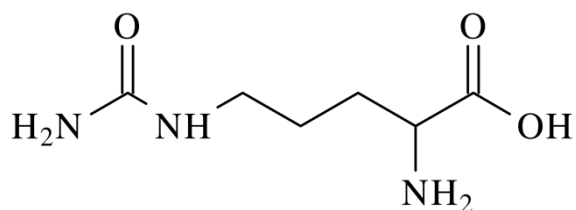


Figure 2.2.1-1 The structural formula of L-citrulline.

2.2.2 Occurrence

L-citrulline is found in high levels in certain Cucurbitacea, including watermelon, cucumber, pumpkin and courgette, and in certain algae such as *Grateloupia vulgaris*. It is also present in fish, meat, pulses and milk, and in vegetables such as onions and garlic (AECOSAN, 2015).

In EU, citrulline can be used in cosmetic products, and there are no restrictions with regard to either product type or use concentrations. Citrulline is used as skin conditioner with the purpose to maintain the skin in good condition (CosIng, 2015).

2.3 Absorption, distribution, metabolism and excretion (ADME)

L-citrulline from the diet is absorbed by the intestine. The pharmacokinetic parameters of increasing loads of L-citrulline were studied in 8 healthy male volunteers (27.6 ± 1.5 years; BMI 22.3 ± 0.5 kg/m²). The volunteers received four oral loads consisting of 2, 5, 10 or 15 g L-citrulline administered in random order. Each load was separated by a washout period of 15 days. L-citrulline was dissolved into 150 ml water. Following the L-citrulline loads, plasma L-citrulline concentration increased from 10-fold for the 2 g load to 100-fold for the 15 g load. The times of maximum concentration (t_{max}) were 0.64, 0.71, 0.72 and 0.94 hours for a load of 2, 5, 10 and 15 grams, respectively. The plasma concentrations returned to baseline values within 5-8 hours post-loading. The maximum concentrations (C_{max}) values for plasma citrulline were 515, 1314, 2756 and 3849 μ mol/l after a load of 2, 5, 10 and 15 grams, respectively. The pharmacokinetic parameters suggest that saturation begins to occur at a load of 15 g. After the L-citrulline loads, there was also an increase of plasma arginine and ornithine.

Most of the endogenous circulating L-citrulline is synthesized from ornithine and glutamine in enterocytes. Other amino acids that can act as L-citrulline precursors are glutamate, proline and, under some circumstances, arginine. In newborn mammals, proline seems to be the main source of L-citrulline. About 83% of L-citrulline released by the enterocytes is metabolized within the kidney (Curis et al., 2005).

In mammals, L-citrulline is a precursor for arginine. The metabolism of free L-citrulline occurs at different levels and occurs via three interconnected pathways: 1) arginine biosynthesis, which involves L-citrulline exchanges at whole body level; 2) nitric oxide (NO) cycle, which can involve local recycling of L-citrulline; 3) the complete urea cycle, which takes place in the liver (Curis et al., 2005).

Three enzymes are important for the metabolism of L-citrulline: ornithine carbamoyltransferase (OCT) and NO-synthase that produce L-citrulline, and argininosuccinate synthase (ASS) that transforms L-citrulline to arginine (AECOSAN, 2015; Curis et al., 2005).

In the majority of tissues that produce NO, L-citrulline is recycled to arginine through a process involving ASS and argininosuccinate lyase (ASL) to increase the availability of arginine for the production of urea (Figure 2.3-1).

L-citrulline not used in the NO metabolism is mainly metabolized in the kidney where it is converted to arginine through a partial urea cycle involving ASS and ASL. In adults, this conversion is enough to provide the body's full arginine requirements. However, in newborns, the *de novo* synthesis is not enough and a citrulline-to-arginine reaction is also observed in the intestinal mucosa (this activity disappears with weaning) (Curis et al., 2005).

In the urea cycle taking place in the liver, L-citrulline is synthesized by OCT from ornithine and catabolised by ASS to arginosuccinate for subsequent production of urea (Figure 2.3-1). All synthesized L-citrulline is converted to argininosuccinate and there is no L-citrulline release into the general circulation. The hepatocytes involved in the urea cycle do not take up L-citrulline from the portal circulation and there is only minimal uptake via arterial circulation. Thus, L-citrulline metabolism in the liver is a compartmentalized metabolism (Curis et al., 2005).

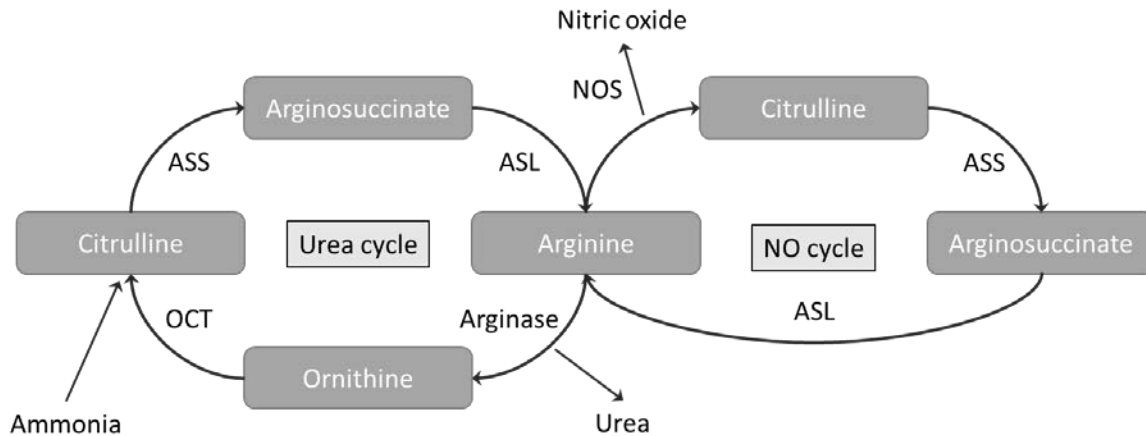


Figure 2.3-1 Citrulline-arginine cycle. Figure from Curis et al. (2005). OCT: ornithine carbamoyltransferase; ASS: arginosuccinate synthase; ASL: arginosuccinate lyase; NOS: nitric oxide synthase; NO: nitric oxide.

The L-citrulline metabolism is split between the kidneys and the liver mainly due to the efficacy of the capture of arginine by the liver. Without metabolic adaptation, almost all of the dietary arginine would be withdrawn from the portal blood by the liver, leaving only low amounts of available arginine for other organs (dietary arginine is degraded by the intestine to yield ornithine and proline, and in the liver arginine is a substrate for ureagenesis). In addition, as arginine is a positive regulator of ureagenesis other amino acids could be over-metabolized. The liver is unable to take up L-citrulline from portal circulation, and it can be seen as a masked form of arginine to bypass the liver. The main feature of L-citrulline is to be taken up by the kidney and metabolized into arginine. Thus, the conversion of L-citrulline into arginine is important in nitrogen homeostasis in the body in that there is an inter-organ cycle of arginine-citrulline-arginine which can be seen as a mechanism for protecting dietary arginine from excessive liver degradation and thus maintaining protein homeostasis (Moinard et al., 2008).

No L-citrulline-specific transporters have been found in any cell type, but various cell types are able to take up or release L-citrulline. There are studies demonstrating that L-citrulline can be transported by generic amino acid transporters (Curis et al., 2005).

In the study by Moinard et al. (2008), the excretion of L-citrulline increased significantly during the study period (0-8 hours) and this increase was related to the load administered. The urinary output returned to physiological values later on (i.e. 8-24 hours). The mass balance between the L-citrulline load and the urinary excretion revealed that only a small fraction of the citrulline load was excreted as shown by the high retention percentages (98.6, 95.8, 92.1 and 86.7 for a load of 2, 5, 10 and 15 gram, respectively). Thus, renal absorption of L-citrulline appears efficient because urinary loss is very low even at high (up to 15 g) L-citrulline intake.

2.4 Toxicological data/Adverse effects

2.4.1 Human studies

An overview of human studies investigating adverse health effects caused by L-citrulline is given in Table 2.4.1-1. In the studies where the authors have not defined citrulline as being L-citrulline, the name citrulline is used in the text below.

Table 2.4.1-1 An overview of human studies investigating L-citrulline and adverse health effects.

Reference	Study design/participant characteristics	Country	Number in treatment group		Dose*	Main endpoint	Duration of the study	Adverse effect
			L-citrulline	Control				
Cormio et al. (2011)	Randomized placebo-controlled, single blinded study N=24 men with mild erectile dysfunction and common concomitant medical conditions Mean age 56.5 ± 9.8 years	Italy	1 st month: placebo 2 nd month: L-citrulline		1.5 g/day (corresponding to 21 mg/kg bw per day for a 70 kg person)	Erection hardness score Number of intercourses per month Treatment satisfaction Adverse events	2 months, of which one month on L-citrulline	No adverse effects were observed.

Reference	Study design/participant characteristics	Country	Number in treatment group		Dose*	Main endpoint	Duration of the study	Adverse effect
			L-citrulline	Control				
Moinard et al. (2008)	Randomized crossover design N=8 healthy male volunteers Mean age 27.6 ± 1.5 years BMI 22.3 ± 0.5 kg/m ²	France	Citrulline administered in random order in 4 loads. Each load separated by a 15-day washout period	None	2, 5, 10 or 15 g (corresponding to 29-214 mg/kg bw per day)	Plasma kinetics of citrulline and other amino acids Haematological markers (leucocytes, polymorphonuclear cells, lymphocytes, monocytes, erythrocytes, hemoglobin) Biochemical markers (Ca, total protein, albumin, C-reactive protein, urea, creatinine, glucose, cholesterol) Arterial pressure Electrocardiogram	64 days	None suffered nausea or diarrhoea or any other side effects. No effect on haematological or biochemical markers nor on blood pressure, and no clinical symptoms.

Reference	Study design/participant characteristics	Country	Number in treatment group		Dose*	Main endpoint	Duration of the study	Adverse effect
			L-citrulline	Control				
Smith et al. (2006)	<p>RCT. Randomized placebo-controlled, double-blinded study</p> <p>N=40</p> <p>Children undergoing cardiopulmonary bypass and at risk for pulmonary hypertension</p> <p>Citrulline group: 50/50 male/female; median age (interquartile range): 12 months (0.3-29)</p> <p>Placebo group: 60/40 male/female; median age (interquartile range): 8 months (4-29)</p>	USA	Citrulline N=20	Placebo (distilled water) N=20	<p>3.8 g/m² per day (administered in 5 doses of 1.9 g/m² given every 12 hours) Total dose 9.5 g/m²</p> <p>(corresponding to 179 mg/kg bw per day and a total dose of 447 mg/kg bw in 12 months old children)[#]</p>	<p>Safety of perioperative oral citrulline supplementation</p> <p>Postoperative pulmonary hypertension</p> <p>An adverse event: a greater than 25% decrease in systemic mean blood pressure from baseline</p>	<p>Dosing: first dosing preoperative, second dose immediately on arrival to the pediatric critical care unit, followed by three doses 12, 24 and 36 hours postoperative</p> <p>Observation: 48 hours postoperative</p>	Mean blood pressure did not differ between the treatment groups.

Reference	Study design/participant characteristics	Country	Number in treatment group		Dose*	Main endpoint	Duration of the study	Adverse effect
			L-citrulline	Control				
Waugh et al. (2001)	<p>Phase II clinical trial during steady states in homozygous sickle cell disease patients (n=4) and sickle cell-hemoglobin C disease patient (n=1)</p> <p>Age 10-18 years</p> <p>Four males, one female</p>	USA	<p>N=5 in the initial 28-days trial</p> <p>N=4 in subsequent trial</p>	None	<p>Initial trial: Twice daily in daily dosages of 0.09-0.13 g/kg (corresponding to 2.1-3.0 and 1.5-2.1 mg/kg bw per day in children aged 10 to <14 years and adolescents aged 14 to <18 years, respectively)</p> <p>Subsequent trial: For 3 patients, serial 4- to 6-weeks test periods were performed, after at least 12 weeks of discontinued citrulline therapy. One patient remained on continuous citrulline supplementation</p>	<p>Symptoms of illness</p> <p>Cardiovascular measurements</p> <p>Hematologic and biochemical measurements</p>	28 days followed by 5 or 9 periods of 4- or 6- weeks duration	Side effects or toxicity from L-citrulline were not experienced.

Reference	Study design/participant characteristics	Country	Number in treatment group		Dose*	Main endpoint	Duration of the study	Adverse effect
			L-citrulline	Control				
Mizutani et al. (1984)	<p>Case-control study</p> <p>Two male siblings (7 and 14 years) with characteristic trait of lysinuric protein intolerance</p> <p>5 healthy controls (7-12 years)</p> <p>Loading tests were performed to study the preventive effect of arginine or citrulline supplement against hyperammonemia induced by intravenous amino nitrogen load</p> <p>A long term trial of ingestion of L-arginine or L-citrulline followed the loading trial</p>	Japan	<p><u>Loading test</u> (n=7)</p> <p>Intravenous load of L-alanine, 2) oral load or intravenous load of L-arginine plus intravenous load of L-alanine, 3) oral load of L-citrulline</p> <p><u>Long term trial</u> (n=1) older patient</p>	<p><u>Long term trial</u> (n=1) younger patient: 5.0 g L-arginine/day</p>	<p><u>Loading test</u></p> <p>1.0 mmol/kg bw (corresponding to 175 mg/kg bw per day)</p> <p><u>Long term trial in older patient</u></p> <p>8.0 g L-citrulline/day (corresponding to 131 mg/kg bw per day)</p>	<p><u>Loading test</u></p> <p>Amino acids in plasma and urine, plasma urea and blood ammonia levels</p> <p><u>Long term trial</u></p> <p>Amino acids in plasma and urine, plasma urea and blood ammonia levels, body weight and height</p>	<p><u>Loading test</u></p> <p>4 hours</p> <p><u>Long term trial</u></p> <p>2 years</p>	<p><u>Loading test</u></p> <p>Adverse effects were not reported.</p> <p><u>Long term trial</u></p> <p>No negative effects such as diarrhoea or vomiting were observed.</p>

Reference	Study design/participant characteristics	Country	Number in treatment group		Dose*	Main endpoint	Duration of the study	Adverse effect
			L-citrulline	Control				
Rajantie et al. (1980)	Clinical study N=19 6 male and 13 female patients with clinical findings of lysinuric protein intolerance (6 males and 13 females), age 1.9-32.7 years	Finland	N=19 Daily intake of 2.0-2.8 g citrulline (5-7 tablets containing 0.4 g citrulline each, with or without 4 to 6 lysine-HCl tablets (0.2 g) daily at meals according to body weight)	None	The daily intake corresponds to 87-121 mg/kg bw per day in children <10 years; 46-64 mg/kg bw per day in children 10-<14 years; 33-46 mg/kg bw per day in adolescents; 29-40 mg/kg bw per day for adults	Parameters such as diamino acids, citrulline, valine and ½-cystine, blood ammonia, urea-N, haemoglobin, albumin, serum immunoglobulins, complement fraction C3, lactate dehydrogenase, orotic acid excretion, SDSe (standard deviation score of deviation of height from the expected height), height velocity SDS (standard deviation score) and bone age lag were measured, as well as protein tolerance and intake and hair quality	2 years	Citrulline provoked no apparent negative effects.

**When daily doses in mg/kg bw were not stated in the paper, default body weights (bw) for each age group as determined by EFSA were used for calculation of daily intake per kg bw: 3 to <10 years; 23.1 kg, 10 to <14 years; 43.4 kg, 14 to <18 years; 61.3 kg and adults; 70.0 kg (EFSA, 2012).

#See appendix 9.2 for conversion from g/m² to g/kg bw.

§See appendix 9.3 for conversion from mmol/kg bw to mg/kg bw.

In a single-blinded, placebo-controlled, prospective study aiming to determine whether oral L-citrulline supplementation improved erection hardness in patients with mild erectile dysfunction, 24 men (age 56.5 ± 9.8 years) were included (Cormio et al., 2011). The patients received placebo (content of placebo was not defined) for the first month and L-citrulline, 1.5 g/day (21 mg/kg bw per day) for the second month. The patients kept a sexual diary where also possible adverse events were recorded. Follow-up visits were scheduled at the end of each month. Possible adverse events were evaluated at each follow-up. All patients concluded the study without adverse events. The most common concomitant medical conditions were consistent with those generally associated with erectile dysfunction including hypertension (37.5%), hypercholesterolemia (21%), benign prostatic hyperplasia (12.5%) and diabetes mellitus (12.5%).

The tolerance and pharmacokinetic parameters of increasing loads of citrulline was studied in 8 healthy male volunteers (27.6 ± 1.5 years; BMI 22.3 ± 0.5 kg/m²) (Moinard et al., 2008). All volunteers received four oral loads consisting of 2, 5, 10 or 15 g (29-214 mg/kg bw) citrulline administered in random order, each load being separated by a washout period of 15 days. Citrulline was dissolved into 150 ml water and then rapidly drunk. Blood samples were taken before administration and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5 and 8 hours after the loads. Urine samples were collected during the 0-8 hour period and then at 16 and 24 hour post-administration. Following the citrulline loads, plasma L-citrulline concentration increased from 10-fold for the 2 g load to 100-fold for the 15 g load. The plasma concentrations returned to baseline values within 5-8 hours post-loading (t_{max} : 0.64, 0.71, 0.72 and 0.94 hours for a load of 2, 5, 10 and 15 grams, respectively). The C_{max} values for plasma citrulline were 515, 1314, 2756 and 3849 $\mu\text{mol/l}$ after a load of 2, 5, 10 and 15 grams, respectively. After the citrulline loads, there was also an increase in plasma arginine and ornithine concentrations (arginine C_{max} of 146, 209, 280 and 303 $\mu\text{mol/l}$ and ornithine C_{max} of 81, 114, 152 and 179 $\mu\text{mol/l}$ for 2, 5, 10 and 15 g of citrulline, respectively) that reached a maximum (arginine t_{max} of 1.17, 1.44, 1.67 and 2.29 hours and ornithine t_{max} of 1.38, 1.35, 1.57 and 1.79 hours for 2, 5, 10 and 15 g of citrulline, respectively) and then decreased without reaching baseline values at the end of the 8 hour period. According to the authors, arginine production at the highest citrulline dose (15 g) was not related to the dose administered (i.e. was lower than expected). Plasma citrulline concentration is the primary factor which determines arginine production by the kidney. Thus, it appears that renal arginine synthesis becomes saturated. This was confirmed by an increase in urinary arginine excretion and a decrease in both citrulline retention percentage and fractional reabsorption rate at the citrulline intake of 15 g. Administration of citrulline did not result in significant changes in other amino acid concentrations compared with baseline values. The authors stated that this was in agreement with their previous studies and that it indicated that citrulline is a "neutral" amino acid that performs a specific job in terms of arginine metabolism. Haematological markers (leucocytes, polymorphonuclear cells, lymphocytes, monocytes, erythrocytes, hemoglobin (Hb)) and biochemical markers (Ca, total protein, albumin, C-reactive protein, urea, creatinine, glucose and cholesterol) were determined before and after the study period. Safety was evaluated by measurement of arterial pressure and electrocardiogram

was performed before and at 1, 2, 4 and 8 hours after citrulline administration. None of the volunteers suffered nausea or diarrhoea or any other negative effects. Furthermore, citrulline administration had no effect on haematological or biochemical markers or on blood pressure, and no clinical symptoms were noticed during the study.

A randomized, placebo-controlled, double-blinded study was performed on 40 infants or children (<6 years of age) undergoing one of six surgical procedures for correction of congenital heart lesion at Vanderbilt Children's hospital in USA (Smith et al., 2006). The purpose of the study was to determine whether perioperative oral citrulline supplementation was 1) safe in patients after heart surgery, 2) efficacious in increasing plasma citrulline and arginine concentrations, and 3) associated with development of postoperative pulmonary hypertension. Measurements of systemic blood pressure and presence of pulmonary hypertension were collected. The patients were randomized to receive perioperative doses of either citrulline (100 mg/ml (10%) solution with distilled water) (n=20; 50% males; 12 months old (0.3-29 months)) or placebo (n=20; 60% males; 8 months old (4-29 months)). Citrulline was administered in five doses of 1.9 g/m² given every 12 hours for a daily dose of 3.8 g/m² (179 mg/kg bw) and for a total dose of 9.5 g/m² (447 mg/kg bw). The first dose was administered through an orogastric feeding tube after induction of anesthesia and intubation, but before the surgery. The last doses were given enterally through a nasogastric feeding tube immediately on arrival in the pediatric critical care unit and 12, 24 and 36 hours postoperatively. Systemic blood pressure was monitored continuously during the 48-hour study period because of the theoretic risk of hypotension associated with citrulline administration. An adverse event was defined as a greater than 25% decrease in systemic mean blood pressure from baseline (measured before cardiopulmonary bypass). Mean blood pressure did not differ between the citrulline and placebo groups. Furthermore, no deaths occurred within the 48-hour study period. Three patients died of postoperative complications (2 in the citrulline group vs. 1 in the placebo group). The deaths were found to be unrelated to the study drug administration.

Five African-American children with either homozygous sickle cell disease (3 males aged 14, 17 and 18 years; 1 female aged 12 years) or sickle cell-hemoglobin C disease (n=1; male aged 10 years) were included in a Phase II Clinical trial (Waugh et al., 2001). All patients had been in a steady state (not painful crisis state) for at least four weeks. Capsules with L-citrulline were given orally twice daily for 28 days in daily dosages of 0.09-0.13 g/kg (corresponding to 2.1-3.0 and 1.5-2.1 mg/kg bw per day in children aged 10 to <14 years and adolescents aged 14 to <18 years, respectively). On the first day, before citrulline was given, focused histories, physical examinations, two-dimensional echocardiographic examinations and blood specimens were obtained. Brachial blood pressures were measured and mean arterial blood pressures estimated. Similar 4-week evaluations were performed at the end of the first 28-days on citrulline. Subsequent to the initial 28-day trials in these patients, in three of the four who agreed to continue taking citrulline, serial 4- to 6-weeks test periods were performed, after at least 12 weeks of discontinued citrulline therapy. A fourth patient remained on continuous citrulline supplementation. Echocardiograms were not

done during these subsequent trial periods. Side effects or toxicity from the citrulline treatment were not experienced.

In two male sibling patients (7 and 14 years) with lysinuric protein intolerance (LPI), the therapeutic effect of oral supplement of arginine and citrulline on postprandial hyperammonemia was investigated (Mizutani et al., 1984). Five healthy control subjects aged 7-12 years were also included in the study. Intravenous load of L-alanine (6.6 mmol/kg bw), and oral load (via naso-gastric tube) of L-arginine (0.8 mmol/kg bw) or L-citrulline (1.0 mmol/kg bw) during intravenous load of L-alanine were given. The citrulline dose corresponds to 175 mg/kg bw per day. Blood samples were collected at 0, 2, 3 and 4 hours after the load. Amino acids in plasma and urine, plasma urea and blood ammonia levels were analysed. Hyperammonemia induced by the intravenous load of L-alanine was prevented and plasma urea improved by the oral administration of L-citrulline in both patients. Thus, the two patients were put on a regimen of long-term ingestion of L-citrulline or L-arginine (older patient: 8.0 g L-citrulline/day; younger patient: 5.0 g L-arginine/day). The L-citrulline dose corresponds to 131 mg/kg bw per day. During the two-year observation, no negative effects, such as diarrhoea or vomiting, were observed.

In 19 patients with clinical findings of LPI, the status after a two-year treatment with citrulline with or without lysine was investigated (Rajantie et al., 1980). Of the patients, 8 were children less than 10 years of age, 5 were children aged 10 to <14 years, 2 were adolescents (14 to <18 years) and 4 were adults. The patients were examined at the start of the citrulline regimen and then every six months. The regimen consisted of an isocaloric diet supplemented with daily intake of 5 to 7 citrulline tablets containing 0.4 g each, with or without lysine-HCl tablets, according to the patients' body weight. Thus, the participants received a daily intake of 2.0-2.8 g citrulline. This corresponds to 87-121 and 46-65 mg/kg bw per day in children aged <10 years and 10 to <14 years, respectively, 33-46 mg/kg bw per day in adolescents (14 to 18 years), and 29-40 mg/kg bw per day in adults (estimated using default body weights for each age group as determined by EFSA). Clinical examinations were performed by the same medical doctor. Fasting blood samples and 24-hour urine samples were collected. Parameters such as diamino acids, citrulline, valine and ½-cystine, blood ammonia, urea-N, haemoglobin, albumin, serum immunoglobulins, complement fraction C3, lactate dehydrogenase, orotic acid excretion, SDSe (standard deviation score of deviation of height from the expected height), height velocity SDS (standard deviation score) and bone age lag were measured, as well as protein tolerance and intake, and hair quality. Citrulline provoked no apparent negative effects during the study period.

2.4.1.1 Interactions

There was no information in humans concerning interactions in the literature reviewed in the present risk assessment. The absence of information in the selected literature does not document an absence of interactions.

2.4.1.2 Allergic sensitisation (including adjuvant effects)

There was no information concerning allergic sensitisation or allergy adjuvant effects in the literature reviewed in the present risk assessment. The absence of information in the selected literature does not document an absence of allergic sensitisation or allergy adjuvant effects.

2.4.2 Animal studies

Tain et al. (2015) intended to examine whether L-citrulline could prevent NO deficiency-induced programmed hypertension in the offspring exposed to maternal L-N^G-nitro-arginine-methyl ester (L-NAME) administration and to quantify the abundance of RNA transcripts in the offspring kidney. Female Sprague Dawley rats (10 week-old) were caged individually with a male until mating was confirmed. Pregnant Sprague-Dawley rats were assigned to three groups: control, L-NAME and L-NAME + citrulline. Pregnant rats received L-NAME administration at 60 mg/kg per day by a subcutaneous osmotic pump during the whole pregnancy period, whereas pregnant controls received iso-osmotic saline. Half of the L-NAME treated rats received 0.25% L-citrulline solution dissolved in the drinking water during the entire pregnancy and lactation period. After birth, the litters were culled to 8 pups to standardize the received quantity of milk and maternal pup care. Each litter was left with the mother until weaning. Male offspring were sacrificed at 12 weeks of age. Litter sizes were not significantly altered by L-NAME or L-citrulline. Male pup mortality rates, body weight, kidney weight and heart weight were not different among the three groups. The systolic blood pressure and mean arterial pressure of the L-NAME group were significantly higher than those in the control, which maternal L-citrulline therapy prevented. Since the daily intake of drinking water was not reported by the authors, a daily intake of L-citrulline cannot be calculated. Thus, this study is not used further for the risk characterization.

Pradilla et al. (2012) performed a toxicity and dose-escalation study where C57Bl6 mice received an intraperitoneal injection of 30, 50, 100 or 200 mg/kg (n = 3 in each group) every 8 hours of L-citrulline (for 24 hours or 90 days?). They were evaluated daily for weight and neurological function using a posture, grooming and ambulation (PGA) scale for 90 days. After euthanasia, a histopathological evaluation was performed. The animals did not exhibit signs of systemic or neurological toxicity with doses up to 200 mg/kg bw over 8 hours. All mice showed adequate progressive weight gain throughout the study period, and postmortem histopathological analyses did not reveal any signs of central nervous system or systemic toxicity (only data for weight gain was shown in the study report). Since there were only three mice in each dose group, the number of L-citrulline doses and exposure period not clearly stated by the authors, and the data were not shown for other outcomes than body weight gain, a NOAEL was not defined based on this study. Thus, this study was not used further for the risk characterization.

2.4.2.1 Interactions

There was no information in animals concerning interactions in the literature reviewed in the present risk assessment. The absence of information in the selected literature does not document an absence of interactions.

2.4.2.2 Allergic sensitisation (including adjuvant effects)

There was no information concerning allergic sensitisation or allergy adjuvant effects in the literature reviewed in the present risk assessment. The absence of information in the selected literature does not document an absence of allergic sensitisation or allergy adjuvant effects.

2.4.3 Vulnerable groups

Persons with citrullinemia (increased levels of citrulline in the blood) are potentially vulnerable to intake of additional L-citrulline from supplements. Citrullinemia is an autosomal recessive disorder and urea cycle disease that shows neuropsychiatric symptoms and elevated concentrations of serum citrulline and ammonia (Woo et al., 2014). The disease is classified as type I (CTLN1) and II (CTLN2) based on molecular pathogenesis. CTLN1, is an argininosuccinate synthetase (ASS) deficiency, which is caused by mutations in the *ASS1* gene and show very heterogeneous clinical manifestations, such as hyperammonemic encephalopathy, including lethargy, failure to thrive, seizure, loss of consciousness and death early in life (classic neonatal onset). CTLN2 results from citrin deficiency, resulting from mutations in the *SLC25A13* gene and show recurring sudden encephalopathic symptoms, including headache, abnormal behavior and disorientation related to hyperammonia and steatohepatitis (adult onset). Numerous mutations have been reported in these genes. There are also other disorders associated with high citrulline concentration, such as argininosuccinic aciduria and pyruvate carboxylase deficiency.

The levels of citrulline circulating in plasma are remarkably elevated in humans with chronic renal failure. Sodium-dependent dicarboxylate cotransporters, including human organic anion transporter 1 (OAT1), seems to be major contributors to renal basolateral uptake of citrulline, and impaired activities of this or similar transporters may contribute substantially to an increase in plasma citrulline in renal failure (Nakakariya et al., 2009). Two mechanisms may contribute to elevation of plasma citrulline concentration in renal failure, i.e. upregulation of absorptive transporters and downregulation of secretory transporters. Thus, at least in theory, humans with defects in such transporters may be potentially vulnerable to supplementation of L-citrulline.

2.5 Summary of hazard identification and characterisation

L-citrulline is classified as a non-protein amino acid. Following oral intake of L-citrulline, plasma L-citrulline concentration increases rapidly but returns to baseline values within 5-8

hours post-exposure. L-citrulline from the diet is absorbed by the intestine. About 83% of L-citrulline released by the enterocytes is metabolized within the kidney. There are three interconnected metabolic pathways for L-citrulline: 1) arginine biosynthesis, which involves L-citrulline exchange at whole body level, 2) nitric oxide (NO) cycle, which can involve local recycling of L-citrulline, and 3) the complete urea cycle, which takes place in the liver. Renal L-citrulline reabsorption appears very efficient because urinary loss is very low even at high L-citrulline intake. The conversion of L-citrulline into arginine is important in nitrogen homeostasis in the body as there is an inter-organ cycle of arginine-citrulline-arginine which can be seen as a mechanism for protecting dietary arginine from excessive liver degradation and thus maintaining protein homeostasis.

No adverse health effects of L-citrulline were observed in the six human studies covering the ages 12 months to 56 years, with L-citrulline exposure lengths varying from less than one day (acute doses) to 2 years. The doses varied from 2.1-179 mg/kg bw per day for children (<14 years), 1.5-175 mg/kg bw per day for adolescents (14 to <18 years) and 21-214 mg/kg bw per day in adults, respectively (Table 2.5-1).

Table 2.5-1 Summary of doses, exposure periods and number of participants receiving L-citrulline in the human studies.

Age groups	Daily doses (mg/kg bw per day)*	Length of L-citrulline exposure	No. in L-citrulline group	References
Children (<10 years)	179	36 hours	20	Smith et al. 2006
	175	4 hours	6 [#]	Mizutani et al. 1984
	87-121	2 years	8	Rajantie et al. 1980
Children (10 to <14 years)	2.1-3.0	28 days – 20/54 weeks	2	Waugh et al. 2001
	46-65	2 years	5	Rajantie et al. 1980
Adolescents (14 to <18 years)	1.5-2.1	28 days – 20/54 weeks	3	Waugh et al. 2001
	175	4 hours	1	Mizutani et al. 1984
	131	2 years	1	Mizutani et al. 1984
	33-46	2 years	2	Rajantie et al. 1980
Adults (≥18 years)	21	1 month	24	Cormio et al. 2011
	29-40	2 years	4	Rajantie et al. 1980
	29-214	(acute)	8	Moinard et al. 2007

*When daily doses in mg/kg bw were not stated in the paper, default body weights (bw) for each age group as determined by EFSA were used for calculation of daily intake per kg bw: 3 to <10 years; 23.1 kg, 10 to <14 years; 43.4 kg, 14 to <18 years; 61.3 kg and adults; 70.0 kg (EFSA, 2012).

[#]Includes five healthy controls aged 7-12 years (exact ages are unknown).

The human studies available had low number of participants and, with the exception of one study, included non-healthy populations. However, none of the studies observed any adverse effects of oral intake of L-citrulline. The study by Rajantie et al. (1980) (19 patients with LPI, ages 1.9-32.7 years, 2 years of treatment), reported that daily intakes of 65 mg/kg bw in

children (10 to <14 years), 46 mg/kg bw in adolescents (14 to <18 years) and 40 mg/kg bw in adults (highest doses administered) did not cause adverse effects. Thus, VKM has used the 2-year clinical study by Rajantie et al. (1980) in the risk characterization.

The patients in the study by Rajantie et al. (1980) had LPI, a rare autosomal recessive disorder caused by defective cationic amino acid (lysine, arginine, and ornithine) transport over cell membranes. This mainly affects their intestinal absorption and renal reabsorption, but may also lead to entrapment of arginine in cells. The citrulline transport, on the other hand, is not affected in LPI patients. Plasma concentrations of cationic amino acids are usually below normal for age but may also be within the normal range in these patients. They have an elevated urinary excretion of arginine, ornithine and lysine. In addition, plasma concentrations of citrulline, serine, glycine, proline, alanine and glutamine are often increased (de Baulny et al., 2012; Sebastio and Nunes, 1993; Sebastio et al., 2011). At high intake levels of citrulline in LPI patients, there is a risk that the arginine entrapment may lead to high concentrations of intracellular NO. Even though LPI patients have different plasma levels of amino acids such as arginine and citrulline compared to healthy individuals, VKM considers that the results from Rajantie et al. (1980) can be used for the risk characterisation of the general population based on the following arguments:

1) In theory, LPI patients could tolerate higher doses of L-citrulline due to their lower plasma levels of arginine compared to healthy individuals. However, in healthy individuals the plasma levels of arginine is tightly regulated. It appears that renal arginine synthesis becomes saturated after oral intake of citrulline combined with an increase in urinary arginine excretion and a decrease in both citrulline retention percentage and fractional reabsorption rate (Moinard et al., 2008).

2) High citrulline intakes may, due to the impaired arginine transport, give increased levels of intracellular arginine in LPI patients making them more vulnerable to adverse effects of oral supplementation of citrulline compared to healthy individuals. However, no adverse effects were observed during the two-year study period. In addition, oral supplementation of citrulline was well tolerated in LPI patients even though they often have higher plasma concentrations of citrulline than healthy individuals.

Oral L-citrulline supplementation for 4 weeks of 6 (n=17, age 21.6 ± 0.9 years) or 2 weeks of 7-11 g/day (n=16, 23 ± 3 years) was given to healthy young males in randomized placebo-controlled cross-over studies of blood pressure (Figuroa et al., 2010; Sanchez-Gonzalez et al., 2013). Six g/day was given for 8 weeks to obese postmenopausal women with high blood pressure (n=14, 58 ± 1 years) in a study with randomized parallel design studying arterial stiffness and leg muscle function (Figuroa et al., 2015). In these studies, there were no mentioning of negative health effects and no participants left the study because of negative effects, indicating that a dose of L-citrulline of approximately 86 mg/kg bw/day (6 g/70 kg bw) was well tolerated for 2-8 weeks. However, none of these studies reported that negative health effects outside the end points of interest in the studies had actually been looked for.

Persons with citrullinemia caused by mutations in enzymes involved in citrulline metabolism are potentially vulnerable to intake of additional L-citrulline from supplements. In addition, humans with chronic renal failure and/or mutations in renal citrulline transporters are potentially vulnerable to supplementation of L-citrulline.

The values used for comparisons with the estimated exposure in the risk characterization are 65 mg/kg bw per day for children (10 to <14 years) 46 mg/kg bw for adolescents (14 to <18 years) and 40 mg/kg bw per day for adults (\geq 18 years) based on the two-year clinical study of patients with LPI by Rajantie et al. (1980).

3 Exposure / Intake

NFSA requested VKM to perform a risk assessment of 1000, 1500 and 2000 mg/day of L-citrulline in food supplements for children (10 to <14 years), adolescents (14 to <18 years) and adults. The default body weights (bw) for these groups as determined by EFSA were used: 10 to <14 years; 43.4 kg, 14 to <18 years; 61.3 kg and adults; 70.0 kg (EFSA, 2012).

The estimated exposure to L-citrulline from food supplements for the various age groups is presented in Table 3-1.

From a daily dose of 1000 mg L-citrulline, the calculated intake levels are 23.0, 16.3 and 14.3 mg/kg bw per day for children (10 to <14 years), adolescents (14 to <18 years) and adults (≥ 18 years), respectively. From a daily dose of 1500 mg, the calculated intake levels are 34.6, 24.5 and 21.4 mg/kg bw per day for children (10 to <14 years), adolescents (14 to <18 years) and adults (≥ 18 years), respectively. From a daily dose of 2000 mg, the calculated intake levels are 46.1, 32.6 and 28.6 mg/kg bw per day for children (10 to <14 years), adolescents (14 to <18 years) and adults (≥ 18 years), respectively.

Table 3-1 Estimated daily intake of L-citrulline (mg/kg bw per day) from food supplements for the various age groups.

Intake (mg/kg bw per day)	Daily doses (mg)		
	1000	1500	2000
Children (10 to <14 years)	23.0	34.6	46.1
Adolescents (14 to <18 years)	16.3	24.5	32.6
Adults (≥ 18 years)	14.3	21.4	28.6

3.1 Other sources

L-citrulline occurs naturally in food. Watermelon is unusually rich in citrulline. However, very little citrulline is contained in a normal diet (Fike et al., 2014). Human milk, infant formulas and parenteral nutrition solutions contain minimal or no citrulline. No quantitative data on dietary intake of L-citrulline has been found.

In EU, citrulline can be used in cosmetic products, and there are no restrictions with regard to either product type or use concentrations. Citrulline is used as skin conditioner with the purpose to maintain the skin in good condition (CosIng, 2015).

4 Risk characterisation

No adverse health effects of L-citrulline were observed in the six human studies covering the ages 12 months to 56 years, with L-citrulline exposure varying from acute doses to 2 years. Since L-citrulline is synthesised endogenously, occurs naturally in foods and plasma L-citrulline concentration returns to baseline values within 5-8 hours after oral exposure, no safety factor was applied to the intake values of 65 mg/kg bw per day for children, 46 mg/kg bw per day for adolescents and 40 mg/kg bw per day for adults (Rajantie et al., 1980).

NFSA requested VKM to perform a risk assessment of the doses of 1000, 1500 and 2000 mg/day of L-citrulline in food supplements for the general population, ages 10 years and above. The estimated exposures are presented in Table 3-1.

For children, from a daily intake of 1000, 1500 and 2000 mg the estimated exposures were 23.0, 34.6 and 46.1 mg/kg bw per day, respectively. These intake values are below 65 mg/kg bw per day. VKM considers it unlikely that a daily intake of 1000, 1500 or 2000 mg L-citrulline from food supplements causes adverse health effects in children (10 to <14 years).

For adolescents, from a daily intake of 1000, 1500 and 2000mg the estimated exposures were 16.3, 24.5 and 32.6 mg/kg bw per day, respectively. These intake values are below 46 mg/kg bw per day. VKM considers it unlikely that a daily intake of 1000, 1500 or 2000 mg L-citrulline from food supplements causes adverse health effects in adolescents (14 to <18 years).

For adults, from a daily intake of 1000, 1500 and 2000 mg the estimated exposures are 14.3, 21.4 and 28.6 mg/kg bw per day, respectively. These intake values are below 40 mg/kg bw per day. VKM considers it unlikely that a daily intake of 1000, 1500 or 2000 mg L-citrulline from food supplements causes adverse health effects in adults (≥ 18 years).

5 Uncertainties

5.1 Hazard identification and characterisation

The human studies were specifically designed to investigate the positive effects of L-citrulline, and not negative effects.

In some studies, the authors have not defined the isoform of citrulline used.

The human studies available had low number of participants and, with the exception of one study, included non-healthy populations. Nevertheless, in none of the studies adverse effects were observed after treatment for up to two years.

The values for comparison used in this opinion is based on the highest doses of L-citrulline given in the study by Rajantie et al. (1980). This study included patients with LPI. Since LPI patients have a different intestinal absorption and renal reabsorption of cationic amino acids than healthy individuals, it is uncertain whether doses given to LPI patients can be directly extrapolated to healthy individuals.

5.2 Exposure estimates

With use of the default (mean) body weight of an age (population) group, the variance in all individuals in the group will not be covered. Dividing children, adolescents and adults in different age groups minimizes the uncertainty. For individuals with a body weight below the default body weight the actual exposure will be higher than the calculated, whereas for individuals with body weight above the default body weight the actual exposure will be below the calculated. Individuals with body weights less than the default estimate in a given age group are not fully covered in the risk estimate.

6 Conclusions with answers to the terms of reference

The Norwegian Scientific Committee for Food Safety (VKM) has at the request of the Norwegian Food Safety Authority, assessed the risk of L-citrulline (1000, 1500 and 2000 mg/day) in food supplements. L-citrulline is found in high levels in certain Cucurbitacea, including watermelon, cucumber, pumpkin and courgette, and in certain algae such as *Grateloupia vulgaris*. It is also present in fish, meat, pulses and milk, and in vegetables such as onions and garlic.

The human studies available had low number of participants and, with exception of one study, included non-healthy populations. However, none of the studies observed any adverse effects of L-citrulline. The study by Rajantie et al. (1980) (19 patients with lysinuric protein intolerance (LPI), ages 1.9-32.7 years, 2 years treatment) reported that daily intakes of 65 mg/kg bw in children (10 to <14 years), 46 mg/kg bw in adolescents (14 to <18 years) and 40 mg/kg bw in adults (highest doses applied) did not cause adverse effects. These age specific reference points were used for comparison with the estimated exposures in the risk characterization.

For children, from a daily intake of 1000, 1500 and 2000 mg the estimated exposures are 23.0, 34.6 and 46.1 mg/kg bw per day, respectively. These intake values are below 65 mg/kg bw per day. VKM therefore considers it unlikely that a daily intake of 1000, 1500 or 2000 mg L-citrulline from food supplements causes adverse health effects in children (10 to <14 years).

For adolescents, from a daily intake of 1000, 1500 and 2000 mg the estimated exposures are 16.3, 24.5 and 32.6 mg/kg bw per day, respectively. These intake values are below 46 mg/kg bw per day. VKM therefore considers it unlikely that a daily intake of 1000, 1500 or 2000 mg L-citrulline from food supplements causes adverse health effects adolescents (14 to <18 years).

For adults, from a daily intake of 1000, 1500 and 2000 mg the estimated exposures are 14.3, 21.4 and 28.6 mg/kg bw per day, respectively. These intake values are below 40 mg/kg bw per day. VKM therefore considers it unlikely that a daily intake of 1000, 1500 or 2000 mg L-citrulline from food supplements causes adverse health effects in adults (≥ 18 years).

Since LPI patients have a different intestinal absorption and renal reabsorption of cationic amino acids than healthy individuals, it is uncertain whether doses given to LPI patients can be directly extrapolated to healthy individuals.

Persons with citrullinemia caused by mutations in enzymes involved in citrulline metabolism are potentially vulnerable to intake of additional L-citrulline from supplements. In addition, humans with chronic renal failure and/or mutations in renal citrulline transporters are potentially vulnerable to supplementation of L-citrulline.

An overview of the conclusions is presented in Table 6.1. Estimated exposures unlikely to cause adverse health effects (below the values for comparison) are shown in green.

Table 6.1 An overview of the conclusions on L-citrulline in food supplements. Green: estimated exposure to L-citrulline is unlikely to cause adverse health effects.

L-citrulline			
Food supplement Age groups	Dose (mg/day)		
	1000	1500	2000
Children (10 to <14 years)			
Adolescents (14 to <18 years)			
Adults (≥18 years)			

7 Data gaps

- There are few human and animal studies on negative health effects of L-citrulline.
- No studies are found that include effects of L-citrulline in lactating or pregnant women.
- There is lack of sub-chronic and chronic toxicity studies with a sufficient number of healthy subjects.

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9 Appendix

9.1 Appendix 1 – Literature search

Search Strategy in Ovid Medline and Embase (from 1946 and 1974, respectively, to December 2nd 2015).

- 1 citrulline*.ti. (2419)
- 2 (risk* or safety or adverse or side-effect*1 or hazard* or harm* or negative or contraindicat* or contra-indicat* or interact* or toxicity or toxic).tw. (9659232)
- 3 1 and 2 (284)
- 4 (conference abstract* or letter* or editorial*).pt. (4846385)
- 5 3 not 4 (236)
- 6 limit 5 to (danish or english or norwegian or swedish) (228)
- 7 remove duplicates from 6 (123)

9.2 Appendix 2 – Conversion of g/m² to g/kg bw for 12 months old children

9.2.1 Formulas

Calculation of body surface area (BSA): $BSA (m^2) = \sqrt{\frac{\text{body weight (kg)} \times \text{body length (cm)}}{3600}}$

Calculation of K_m (correction factor): $Km = \frac{bw}{BSA}$

Where bw = body weight

Calculation of g/kg bw: $g/kg bw = \frac{g/m^2}{Km}$

9.2.2 Calculations for 12 months old children

Body surface area (BSA)

bw = 9.4 kg and body length = 75 cm

$$BSA = \sqrt{\frac{9.4 \text{ kg} \times 74 \text{ cm}}{3600}} = 0.4425 \text{ m}^2$$

K_m Correction factor

bw = 9.4 kg and BSA = 0.4425 m²;

$$K_m = 9.4 \text{ kg} / 0.4425 \text{ m}^2 = 21$$

g/kg bw and mg/kg bw

Daily dose = 3.8 g/m² and total dose = 9.5 g/m²

$$K_m = 21$$

$$\text{Daily dose} = \frac{3.8 \text{ g/m}^2}{21 \text{ kg/m}^2} = 0.179 \text{ g/kg bw} = 179 \text{ mg/kg bw}$$

$$\text{Total dose} = \frac{9.5 \text{ g/m}^2}{21 \text{ kg/m}^2} = 0.447 \text{ g/kg bw} = 447 \text{ mg/kg bw}$$

9.3 Appendix 3 – Conversion of mmol/kg bw to mg/kg bw

Molecular weight of citrulline=175 g/mol

Dose administered = 1 mmol/kg bw

Molecular weight of L-citrulline: 175 g/mol

$$1 \text{ mmol} = \frac{175 \text{ g}}{1000 \text{ mol}}$$

Thus, 1 mmol/kg bw = 0.175 g / kg bw = 175 mg / kg bw