Food and Agriculture Organization of the United Nations

The Joint FAO/WHO Expert Meetings on Nutrition (JEMNU)

Nitrogen to protein conversion factors for soy-based and milk-based ingredients used in infant formula and follow-up formula

Report of the meeting of the expert panel
Geneva, Switzerland, 16–17 July 2019
The Joint FAO/WHO Expert Meetings on Nutrition (JEMNU)

NITROGEN TO PROTEIN CONVERSION FACTORS FOR SOY-BASED AND MILK-BASED INGREDIENTS USED IN INFANT FORMULA AND FOLLOW-UP FORMULA

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# CONTENTS

Acknowledgements v
Abbreviations and acronyms vi
Executive summary 1
1 Introduction 5
2 Background 7
3 The GRADE framework for assessing quality of evidence 9
4 Results of systematic review on nitrogen to protein conversion factors 11
5 Discussion of the systematic review 15
6 Quality of evidence for nitrogen to protein conversion factors: GRADE assessments 20
7 Final determination of recommended nitrogen to protein conversion factors 23
8 Conclusions 25

**Annexes**

Annex 1 List of participants 31
Annex 2 Call for data on nitrogen to protein conversion factors for soy-based and milk-based ingredients 33
Annex 3 Call for experts for the Joint FAO/WHO Expert Meetings on Nutrition (JEMNU) on nitrogen to protein conversion factors for soy-based and milk-based ingredients used in infant formula and follow-up formula 35
Annex 4 Discussion paper prepared by Canada and the United States of America 38
Annex 5 The final GRADE evidence profiles 42
Annex 6 Methods for amino acid analysis – best practice 52
ACKNOWLEDGEMENTS

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## ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CAC</td>
<td>Codex Alimentarius Commission</td>
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<td>CCNFSU</td>
<td>Codex Committee on Nutrition and Foods for Special Dietary Uses</td>
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<td>CRE</td>
<td>WHO Office of Compliance, Risk Management and Ethics</td>
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<td>DOI</td>
<td>declaration of interests</td>
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<td>IDF</td>
<td>International Dairy Federation</td>
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<td>ISO</td>
<td>International Organization for Standardization</td>
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<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
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<tr>
<td>GRADE</td>
<td>Grading of Recommendations, Assessment, Development and Evaluation</td>
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<td>JEMNU</td>
<td>Joint FAO/WHO Expert Meetings on Nutrition</td>
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<td>PICO</td>
<td>population, intervention, comparator and outcome</td>
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<td>WHO</td>
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EXECUTIVE SUMMARY

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) established the Joint FAO/WHO Expert Meetings on Nutrition (JEMNU) in 2009 to provide scientific advice to the Codex Alimentarius Commission (CAC) and its subsidiary bodies, as well as Member Countries. JEMNU aims to provide relevant scientific advice in an independent, timely and cost-effective manner, and is convened in response to requests from specific Codex committees or Member Countries.

As part of its work on updating the Codex standards for infant formula and follow-up formula, the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) is currently discussing the most appropriate nitrogen to protein conversion factor (or factors) to use in estimating protein content of soy-based and milk-based ingredients used in infant formula and follow-up formula. In 2017, the CCNFSDU requested that JEMNU be convened to review the evidence and develop evidence-informed guidance on this topic.

Thus, the requested work by JEMNU was initiated and a meeting was convened by FAO and WHO in Geneva, Switzerland, from 16 to 17 July 2019. Thirteen experts participated in the meeting, along with an external resource person, and representatives of the Codex Secretariat.

The objectives of the meeting were to:

- review and interpret the evidence of a systematic review commissioned to compile and analyse the available data on nitrogen to protein conversion factors for foods containing soy-based or milk-based ingredients;
- determine appropriate conversion factors for soy-based and milk-based ingredients used in infant formula and follow-up formula;\(^1\) and
- provide any additional relevant information to be included in the report to be submitted to the 41st Session of CCNFSDU, to be held in Düsseldorf, Germany, from 24 to 29 November 2019.

The systematic review that was commissioned summarized relevant analytical methods, and compiled and calculated pooled estimates for different types of conversion factor from published sources and from unpublished sources (via a call for data). The JEMNU expert panel discussed the findings of the systematic review and then applied the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) framework\(^2\) to assess the certainty in the evidence for each of the pooled conversion factor estimates.

\(^1\) The discussion in this paper relates to products covered by the Codex standards on infant formula and follow-up formula, which include formula products intended for infants, older infants and young children up to the age of 36 months. Where the terms ‘formula’, ‘formulae’ and ‘formula products’ are used without specifying ‘infant formula’ or ‘follow-up formula’, they refer to the full range of products for infants and children from birth to 36 months.

\(^2\) GRADE is used by WHO and many other organizations to assess the quality of evidence informing their recommendations and guidelines. More information on GRADE can be found at http://www.gradeworkinggroup.org.
In discussing the findings of the review, three major themes emerged, as outlined below:

- It is difficult to accurately estimate the protein content of foods directly, because the most commonly used methods have numerous shortcomings. However, correction of the crude protein content of a food (based on analysis of total nitrogen) for measured non-protein nitrogen content allows an accurate determination of food proteins.

- The shortcomings of the commonly used protein estimation methods, together with a relative paucity of data for formulas and formula ingredients – resulting in low to moderate confidence in the conversion factors recommended by the expert panel – should be the impetus for a call to action by industry and other relevant stakeholders to generate relevant conversion factors for formulas using best available methods, possibly as part of a multinational consortium.

- Selecting the appropriate conversion factor for a particular food or ingredient depends greatly on the nature of the protein and nitrogen in the sample, and how the two elements are defined and characterized.

Defining protein in the context of the derivation and use of conversion factors is particularly important because the relevance of a particular conversion factor in estimating the protein content of a food or ingredient varies, depending on whether the intention is to estimate the amount of amino acids in a food or the total protein content. For the purposes of deriving and using conversion factors, protein can be considered in terms of amino acid content only or of amino acids plus prosthetic groups that may also be present. These prosthetic groups commonly include phosphoryl groups (from phosphorylation of amino acids) and oligosaccharides (from glycosylation of amino acids).

Consideration of protein as the sum of amino acids (without the prosthetic groups) keeps the focus on delivery of amino acids; in contrast, inclusion of prosthetic groups considers the whole protein, but may provide a higher estimate of protein content for the same amino acid content. A more holistic view of protein acknowledges that prosthetic groups are part of protein, and contends that focusing on amino acids alone ignores the other components of protein that could provide nutrition or other health benefits, even if their roles are not yet fully understood. Dairy proteins usually contain prosthetic groups, whereas any such groups associated with soy proteins are generally stripped off during the processing used to inhibit antinutritional activity. Prosthetic groups are an integral part of dairy proteins when used as ingredients in formulas.

It was unclear whether the recommended ranges of protein provided in the relevant Codex standards are intended to ensure adequate delivery of amino acids or of total protein (including prosthetic groups). Thus, it was agreed that two sets of conversion factors would be provided for consideration by CCNFSDU.

On the basis of the results of the systematic review and the confidence in these results as assessed by the expert panel via the GRADE assessment process, the following conversion factors were proposed:

Option 1: When protein is defined as being only the sum of the constituent amino acids (i.e. ensuring delivery of amino acids is the primary aim), the recommended conversion factor

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1 Many proteins are post-translationally modified (i.e. modifications to the protein which occur after it has been synthesized) resulting in the addition of prosthetic groups to the amino acids and an increase in the mass of the protein.
for dairy-based ingredients is 6.1 and for soy-based ingredients is 5.7. There was moderate certainty in the evidence underlying the conversion factor for dairy, and low certainty in the evidence underlying the conversion factor for soy.\(^1\)

**Option 2: When protein is defined as being the sum of amino acids and prosthetic groups (i.e. ensuring nutrition and potential health effects of the total protein is the primary aim), the recommended conversion factor for dairy-based ingredients is 6.3 and for soy-based ingredients is 5.7.** There was moderate certainty in the evidence underlying the conversion factor for dairy, and very low certainty in the evidence underlying the conversion factor for soy.

Importantly, the recommended conversion factors in Option 1 are based on conversion factors derived from amino acid composition analyses in which prosthetic groups were not included, and those in Option 2 on conversion factors derived from various analyses in which prosthetic groups were either implicitly or explicitly included. The factors recommended above are based largely on data from non-formula dairy and soy foods, and to a lesser extent on whole, finished formulas rather than formula ingredients, for which there is a paucity of data. This situation is acknowledged in the expert panel’s confidence in these factors, as formalized in the certainty of the evidence assessed via GRADE. The expert panel stressed that the recommended conversion factors are specifically for formulas and not for other dairy-based or soy-based ingredients or foods.

The selection of conversion factors (i.e. deciding on Option 1 or Option 2) ultimately depends on whether the primary aim of determining protein content is to ensure adequate delivery of amino acids or delivery of total protein. However, in terms of the strength of the supporting evidence, the expert panel considered Option 1 to be the preferred option, because the data underpinning the corresponding conversion factors are more robust (i.e. there are more data available for Option 1 at this time), and therefore the expert panel had greater confidence in the data for Option 1, as indicated in the overall higher certainty of evidence as assessed by GRADE. It was further noted that if CCNFSDU decides to use Option 2, further work will probably be needed to improve the accuracy of the estimates of the conversion factors, to be able to have greater confidence that the estimates reflect the true values.

The expert panel considered it critical that CCNFSDU select only one of the two options to disseminate. They are not intended as options to be presented to and decided on by manufacturers. It was felt that disseminating two different sets of factors and providing the option of picking one or the other for different applications, or otherwise mixing or swapping factors, would create considerable confusion and uncertainty.

The expert panel noted that the conversion factor of 6.25 currently used in the standards for infant formula and follow-up formula has not been empirically determined and agreed that its application to a wide variety of proteins is highly inappropriate. Therefore, the proposed conversion factors represent a considerable improvement over the current situation.

Nonetheless, confidence in the evidence underlying the proposed conversion factors is not high, reflecting shortcomings in the analytical methods most often used in deriving conversion factors (e.g. hydrolysis of protein), and the need to improve their accuracy. There

\(^1\) The conversion factors recommended in Option 1 are based on those that were derived by assessing protein nitrogen only (i.e. non-protein nitrogen was not included in the denominator of the equation for \(K_A\) as shown in Section 4.4). Therefore, when using the recommended conversion factors in Option 1, determination of protein nitrogen in the sample (i.e. total nitrogen minus non-protein nitrogen) and use of this value with the conversion factors should generally provide the most accurate estimate of protein content.
is considerable scope to determine more accurate factors (for both Options 1 and 2) through further research and the use of amino acid analysis with improved analytical methods.

The expert panel agreed that there is a very strong case for the relevant industries to invest in further collaborative efforts to improve the accuracy of the conversion factor estimates, using the best available methods to conduct amino acid analysis, and focusing on ingredients that are specifically used in formula products.

The expert panel also felt that it was important to retain the option for manufacturers to propose different conversion factors for particular ingredients where scientifically justified, as per footnote 2 in the Codex Alimentarius infant formula standard (CODEX STAN 72–1981). Such conversion factors should be determined using a prespecified, standardized amino acid methodology.
1. **INTRODUCTION**

The Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) established the Joint FAO/WHO Expert Meetings on Nutrition (JEMNU) in 2009 to provide scientific advice to the Codex Alimentarius Commission (CAC) and its subsidiary bodies, as well as Member Countries. JEMNU aims to provide relevant scientific advice in an independent, timely and cost-effective manner, and is convened in response to requests from specific Codex committees or Member Countries.

Breastfeeding is the cornerstone of optimal health and development for infants and young children, and remains the gold standard for infant feeding.\(^1\) Nonetheless, when infants and children are fed with breast-milk substitutes (including infant formula, follow-up formula and formula products for young children), it is important that these products comply with the relevant standards for composition and quality.

As part of deliberations on such standards, the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) has been considering the most appropriate nitrogen to protein conversion factors to employ in estimating the protein content of soy-based and milk-based ingredients used in infant formula and follow-up formula. To provide guidance on this topic, at its 39th Session in 2017, CCNFSDU requested that JEMNU be convened to review the evidence and develop evidence-informed guidance regarding nitrogen to protein conversion factors.

The requested work by JEMNU was thus initiated, and a meeting was convened by FAO and WHO in Geneva, Switzerland from 16 to 17 July 2019, to consider the specific issue of nitrogen to protein conversion factors for soy-based and milk-based ingredients used in infant formula and follow-up formula. Thirteen experts\(^2\) participated in the meeting, along with an external resource person, and representatives of the Codex Secretariat (see Annex 1 for the list of participants). As part of the requested work by JEMNU, a systematic review of the literature on nitrogen to protein conversion factors in soy-based and dairy-based foods and ingredients was commissioned. Also, because it was expected that there would be a significant number of relevant conversion factors not published in scientific literature databases, a call for data on conversion factors was issued in November 2018 (Annex 2).

The objectives of the meeting held on 16–17 July 2019 were to:

- review and interpret the evidence of the systematic review commissioned to compile and analyse the available data on nitrogen to protein conversion factors for foods containing soy-based and/or milk-based ingredients;

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\(^{1}\) WHO recommends early initiation of breastfeeding within 1 hour of birth, exclusive breastfeeding for the first 6 months of life, and introduction of nutritionally adequate and safe complementary foods at 6 months together with continued breastfeeding up to 2 years of age or beyond.

\(^{2}\) Members of the JEMNU expert panel were identified through a call for experts issued in February 2019 (Annex 3). As part of the application process, applicants were required to complete and submit a declaration of interest (DOI) form. DOI forms from experts who were provisionally selected based on their expertise were reviewed and discussed with the WHO Office of Compliance, Risk Management and Ethics (CRE). In addition, due diligence was conducted in the form of online searches for each expert going back 4 years. Based on these assessments, a possible conflict of interest was noted for one expert, Dr Elaine Krul. On the advice of CRE, Dr Krul therefore participated in the meeting in a modified capacity, in that she was allowed to participate in all discussions except for the final determination of recommended nitrogen to protein conversion factors (Agenda item 6, Annex 4). All other experts participated fully in all discussions.
• determine appropriate conversion factors for soy-based and milk-based ingredients used in infant formula and follow-up formula; and

• provide any additional, relevant information to be included in the meeting report to be submitted to the 41st Session of CCNFSDU, to be held in Düsseldorf, Germany, from 24 to 29 November 2019.

At the meeting, the members of the JEMNU expert panel introduced themselves, outlined their areas of expertise and declared their interests. Dr Richard Cantrill was proposed and accepted as chair, and he chaired the discussion for the remainder of the meeting.

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1 The discussion in this paper relates to products covered by the Codex standards on infant formula and follow-up formula, which includes formula products intended for infants, older infants and young children up to the age of 36 months. Where the terms “formula”, “formulas” and “formula products” are used without specifying “infant formula” or “follow-up formula”, they refer to the full range of products for infants and children from birth to 36 months.
2. BACKGROUND

Dr Chizuru Nishida, Department of Nutrition for Health and Development, WHO, summarized the background to the meeting and the JEMNU process.

Following the lapse of an earlier Joint FAO/WHO Expert Committee on Nutrition, CCNFSDU has expressed the need for a joint FAO/WHO mechanism to provide scientific advice on nutrition to the Codex and its subsidiary bodies, in addition to the provision of scientific advice being provided independently by FAO and WHO. After extensive reviews and discussions between FAO and WHO, the establishment of JEMNU, and the procedures and mechanisms for its operation, were proposed to CCNFSDU in 2009 and to the Codex Committee on Food Labelling in 2010, and have been included in the Codex Procedural Manual since the 40th CAC in 2017. The JEMNU mechanism involves the identification of relevant experts, taking into consideration geographical representativeness and gender balance, and the conduct of systematic reviews, including objective assessment of the certainty in the available evidence. The JEMNU process was activated for the first time with a request issued at the 39th Session of CCNFSDU in December 2017 for scientific advice on nitrogen to protein conversion factors for soy-based and milk-based ingredients used in infant formula and follow-up formula.

Ms Maria Xipsiti, Nutrition and Food Systems Division, FAO, provided an overview of the relevant Codex standards, including the standard on infant formula and formulas for special medical purposes (CODEX STAN 72-1981) and the standard for follow-up formula (CODEX STAN 156-1987). Since Codex standards often serve as the basis for national legislation, these standards can have a profound impact on infant and young child nutrition and health. CCNFSDU completed a review and updating of the infant formula standard in 2007, and a review of the standard for follow-up formula is currently ongoing. This review of the standard for follow-up formula will make a distinction between products targeted at older infants (aged 6–12 months) and those aimed at young children (aged 12–36 months).

The infant formula standard sets minimum and maximum protein levels, based on a nitrogen conversion factor of 6.25. The standard also indicates that:

- a conversion factor of 6.38 is generally used for other milk products, whereas 5.71 is generally used in other soy products; and
- for an equal energy value, the formula must contain an available quantity of each essential and semi-essential amino acid at least equal to the amount contained in breast milk (the reference protein, defined in Annex 1 of the standard).

Similarly, the protein levels in the standard on follow-up formula are currently based on a conversion factor of 6.25. This factor was originally based on the premise that all nitrogen present was in the form of amino acids, but once again the standard notes the general use of 6.38 as a conversion factor for other milk products.

Following discussion during this standard review process about the accuracy and appropriateness of conversion factors, CCNSFDU requested scientific advice on the establishment of the most appropriate nitrogen to protein conversion factors for soy-based and milk-based ingredients used in infant formula and follow-up formula.
As per the JEMNU terms of reference, this request was formulated in PICO (population, intervention, comparator and outcome) question format.\(^1\) To increase the specificity of the questions – which then provides more precise guidance in conducting the systematic review and facilitates assessment of evidence via the Grading of Recommendations, Assessment and Evaluation (GRADE) approach\(^2\) – the original PICO questions were further refined, in particular to acknowledge the different ways in which “protein” can be considered. The final questions were as follows:

1. When using the equation \( \text{amount of protein (P)} = \text{nitrogen to protein conversion factor (K)} \times \text{amount of nitrogen (N)} \) to estimate the protein content of dairy-based ingredients used in infant formula and follow-up formula, which value of K most closely estimates the true amount of protein (P), \( \text{where “protein” is defined as amino acid content only} \)?

2. When using the equation \( \text{amount of protein (P)} = \text{nitrogen to protein conversion factor (K)} \times \text{amount of nitrogen (N)} \) to estimate the protein content of soy-based ingredients used in infant formula and follow-up formula, which value of K most closely estimates the true amount of protein (P), \( \text{where “protein” is defined as amino acid content only} \)?

3. When using the equation \( \text{amount of protein (P)} = \text{nitrogen to protein conversion factor (K)} \times \text{amount of nitrogen (N)} \) to estimate the protein content of dairy-based ingredients used in infant formula and follow-up formula, which value of K most closely estimates the true amount of protein (P), \( \text{where “protein” is defined as amino acids plus prosthetic groups} \)?

4. When using the equation \( \text{amount of protein (P)} = \text{nitrogen to protein conversion factor (K)} \times \text{amount of nitrogen (N)} \) to estimate the protein content of soy-based ingredients used in infant formula and follow-up formula, which value of K most closely estimates the true amount of protein (P), \( \text{where “protein” is defined as amino acids plus prosthetic groups} \)?

This set of PICO questions served as a framework for the commissioned systematic review and for the expert panel’s discussions.

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\(^1\) PICO questions were originally prepared by representatives from Canada and the United States of America, with guidance from FAO and WHO. The original PICO questions can be found in Annex 5.

\(^2\) More information on GRADE can be found at the GRADE working group’s website http://www.gradeworkinggroup.org/.
3. THE GRADE FRAMEWORK FOR ASSESSING QUALITY OF EVIDENCE

Professor Joerg Meerphol, University of Freiburg, Germany, presented an overview of the GRADE approach to assessing the quality of evidence generated via systematic review and meta-analysis.

The GRADE approach has been developed primarily to assess the quality of evidence generated by clinical and public health interventions, but is now also proving valuable in other areas, including diagnostics and prognostics. It provides a framework for assessing the certainty in a body of evidence; that is, the extent to which expert reviewers are confident that an estimate of an effect or association derived from one or more studies is close to the true effect in a population. There are four levels of certainty, ranging from high to very low. In the context of clinical or public health guideline development, assessing the quality of the evidence is the first step, followed by a structured approach to translating the evidence into guidance.1

Using GRADE, certainty in a body of evidence can be downgraded or upgraded based on a number of factors related to how well the studies were conducted and the quality of the results. Evidence can be downgraded for the following reasons:

- systematic study limitations that could lead to bias in the results (risk of bias);
- unexplained inconsistencies or variability in the data across studies (inconsistency);
- evidence that is indirectly, rather than directly, related to the question being asked (indirectness); and
- imprecision due to small sample size or wide confidence intervals around the estimated effect (imprecision).

Certainty can also be downgraded if there appears to be publication bias.

The rating of the certainty in the evidence can also be upgraded as a result of mitigating factors that are sometimes observed in epidemiological studies; however, such factors were not relevant to the analytical studies included in the systematic review on conversion factors. Upgrading was therefore not employed in the assessment of the quality of the evidence in this case.

3.1 GRADE evidence profiles used in the systematic review

The judgements made for risk of bias, inconsistency, indirectness, imprecision, publication bias and any possible upgrading are summarized in GRADE evidence profiles. The evidence profiles generated for the work of JEMNU provided a useful framework to inform the decisions of the expert panel on the advice to provide to CCNFSDU; the final versions, as modified following the discussions of the expert panel, are provided in Annex 5. For the JEMNU work, the assessments of certainty in the evidence can be considered as follows:

1 Translating evidence into guidance was not relevant to the expert panel’s discussions. However, it was noted that in the course of its deliberations on the scientific advice received from JEMNU, CCNFSDU may touch on certain elements normally considered in translating evidence to guidance, such as resource implications and feasibility of implementation.
• **High:** Very confident that the true conversion factor lies close to that of the estimate.

• **Moderate:** Moderately confident that the true conversion factor is likely to be close to that of the estimate, but there is a possibility that it is substantially different.

• **Low:** Confidence in the estimate is limited: the true conversion factor may be substantially different from the estimate.

• **Very low:** Very little confidence in the estimate: the true conversion factor is likely to be substantially different from the estimate.

### 3.2 Discussion of the GRADE framework

There was discussion about whether evidence should automatically be downgraded if it came from old studies, with the implication being that older studies may have used methods that are less robust or accurate than those used in more recent studies. The expert panel noted that the date of publication is not important per se; however, it is important to consider how different analytical methods might affect confidence in the results (e.g. through risk of bias, which may or may not loosely correlate with date of publication).
4. RESULTS OF SYSTEMATIC REVIEW ON NITROGEN TO PROTEIN CONVERSION FACTORS

Professor Daniel Tomé, AgroParis Tech, France, presented the draft systematic review and modelling analysis commissioned by FAO and WHO to provide a full picture of the evidence.1

The most commonly used method for calculating protein content of food products is to measure nitrogen content and then convert this to protein using a conversion factor (K). The conversion factor has traditionally been set at 6.25 on the assumption that proteins contain a fixed amount of nitrogen (16%), based on the average nitrogen content of amino acids commonly found in proteins, and the assumption that all or most of the nitrogen in food is derived from protein. This approach can introduce significant errors, because:

- different proteins contain different ratios of amino acids;
- nitrogen content varies between different amino acids; and
- foods may contain nitrogen from sources other than protein (non-protein nitrogenous compounds).

Hence, the actual nitrogen content of protein can vary from about 13% to 19%, meaning that a default conversion factor of 6.25 may not be appropriate for all protein sources. Specific values may be appropriate for different foods and ingredients, but there are no standardized methods established for deriving these values.

In early studies, conversion factors were based on the purification of crude protein fractions from foods or food ingredients, and conversion factors were calculated from the ratio of the crude protein mass to the mass of the nitrogen measured in the purified crude protein fraction (i.e. protein mass divided by nitrogen mass). Newer approaches rely on more accurate conversion factors that are based on knowledge of the amino acid composition obtained through amino acid analysis or amino acid sequencing.

Another issue is that many proteins undergo post-translational modification (i.e. modifications that occur after a protein has been synthesized) by processes such as phosphorylation or glycosylation, which result in the addition of prosthetic groups to the amino acids and increase the mass of the protein. Hence, for deriving and using nitrogen to protein conversion factors that are dependent on the assessed weights of nitrogen and protein, protein can be defined in two different ways, which have different effects on the weight of protein. The two definitions of protein are:

- the sum of weight of amino acid residues only; or
- the sum of weight of amino acid residues plus the sum of weight of prosthetic groups.

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4.1 Other key issues for consideration in the derivation of nitrogen to protein conversion factors

In addition to the issue of how to define “protein” to be measured, other key issues relevant to different approaches for derivation of nitrogen to protein conversion factors are the:

- anhydrous weight of amino acids;
- contribution of amide amino acids to protein nitrogen content; and
- contribution of non-protein nitrogen to total nitrogen.

These issues are discussed below.

Anhydrous weight of amino acids

The anhydrous weight of amino acids (i.e. the weight of the free amino acid residue minus the weight of one water molecule) should be used to determine protein weight, because each amino acid in a polypeptide chain loses one water molecule during polymerization. Using the summated weight of free amino acids in the derivation of conversion factors would therefore greatly overestimate the protein weight for longer polypeptide chains.

Contribution of amide amino acids to protein nitrogen content

During standard amino acid analysis (with acid hydrolysis), glutamine is converted to glutamic acid and asparagine to aspartic acid. If the ratio of amide to acid forms is not taken into consideration, this can introduce a major error to the calculation of total nitrogen in amino acid residues. It is important, therefore, to use specific analytical conditions to help preserve the amide nitrogen, or to use methods to calculate an estimate of the amide nitrogen content. An alternative is to assume a fixed, arbitrarily assigned, ratio between the acid and amide forms for each protein source; the ratios 75/25 or 50/50 are often applied.

Contribution of non-protein nitrogen to total nitrogen

Foods and food ingredients can contain highly variable amounts of other nitrogenous compounds such as nucleic acids, amines, urea, ammonia, nitrites, vitamins, alkaloids, phospholipids and nitrogenous glycosides. If non-protein nitrogen is not corrected for, it can result in an overestimation of protein content in foods containing appreciable amounts of non-protein nitrogen. When using conversion factors based on amino acid composition (in which nitrogen is generally assessed as protein nitrogen only, and non-protein nitrogen is not accounted for), this correction can be achieved by determining protein nitrogen in the sample (achieved by subtracting the non-protein nitrogen from the total nitrogen) and using this value in calculating protein content via the conversion factor. This approach not only minimizes possible overestimation of protein content, but also has the potential to expose contamination or adulteration of foods and ingredients with nitrogenous compounds, such as melamine.

The different components and ways of considering nitrogen and protein are summarized in Fig. 1.
4. RESULTS OF SYSTEMATIC REVIEW ON NITROGEN TO PROTEIN CONVERSION FACTORS

Fig. 1 Protein and nitrogen in foods

<table>
<thead>
<tr>
<th>Protein = sum of weight of amino acid residues and of prosthetic groups</th>
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<tr>
<td>Protein-associated prosthetic groups</td>
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<tr>
<td>Prosthetic groups</td>
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<tr>
<td>Non-protein nitrogenous compounds</td>
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<td>Protein nitrogen</td>
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4.2 Literature review

A total of 3881 records were identified initially. Ultimately, 214 records on analytical methods and 72 records (including five sets of unpublished data) on nitrogen conversion factors were reviewed.

4.3 Analytical methods

The systematic review included a review of the analytical methods used for nitrogen, amino acid and protein content in foods. The methods used to determine total nitrogen include the Kjeldahl procedure and the Dumas method. Amino acid analysis involves sample hydrolysis (typically for 24 hours) to break a protein down into its constituent components, followed by analysis with methods such as ion exchange chromatography, gas chromatography, high-performance liquid chromatography, ultra-high-performance liquid chromatography and electrophoresis.

4.4 Estimates of nitrogen to protein conversion factors

Three types of nitrogen to protein conversion factor were designated in the review:

- \( K' = \frac{\text{sum of the weights of anhydrous amino acid residues + sum of the weights of prosthetic groups}}{\text{weight of protein nitrogen or weight of total nitrogen}} \)
- \( K_A = \frac{\text{sum of the weights of anhydrous amino acid residues}}{\text{weight of protein nitrogen}} \)

Note: this also needs to be adjusted for the percentage of acid and amide versions of relevant amino acids in the sample, whether determined directly or calculated on the basis of a fixed ratio, giving rise to three specific types of \( K_A \):

- \( K_A \) (directly calculated amide to acid ratio)
- \( K_A \) (50/50 amide to acid ratio)
- \( K_A \) (75/25 amide to acid ratio)

- \( K_B = \frac{\text{sum of the weights of anhydrous amino acid residues}}{\text{weight of total nitrogen}} \)

The respective characteristics of these factors can be summarized as follows:

- \( K' \) includes prosthetic groups in the numerator and will, therefore, overestimate the amino acid content of samples when prosthetic groups are present (not relevant when defining protein as sum of amino acids plus prosthetic groups). It may or may not include non-protein nitrogen in the denominator; therefore, sometimes it may overestimate the amount of protein in samples with appreciable amounts of non-protein nitrogen.
- $K_A$ in its various forms does not include prosthetic groups in the numerator, only (anhydrous) amino acids. Also, it does not include non-protein nitrogen in the denominator (it includes protein nitrogen only). It will thus overestimate the amount of protein when used on samples with appreciable amounts of non-protein nitrogen. However, when using this factor, the non-protein nitrogen content of a sample can be determined empirically, and the total nitrogen value can then be corrected to give protein nitrogen only.

- $K_B$ does not include prosthetic groups in the numerator, only (anhydrous) amino acids. It includes total nitrogen (i.e. protein nitrogen plus non-protein nitrogen) in the denominator. However, because non-protein nitrogen content can vary substantially across food products, a particular $K_B$ value may only be relevant when used on the same or very similar foods from which the particular $K_B$ was derived.

The systematic review identified values for these different types of conversion factors for milk protein-based foods and soy-based foods and ingredients. Data for each type of conversion factor were then pooled to generate pooled estimates, which are summarized in Table 1.

### Table 1. Pooled estimates for nitrogen to protein conversion factors

<table>
<thead>
<tr>
<th></th>
<th>DAIRY</th>
<th>SOY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (95% CI)</td>
<td>Range</td>
</tr>
<tr>
<td><strong>FORMULAS ONLY</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K'$</td>
<td>6.38$^a$</td>
<td>–</td>
</tr>
<tr>
<td>$K_A$ 50/50</td>
<td>6.08 (6.05, 6.12)</td>
<td>5.97–6.17</td>
</tr>
<tr>
<td>$K_A$ 75/25</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$K_A$ direct adjust</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>ALL SAMPLES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K'$</td>
<td>6.32 (6.26, 6.38)</td>
<td>6.07–6.39</td>
</tr>
<tr>
<td>$K_A$ 50/50</td>
<td>6.06 (6.00, 6.12)</td>
<td>5.57–6.37</td>
</tr>
<tr>
<td>$K_A$ 75/25</td>
<td>5.83 (5.77, 5.89)</td>
<td>5.66–6.08</td>
</tr>
<tr>
<td>$K_A$ direct adjust</td>
<td>6.03 (5.98, 6.07)</td>
<td>5.83–6.15</td>
</tr>
<tr>
<td>$K_B$</td>
<td>5.55 (5.31, 5.78)</td>
<td>5.33–5.75</td>
</tr>
</tbody>
</table>

CI: confidence interval.

$^a$ Single measurement only.

$^b$ Two measurements, both with a value of 5.42.

The certainty in the evidence for these pooled estimates was then assessed using the GRADE process and included in the review (see Section 6 of this report for more on this process and discussion of the results).
5. DISCUSSION OF THE SYSTEMATIC REVIEW

5.1 Analytical methods

The expert panel discussed the relative merits of the different analytical methods in use and described in the review.

In relation to methods for the determination of nitrogen, both the Kjeldahl method and the Dumas method are long established, and both have advantages and disadvantages. The Kjeldahl method is most commonly used but, unless it is modified appropriately, it generally does not measure all nitrogen in a sample because it does not measure nitrates or nitrites. A benefit of this method is that it is possible to analyse liquid samples with a wide range of volumes. The Dumas method is becoming more common and the expert panel indicated that this method can have several advantages, in that it includes nitrates and nitrites in nitrogen, uses less hazardous chemicals, takes less time and can be automated. It gives a slightly higher nitrogen value because it includes nitrates and nitrites, but it has limitations when assessing liquid samples with low nitrogen concentration.

A common method for measurement of protein is summation of constituent amino acids via amino acid analysis. However, problems associated with the protein hydrolysis step create errors, and specific methods are necessary to minimize those errors (see Annex 6). Duration of hydrolysis is particularly problematic, but a possible solution suggested was an existing methodology that uses curvilinear model fitting. This approach incorporates multiple hydrolysis times to model amino acid release and destruction, and has been validated against amino acid composition derived from sequencing data. However, because of the multiple hydrolysis times, curvilinear model fitting is more expensive than methods employing a single hydrolysis.

The expert panel also discussed the emergence of newer methods (e.g. mass spectrometry) that offer the promise of being able to measure, identify and summate the amino acids in a sample directly and rapidly. Such methods largely obviate the need for conversion factors, save for sporadic spot-checking of samples against standards as part of quality control. It was noted that Codex committees were aware of such approaches, having already mentioned them in various documents. However, much work remains to be done before such approaches are mainstream, as illustrated in an example provided during the meeting of using mass spectrometry on dairy proteins, which provided results that were not necessarily better than amino acid composition analysis and were worse than nitrogen-based methods. Nevertheless, the expert panel suggested that these methods might be applicable for protein analysis once they are fully developed.

The expert panel agreed that there is an urgent need for a concerted international research effort to accurately determine the amino acid content in all milk-based and soy-based ingredients used in formula products, using the best available analytical methods.
5.2 Protein as amino acids only or protein as amino acids plus prosthetic groups

There was a great deal of discussion on the relative merits of determining protein as the sum of amino acids only, or as the sum of amino acids plus the sum of prosthetic side chains. Both options have strengths and weaknesses, and perspectives vary according to different ideas on the most nutritionally appropriate approach and on the purpose of quantifying protein in formula products. There was agreement that, nutritionally, protein is more than the sum of its amino acids, and acknowledgement that a more holistic view of protein recognizes that the other components of protein may have an important biological role to play. However, for many of the expert panel members the key role of protein in formulas is to deliver amino acids for growth and development, and the purpose of protein quantification is to ensure that the amino acid requirements are fulfilled.

To consider protein as the sum of amino acids (without the prosthetic groups) keeps the focus on delivery of amino acids, with protein effectively functioning as a proxy for amino acids. The inclusion of prosthetic groups may complicate the process of protein determination, including increasing the apparent protein content of a sample without increasing the amino acid content. It was argued that this could lead to unfavourable comparisons for products with no prosthetic groups in the protein, or those that have not been extensively studied for such groups, which would then appear to have a lower amino acid content (e.g. acid whey isolate was noted as being a major ingredient in formulas lacking prosthetic groups). Also, this would be likely to lead to variable delivery of amino acids across different foods, depending on the presence, absence or frequency of prosthetic groups, which can be predicted through amino acid sequencing, but does not accurately measure the prosthetic groups that are present and intact in the final processed protein product.

This variability could have implications for the appropriate minimum and maximum levels in the compositional standards for formula products. In particular, the expert panel noted that using conversion factors in which protein is defined as amino acids only would result in an increased delivery of total protein for those proteins with prosthetic groups, and that overconsumption of protein during infancy has been flagged as a concern by some. It was further noted, however, that discussing the amount of protein infants should consume was beyond the scope of the meeting; that is, issues about amount of protein that should be consumed should be addressed not by the conversion factor (which is intended only to provide an estimate of the amount of protein) but by the amount of protein recommended in relevant standards, and would be for another committee to consider if necessary.

An alternative view is that because prosthetic groups are part of protein, to focus on amino acids alone is to ignore the other components of protein that could be beneficial from a nutrition or health perspective. Relatively little is known about prosthetic groups (although more is known about such groups in dairy proteins), and their role in nutrition and health is not yet fully understood. The expert panel acknowledged that whole proteins are likely to have other important properties, such as accompanying bioactive peptides; as with the prosthetic groups, such peptides are not accounted for by conversion factors based solely on the sum of amino acids.

The expert panel also noted that heat treatment and other forms of processing can affect the presence, absence or frequency of prosthetic groups, which can cause variability even across the same food ingredient depending on the level of processing. As an example, although soy
protein contains prosthetic groups, these are destroyed by the heat treatment used to inhibit antinutritional activity.1

Although the importance of total protein in relation to overall health was noted, it was suggested that in terms of nutrition derived from formulas, the delivery of amino acids is the primary and most important role of proteins. It was further suggested that there may be other ways to quantify and address the nutritional role of prosthetic groups separately from protein measurements, similar to the approach taken for vitamins and minerals, for example.

The expert panel noted that further work should be done on assessing prosthetic groups in foods other than dairy, and on further elucidation of nutrition and health effects of total protein containing prosthetic groups.

Because it was unclear whether the recommended ranges of protein provided in the relevant Codex standards are to ensure adequate delivery of amino acids or of total protein, it was agreed that it would be appropriate to provide two sets of conversion factors in the advice to CCNFSDU: one set for sum of amino acids only and one for amino acids plus prosthetic groups. It was also agreed to indicate, if possible, the expert panel’s preferred option, and to emphasize that selection and application of only one of these options (two sets of values, one for dairy and one for soy) will be essential to avoid confusion in the long term.

5.3 Non-protein nitrogen

Non-protein nitrogen can be absorbed and used metabolically. The levels can be highly variable both between and within types of protein products, and data are scarce, despite established methods for determination. For milk and milk products, there is an International Organization for Standardization (ISO) standard2 for determining non-protein nitrogen, but the acid precipitation method described in the standard is specific to dairy protein and may not accurately determine non-protein nitrogen in other protein ingredients. In addition, formula products may also contain free amino acids that would need to be included in the measurement of non-protein nitrogen; some free amino acids might be released during processing; and, for some methods currently used in determining non-protein-nitrogen, there may sometimes be less than complete precipitation of protein. Whenever non-protein nitrogen is determined, the method needs to be optimized to ensure that protein precipitation is complete, and to correct for any free amino acids or peptides present in the solute.

It was suggested that a threshold for non-protein nitrogen content (e.g. 2%) would be appropriate for the direct application of \( K' \) and \( K_A \) conversion factors. It was also suggested that industry be encouraged to carry out the work necessary to determine the non-protein nitrogen in ingredients used in dairy-based and soy-based formula products, based on standardized procedures (ideally using a single method that applies to both dairy and soy proteins, but taking into account the need to optimize recoveries for the particular protein).

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5.4 Anhydrous amino acids

The expert panel noted that, for the purposes of nitrogen to protein conversion, calculations need to be carried out on the basis of anhydrous amino acids.

5.5 Amide to acid ratio of amino acids

The expert panel discussed the difficulties in determining the ratio of amide and acid forms of glutamine/glutamic acid and asparagine/aspartic acid. The ratio can be set arbitrarily (50/50 and 75/25 are commonly used) or assessed directly, with the latter being more time-consuming and costly. However, it was noted that the fixed ratio of 50/50 gives very close agreement with the results of direct determination for dairy and soy protein. Also, the necessary data on the percentage of amide and acid forms of amino acids are rarely provided, and this issue is often ignored by researchers and analysts. When analysis for amino acids in foods is conducted, it is important that the amide forms are measured. Even when the amino acid sequence of the proteins is known, deamidation may occur during processing, which would affect the amide to acid ratio.

5.6 Converting nitrogen to protein for ingredients or for final products

Most of the data used by the expert panel to derive recommended conversion factors for formulas came from food sources other than formulas; where data did come from formulas, they were mostly from whole, finished formulas rather than from ingredients. There was in fact very little data identified for individual ingredients commonly used in formulas. It was stressed, however, that there can be some discrepancies between conversion factors that are appropriate for ingredients and those that are appropriate for final products, depending on the food matrix. Therefore, concern was expressed that the estimates were coming from sources other than ingredients that could have different conversion factors associated with them. This concern was noted, but was also partially captured in the GRADE assessment, in that the relevant conversion factors were downgraded for indirectness because they were not derived primarily from formulas or formula ingredients. It was further noted that although it would be ideal to have ample data for all major formula ingredients, in reality almost no such data were identified. In the absence of these data, the best available data were used. The expert panel noted that there is an opportunity for a multinational effort to fill the gaps in the evidence with high-quality data. Ideally, there would be specific conversion factors for protein sources used in the manufacturing of formulas and for different formulas.

Related to the concern about different ingredients possibly having different relevant conversion factors, a suggestion was raised about the possibility of allowing manufacturers to submit for consideration conversion factors that may be more appropriate for specific ingredients, provided those conversion factors have been derived using a pre-specified, standardized amino acid methodology. This suggestion follows from footnote 2 in the Codex infant formula standard (CODEX STAN 72-1981), which states that the currently recommended conversion factor be used, unless a scientific justification is provided for the use of a different conversion factor for a particular product. Although there was support for this among the expert panel, it was not clear how the standardized method would be identified. It was further suggested that if a request were made to the International Dairy Federation (IDF) and the ISO, they might be able to provide a method. It was further noted that such an IDF/ISO method for several foods – including dairy and soy – is currently being developed. No specific guidance was developed on this matter.
5.7 Protein variation in formulas for different age groups

It is important to recognize differences in the composition of formula products for different age groups, including the non-protein nitrogen components. The differences in how industry characterizes formulas and how they are characterized in Codex Alimentarius documents was discussed.

To get as close as possible to breast milk – which has a higher ratio of whey to casein than cow’s milk – when manufacturing infant formula, the industry relies heavily on whey protein from cheese manufacture. During analysis, lower molecular weight peptides (i.e. macropeptides) and free amino acids in such ingredients may contribute to non-protein nitrogen. Infant formula and follow-up formula (up to 12 months) are similar in composition, and they differ from products for young children. As the target age increases, products become more similar to cow’s milk, in which protein comprises 20% whey and 80% casein. This means that there may be differences in the amount of non-protein nitrogen in formulas for different age groups.

Considering these differences and the evolving nutritional needs of infants and young children as they grow, the compositional standards for formulas for different age groups are adjusted to meet age appropriate nutritional needs. The proposed revisions to the Codex standard for follow-up formula define the formula for infants up to 12 months as “follow-up formula for older infants”, and they distinguish these from products for young children over the age of 1 year (such products have not yet been named). The essential composition and quality requirements for follow-up formula for older infants will closely mirror those for infant formula. The standard will also cover the formula for young children, from the ages of more than 12 months up to 3 years, and the compositional requirements will be different for products for this age group.

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6. QUALITY OF EVIDENCE FOR NITROGEN TO PROTEIN CONVERSION FACTORS: GRADE ASSESSMENTS

Dr Jason Montez, Department of Nutrition for Health and Development, WHO, described how the certainty in the evidence for these conversion factors had been preliminarily assessed using the GRADE process. A separate GRADE evidence profile had been produced for each of the four PICO questions, and included in the draft systematic review. It was noted that the preliminary assessments were a starting point for discussion, and it was expected that the expert panel would discuss, and further refine and modify, both the preliminary assessments and any assumptions made in making those assessments, as necessary, based on the expert panel’s expertise and understanding of the evidence.

6.1 Discussion of the GRADE assessment process

This was a novel application of GRADE and a new process for most of the experts on the panel; hence, panel members needed time to understand and fully adapt to using this methodology. It was suggested that, because this was a novel application of GRADE, it might be more appropriate for the expert panel to thoroughly vet the application before using it for the JEMNU work. It was noted, however, that although the subject matter was novel, the principles of assessing certainty in the evidence used in GRADE had not been modified. Some small adaptations needed to be made in how some of the elements were assessed (e.g. thresholds for making judgements about downgrading or upgrading), but the process itself was unchanged.

There was clear recognition that a major advantage of the GRADE assessment procedure is the transparency that it brings to the process. Although there will always be a degree of subjectivity in the assessments themselves, the transparent process means that all judgements made leading to the assessments can be understood, reviewed and challenged. The expert panel agreed that the process provided a framework for discussing study limitations (which otherwise would have been discussed in an unstructured manner), and was useful for facilitating nuanced discussion, reaching a conclusion and enabling understanding of how such a conclusion had been reached. Also, GRADE highlighted the areas that needed to be improved in order to increase confidence in available conversion factors. All of this, in turn, will allow other expert bodies to review and validate or challenge the approach and conclusions.

There was clarification that there is no weighting of the different criteria (because each of the domains can threaten the validity of the data) and that the inclusion of unpublished data (submitted in response to the call for data) does not necessarily mean that the certainty of evidence needs to be downgraded. Although unpublished data may not have been peer-reviewed as part of a publication process, it was felt that there were no significant issues with the data received through the call for data.
6.2 Discussion of specific elements of the GRADE quality assessment process

The expert panel reviewed the initial assumptions and “rules” (e.g. thresholds for decision-making) for assessing the evidence.

Indirectness

The expert panel confirmed that it was appropriate to downgrade any conversion factor values that were based predominantly on dairy or soy products other than infant or follow-up formulas for indirectness, because some data for conversion factors were derived directly from formulas, and the primary interest was conversion factors for formulas. In addition, in the preliminary assessments, $K_a$ estimates were initially downgraded because they did not take into consideration the possible contribution of non-protein nitrogen; similarly, $K_a$ values based on arbitrarily fixed ratios of 50/50 and 75/25 were downgraded because they were not as accurate as directly adjusted ratios. However, given the variable nature of non-protein nitrogen across foods, participants considered that the differences between arbitrarily set and directly adjusted amide to acid ratios would not be too significant (although they did consider that there was greater confidence in 50/50 than in 75/25, given that the former more closely tracks with directly adjusted values for dairy and soy protein); hence, they did not feel that downgrading for these reasons was appropriate. It was proposed to simply note in the footnote that there would be greatest confidence in the directly assessed amide to acid ratios (and greater confidence in the 50/50 ratio than in the 75/25).

Imprecision

The expert panel discussed the precise rationale for downgrading confidence in results based on a small number of studies. The degree of confidence in the values is highly dependent on the quality of the research and laboratory conducting the studies. Nonetheless, there was broad agreement that the greater the number of studies, the greater the robustness of the values, and thus that some downgrading for a small study number is appropriate.

6.3 Final assumptions and rules used in assessing certainty of evidence

Incorporating feedback from the expert panel, the final assumptions and rules used in assessing the certainty of the evidence were established as follows:

- **Certainty starting point**: Given the inherent uncertainty of the analytical methods, it was decided that all assessments should initially be considered as “moderate” (i.e. should start at moderate), unless the values were determined by amino acid sequencing or multiple hydrolyses, in which case assessments would initially be considered as “high”. These initial assessments were then downgraded for factors that negatively affect the quality of the evidence.\(^1\)

- **Risk of bias**: level of certainty was downgraded if there was any indication of systematic measurement error or reporting error (selective reporting).

- **Inconsistency**: certainty was downgraded if variance around the mean was 10% or more.

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\(^1\) Using GRADE, it is usual to start at “high” certainty of evidence for high-quality randomized controlled trials and “low” certainty for observational studies, and then downgrade or upgrade from that starting point.

\(^2\) There were no instances of upgrading evidence.
• **Indirectness** – certainty was downgraded for values primarily derived from dairy or soy sources other than infant and follow-up formulas. Where the PICO question related to protein defined as amino acids only, those conversion factor values derived from weight of amino acids plus prosthetic groups were downgraded; where the PICO question related to protein defined as amino acids plus prosthetic groups, only those conversion factor values derived solely from weight of amino acids were downgraded.

• **Imprecision** – certainty was downgraded for values based on fewer than 10 measurements and was downgraded twice for values where fewer than five studies contributed to the mean.

The GRADE evidence profiles were updated to reflect the changes discussed (Annex 5). The footnotes provide detailed explanations of the rationale for downgrading or not downgrading the degree of confidence in the evidence.

The final conclusions of the GRADE review seemed closely aligned with the expert panel’s own independent assessments of the certainty in and strength of the evidence, based on panel members’ expertise and knowledge of the field.

The GRADE assessment process highlights that the level of proof is far from ideal, and that there is a need for further investment in research, with improved analytical methods and more emphasis on amino acid analysis. Improving the analytical methods would increase the certainty in the evidence. **Annex 6** provides suggestions on the currently best available analytical methods for amino acid analysis that should be considered for future work.
7. FINAL DETERMINATION OF RECOMMENDED NITROGEN TO PROTEIN CONVERSION FACTORS

On the basis of the revised GRADE evidence profiles (Annex 5), the expert panel discussed the selection of the recommended conversion factors, taking into account the degree of certainty in the quality of the evidence as assessed by GRADE.

The discussion on whether the primary function of Codex standards for protein content in formula products is to deliver amino acids or total protein has already been noted in Section 5.2. It was noted that because previous Codex documents have referred to assessing protein content via methods in which individual amino acid weights are directly summated without consideration of prosthetic groups, this could imply that some Codex committees consider amino acid delivery as the primary function. Nonetheless, FAO and WHO were unable to get clarity prior to or during the meeting on whether Codex protein standards consider protein as amino acids only or as amino acids plus prosthetic groups. Therefore, because it is unclear whether the recommended ranges of protein provided in the relevant Codex standards are intended to ensure adequate delivery of amino acids or total protein, it was agreed to provide the CCNFSDU with two sets of conversion factors in which:

- protein is defined as the sum of amino acids only (Option 1); and
- protein is defined as the sum of amino acids plus the sum of prosthetic groups (Option 2).

The expert panel was clear that it is critical that CCNFSDU select one set of conversion factors to be advanced. Wider dissemination of two sets of conversion factors or any mixing of the two sets of conversion factors could set a dangerous precedent, leading to the proposal of additional conversion factors for different foods and ingredients, which would clearly create further confusion and a worsening of the current situation. The two options are discussed below.

Option 1: Protein defined as the sum of amino acids only

- **Dairy** – the suggested conversion factor is 6.1.
  - Derivation: Two results have a moderate level of confidence (6.08 and 6.06). The mean of these values was calculated (6.07), and was rounded to the nearest tenth, 6.1.

- **Soy** – the suggested conversion factor is 5.7.
  - Derivation: The mean was calculated for the two results with low certainty of evidence (rather than very low), excluding the $K_\alpha$ 75/25 result (which is less preferable to the 50/50 or direct-adjusted values of $K_\alpha$). The values were 5.68 for $K_\alpha$ 50/50 (all samples) and 5.65 for $K_\alpha$ direct adjustment. The calculated mean of these values is 5.67, which was rounded to the nearest tenth, 5.7.
Option 2: Protein defined as the sum of amino acids plus prosthetic groups (K’)

- **Dairy** – The suggested conversion factor is **6.3**.
- Derivation: There was one result for K’, which is 6.32 and for which there is moderate certainty of evidence. This was rounded to the nearest tenth, **6.3**.
- **Soy** – The suggested conversion factor is **5.7**.
- Derivation: There was only one result for K’, which is 5.71 and for which there is a very low level of certainty. This was rounded to the nearest tenth, **5.7**.

The selection of conversion factors (i.e. Option 1 or Option 2) ultimately depends on whether the primary aim of determining protein content is to ensure adequate delivery of amino acids or delivery of total protein. However, in terms of the strength of the supporting evidence, the expert panel considered Option 1 to be the preferred option, because the data supporting the conversion factors in this option are more robust (i.e. there are more data available for Option 1 at this time), and therefore the expert panel had greater confidence in the data for Option 1, as indicated in the overall higher certainty of evidence as assessed by GRADE. It was also noted that if CCNFSDU decides to use Option 2, further work will probably be needed to improve the accuracy of the estimates of the conversion factors, to be able to have greater confidence that they reflect the true values.

7.1 Discussion of determination of recommended nitrogen to protein conversion factors

Given the paucity of data for K’ values for soy, and the similarity between the K’ value and the K values for soy, the question was raised as to whether soy in fact contained any appreciable number of prosthetic groups, which could conceivably lead to different amounts of amino acids being delivered if K’ values were used. In response, it was pointed out that a 2016 review included estimates of factors for total soy proteins (including prosthetic groups).1 A value of 5.91 was calculated specifically for the soy 75 protein ß-conglycinin (based on the assumption that all three subunits of ß-conglycinin are glycosylated) and values of between 5.69 and 5.79 for total soy proteins with different 11S/7S ratios. These estimated values were not included in the final analysis of the commissioned systematic review, however, because they were based on assumptions made in other reports in the literature rather than being directly measured. Also, as noted previously, severe heat treatment has been shown to remove the putative prosthetic groups from proteins, including both dairy and soy. Given that soy proteins are generally heat treated to inactivate antinutritional factors, prosthetic groups are rarely considered for soy proteins when reporting conversion factors.

Concerns that the conversion factors being recommended were largely based on dairy and soy foods other than formulas and formula ingredients were reiterated. As previously noted, data for formulas and formula ingredients are relatively scarce; therefore, the expert panel reviewed the best, currently available evidence, which did include some data for formulas. The fact that the recommended conversion factors were derived largely from non-formula sources was partially captured in the downgrading of the certainty in the evidence owing to indirectness.

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8. CONCLUSIONS

The expert panel discussed the findings of a systematic review that had been commissioned to summarize relevant analytical methods, and to compile and calculate pooled estimates for different types of conversion factor from published sources and from unpublished sources (via a call for data). It then applied the GRADE framework to assess the quality of evidence for each of the pooled conversion factor estimates.

In discussing the findings of the review, three major themes emerged, as outlined below:

- It is difficult to accurately estimate the protein content of foods directly, because the most commonly used methods have numerous shortcomings. However, correction of the crude protein content of a food (based on analysis of total nitrogen) for measured non-protein nitrogen content allows an accurate determination of food proteins.

- The shortcomings of the commonly used protein estimation methods, together with a relative paucity of data for formulas and formula ingredients – resulting in low to moderate confidence in the conversion factors recommended by the expert panel – should be the impetus for a call to action by industry and other relevant stakeholders to generate relevant conversion factors for formulas using the best available methods, possibly as part of a multinational consortium.

- Selecting the appropriate conversion factor for a particular food or ingredient depends greatly on the nature of the protein and nitrogen in the sample, and how the two elements are defined and characterized.

Defining protein is particularly important because the relevance of a particular conversion factor in estimating the protein content of a food or ingredient varies, depending on whether the intention is to estimate the amount of amino acids in a food or the total protein content; that is, it depends on how protein is defined. For the purposes of developing and using conversion factors, protein can be considered in terms of amino acid content only, or of amino acids plus prosthetic groups that may also be present. Consideration of protein as the sum of amino acids (without the prosthetic groups) keeps the focus on delivery of amino acids, whereas inclusion of prosthetic groups considers the whole protein, not just amino acids; however, the latter may provide a higher estimate of protein content for the same amino acid content. A more holistic view of protein acknowledges that prosthetic groups are part of protein, and that focusing on amino acids alone is to ignore the other components of protein that could provide nutrition and other health benefits, even if their roles are not yet fully understood.

Because it is unclear whether the recommended ranges of protein provided in the relevant Codex standards are intended to ensure adequate delivery of amino acids or total protein, it was agreed to provide two sets of conversion factors to CCNFSDU.

On the basis of the results of the systematic review and the confidence in these results as assessed by the expert panel via the GRADE assessment process, the following conversion factors were proposed:

Option 1: When protein is defined as being only the sum of amino acids (i.e. ensuring delivery of amino acids is the primary aim), the recommended conversion factor for dairy-based ingredients is 6.1 and for soy-based ingredients is 5.7. There was moderate certainty
in the evidence underlying the conversion factor for dairy, and low certainty in the evidence underlying the conversion factor for soy. 

Option 2: When protein is defined as being the sum of amino acids and prosthetic groups (i.e. ensuring nutrition and health effects of the total protein is the primary aim), the recommended conversion factor for dairy-based ingredients is 6.3 and for soy-based ingredients is 5.7. There was moderate certainty in the evidence underlying the conversion factor for dairy, and very low certainty in the evidence underlying the conversion factor for soy.

The expert panel stressed that the recommended conversion factors in Option 1 are based on conversion factors derived from amino acid composition analyses in which prosthetic groups were not considered, and that the recommended conversion factors in Option 2 are based on conversion factors derived from various analyses in which prosthetic groups were either implicitly or explicitly included (noting that there was only one value of $K'$ to be considered for soy protein). The factors recommended above are based largely on data from non-formula dairy and soy foods, and to a lesser extent on whole, finished formulas rather than formula ingredients, for which there is a paucity of data. This is acknowledged in the expert panel’s confidence in these factors, as formalized in the certainty of the evidence assessed via GRADE.

Although the selection of conversion factors (i.e. deciding on Option 1 or Option 2) ultimately depends on whether the primary aim of determining protein content is the delivery of amino acids or delivery of total protein, in terms of the strength of the supporting evidence, the expert panel considers Option 1 to be the preferred option. Option 1 is preferred because the data supporting the corresponding conversion factors are more robust (i.e. there are more data for Option 1 at this time), and therefore the expert panel had greater confidence in Option 1, as indicated in the overall higher certainty of evidence as assessed by GRADE. It was also noted that if CCNFSDU decides to use Option 2, further work will likely be needed to improve the estimates of the conversion factors, and therefore have greater confidence that they reflect the true values.

The expert panel considers it critical that CCNFSDU select only one of these options to advance. The options are not intended to be presented to and decided on by manufacturers. Dissemination of two different sets of factors, providing the option of picking one or the other for different applications, or otherwise mixing or swapping between the factors, would create considerable confusion and uncertainty.

The expert panel noted that the conversion factor of 6.25 currently used in the Codex standards for infant formula and follow-up formula has not been empirically determined, and agreed that its application to a wide variety of proteins is highly inappropriate. The proposed conversion factors, therefore, represent a considerable improvement over the current scenario. The expert panel understands that CCNFSDU must consider the practical and nutritional ramifications of changing the current conversion factors, but it was not in the remit of the expert panel to make any recommendations in this regard. The expert panel also felt that it was important to retain the option for manufacturers to propose different

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1 The conversion factors recommended in Option 1 are based on those that were derived by assessing protein nitrogen only (i.e. non-protein nitrogen was not included in the denominator of the equation shown in Section 4.4). Therefore, when using the recommended conversion factors in Option 1, determination of protein nitrogen in the sample (i.e. total nitrogen minus non-protein nitrogen) and use of this value with the conversion factors, should generally provide the most accurate estimate of protein content.
conversion factors for particular ingredients where scientifically justified, as per footnote 2 in the Codex infant formula standard (CODEX STAN 72–1981). The expert panel agreed that such conversion factors should be determined using a prespecified, standardized amino acid methodology. Nonetheless, it is also clear that the level of confidence in the evidence behind the proposed conversion factors is not high. This reflects the need to improve the accuracy of the analytical methods used. There is considerable scope to determine more precise factors (for both Options 1 and 2) through further research, using improved analytical methods for amino acid analysis (see Annex 6).

There is a very strong case, therefore, for the relevant industries to invest in further collaborative efforts to improve the precision of these estimates, using the best available methods to conduct amino acid analysis, with a focus on ingredients that are specifically used in infant and follow-up formula products. In addition, improved amino acid analyses could provide direct measures of protein in formulas and obviate the need for conversion factors, a topic that has been suggested in previous Codex documents pertaining to protein measurements in foods.
LIST OF PARTICIPANTS

JEMNU expert panel members

Professor Jayendra Amamcharla, Associate Professor (Food Science), Kansas State University, Manhattan KS 66506, United States of America (USA)

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Ms Emma Kennedy, Assistant to the Coordinator, NPU/NHD/WHO, 20, Avenue Appia, 1211 Geneva 27, Switzerland

Ms Karen McColl (rapporteur), Independent consultant, United Kingdom of Great Britain And Northern Ireland
ANNEX 2

CALL FOR DATA ON NITROGEN TO PROTEIN CONVERSION FACTORS FOR SOY-BASED AND MILK-BASED INGREDIENTS

Background

The Codex Alimentarius Commission is the Joint FAO/WHO Food Standards Programme; it was established by FAO and WHO to protect consumer health and promote fair practices in food trade, via the adoption of international food standards, guidelines and codes developed by various Codex Committees, including the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU).

Currently being discussed at CCNFSDU is the most appropriate nitrogen to protein conversion factor (or factors) to use in estimating protein content of infant formula and follow-up formula containing soy-based and/or milk-based ingredients. To provide guidance on this topic, at the 39th Session of CCNFSDU, the Committee requested that the Joint FAO/WHO Expert Meetings on Nutrition (JEMNU) to be formed to provide scientific advice and evidence-informed guidance regarding nitrogen to protein conversion factors.

To facilitate the work of JEMNU, a systematic review is currently being conducted to compile and analyse the available data on nitrogen to protein conversion factors for foods containing soy-based and/or milk-based ingredients.

Call for data

To complement published data that will be identified via systematic searching of relevant scientific and analytical databases, FAO and WHO are requesting interested parties to submit available data on nitrogen to protein conversion factors and associated measurements (nitrogen, amino acid, etc.) for soy and/or dairy proteins assessed in foods containing soy-based and/or milk-based ingredients.

When submitting data on nitrogen to protein conversions factors, please provide as much analytical and descriptive information as possible, including the following:

Minimal information

- Nitrogen to protein conversion factor(s) from source materials containing soy-based or milk-based ingredients (not limited to infant formula and follow-up formula)
- Detailed information on source materials, including scientific names, cultivar and other descriptive information, as applicable
- Total nitrogen content as determined according to the Official Methods of Analysis of AOAC International or equivalent method
- Total amino acid content determined by hydrolysis and analysis by chromatography, including sulfur-containing amino acids and tryptophan
- Moisture content of source materials
- Detailed descriptions of all analytical methods used
Additional, important information when available

- Total amino acid content determined by hydrolysis and analysis by chromatography, including sulfur-containing amino acids and tryptophan
- Nitrogen content of precipitated protein (protein nitrogen) and supernatant (i.e. non-protein nitrogen), reported individually

Helpful if available

- Protein content measured by other methods
- Non-protein composition of source material(s) (e.g. carbohydrate content, fat content, etc.)

Ingredients of interest include, but are not limited to, those listed in the following table:

<table>
<thead>
<tr>
<th>MILK-BASED INGREDIENTS*</th>
<th>SOY-BASED INGREDIENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-fat milk solids</td>
<td>Soy protein isolate (hydrolyzed or not)</td>
</tr>
<tr>
<td>Extensively hydrolyzed Casein</td>
<td>Soy protein concentrate</td>
</tr>
<tr>
<td>Casein</td>
<td>Organic soy protein</td>
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<tr>
<td>Dried skim milk/nonfat milk powder</td>
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<td>Milk protein isolate</td>
<td></td>
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<tr>
<td>Milk protein concentrate</td>
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<td>Whey protein isolate</td>
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<td>Skim milk/nonfat milk</td>
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<tr>
<td>Extensively hydrolyzed whey protein</td>
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<td>Partially hydrolyzed whey protein</td>
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<td>Whey protein concentrate</td>
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<td>Calcium caseinate powder</td>
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<td>Sodium caseinate powder</td>
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<tr>
<td>Partially hydrolysed reduced minerals whey protein concentrate</td>
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<tr>
<td>Full fat</td>
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<tr>
<td>Modified milk ingredients</td>
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<tr>
<td>Organic milk and organic skim milk</td>
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</tbody>
</table>

* Milk-based ingredients could originate from cow, goat, sheep, among other mammals

Data must be submitted by 31 December 2018 to be considered for inclusion in the systematic review and subsequent review of this evidence at the forthcoming meeting of JEMNU. Data must be submitted online at the link below. Data submitted by email or in hard copy will not be considered. Data submitted for foods not containing either soy-based or milk-based ingredients will also not be considered.

Questions regarding the call for data should be addressed to NPUinfo@who.int.

This call for data is also cross-posted on the website of the Food and Agriculture Organization of the United Nations (FAO) at http://www.fao.org/nutrition/requirements/proteins/.
ANNEX 3

CALL FOR EXPERTS FOR THE JOINT FAO/WHO EXPERT MEETINGS ON NUTRITION (JEMNU) ON NITROGEN TO PROTEIN CONVERSION FACTORS FOR SOY-BASED AND MILK-BASED INGREDIENTS USED IN INFANT FORMULA AND FOLLOW-UP FORMULA

Background

The Joint FAO/WHO Expert Meetings on Nutrition (JEMNU) was established in 2012 to provide scientific advice to the committees of the Joint FAO/WHO Food Standards Programme (i.e. Codex Alimentarius) or Member Countries. JEMNU aims to provide relevant scientific advice in an independent and cost-effective manner; therefore, the Meetings will be convened when there is a specific request from a Codex Committee or Member Countries.

Currently being discussed at the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) is the most appropriate nitrogen to protein conversion factor (or factors) to use in estimating protein content of soy-based ingredients and milk-based ingredients used in infant formula and follow-up formula. To provide guidance on this topic, at the 39th Session of CCNFSDU in 2017, the Committee requested that JEMNU be convened to review the evidence and develop evidence-informed guidance regarding nitrogen to protein conversion factors. (To facilitate the work of JEMNU, a systematic review is currently being conducted to compile and analyse the available data on nitrogen to protein conversion factors for foods containing soy-based and/or milk-based ingredients.)

FAO and WHO have therefore initiated the convening of JEMNU and are in the process of identifying experts with relevant knowledge and experiences to participate in the expert meeting to be held during 15–19 July 2019 (exact dates to be confirmed). The selected experts will review the evidence to establish appropriate nitrogen to protein conversion factors for soy-based and milk-based ingredients used in infant formula and follow-up formula.

Desired expertise

Successful candidates should meet most or all of the following qualifications:

- Experience in research and application of methodologies for assessing protein quality and quantity in foods, particularly those containing soy- and milk-based ingredients;
- Good knowledge of the English language, both written and oral;
- Ability to prepare scientific documents and to work in an international environment with scientists from various disciplines;
- Recent, relevant scientific publications in peer-reviewed journals is desirable;
- Leadership, or invited participation, in national or international scientific bodies, committees and other expert advisory bodies pertinent to the scope of this work is desirable.
Expert activities

Experts will be expected to actively engage in:

- reviewing and interpreting the evidence;
- establishing recommended conversion factors;
- contributing to the development of a report summarizing the discussion and outcomes of the meeting; and
- reviewing the final report.

The meeting report, including recommended conversion factors, will be made available to the 41st Session of CCNFSDU to be held in November 2019.

Applying

To submit your application, please submit the following documents via the submission form at https://extranet.who.int/dataform/344856:

1. Curriculum vitae, including
   - detailed education background;
   - relevant work experience; and
   - list of peer-reviewed publications.

2. Completed Declaration of Interests (DOI) form
   - PDF and Word versions of the DOI form, along with documents providing guidance on completing the DOI form can be downloaded at https://extranet.who.int/dataform/344856

3. Signed Confidentiality Undertaking
   This document can be downloaded at https://extranet.who.int/dataform/344856

Process for selection of experts

- Curriculum vitae of applicants will be reviewed to assess whether applicants have relevant technical expertise and experience in the specified areas as listed above.
- Declaration of Interest forms will also be reviewed thoroughly to assess, any potential or perceived conflicts of interest disclosed, as required.
- In addition to subject matter expertise, the selection of experts will also take into consideration diversity and complementarities of expertise, a balance of genders and balanced representation from FAO/WHO geographic regions including developing and developed countries.
- Representatives of commercial organizations may not serve as experts.
- Selected experts will be invited to contribute only in their individual capacity as experts and will not represent their government, nor their institution. The names and affiliations of experts participating in the meeting will be included in the report and published on the FAO and WHO websites.
• The meeting will be held in English only and all documents including the background systematic review will be prepared in English. Travel and per diem to attend the meeting will be provided. No honoraria will be provided.

**Documents must be submitted by 1 March 2019** to be eligible for consideration.

Documents can be submitted through the online submission form at https://extranet.who.int/dataform/344856.

Detailed instructions for submitting documents are provided in the online form. This call for experts is also cross-posted at http://www.fao.org/nutrition/requirements/proteins/en/

Questions regarding the call for experts should be addressed to NPUinfo@who.int.
1. **Background**

The current approved Codex methodology to determine protein content is to detect Nitrogen and then convert to protein using the appropriate conversion factor. There is currently some discussion in the scientific community about the appropriate nitrogen to protein conversion factors to use in the various matrices. Across Codex standards, there is no single universally agreed upon nitrogen to protein conversion factor for soy and milk.

At the 37th Session of the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU), the Committee raised the question about the appropriate nitrogen to protein conversion factors to be used for milk and soy protein in infant formula and follow-up formula. The Committee agreed to request advice from the Codex Committee on Methods of Analysis and Sampling (CCMAS) on the accuracy and appropriateness of 5.71 as the nitrogen to protein conversion factor for soy protein isolates used in formulas for infants and young children and to consider the amino acid profile of the isolate. However, at the 37th Session of CCMAS (REP16/MAS), the Committee informed CCNFSDU that appropriate nitrogen to protein conversion factors were not part of the scope of CCMAS and noted that FAO and WHO could convene an expert panel to assess the scientific basis for nitrogen to protein conversion factors to answer the question of appropriate nitrogen to protein conversion factors for use by Codex.

2. **Joint Expert Meetings on Nutrition (JEMNU)**

The Joint Expert Meetings on Nutrition (JEMNU) was established to provide scientific information and advice to the committees of the Joint FAO/WHO Food Standards Programme (i.e. Codex) or Member Countries. At the 38th Session of the CCNFSDU, the Committee was asked to consider the draft proposal prepared by the Secretariat on the amendment to Section 6 “Selection of risk assessor by CCNFSDU”, paragraph 33 of the nutritional risk analysis principles to include JEMNU as a primary source of scientific advice. The Committee agreed to forward the proposed amendments to Section 6, Paragraph 33 to the Commission for adoption.
As evidence reviews and JEMNU meetings are funded through extra budgetary funds, Codex committees or Member Countries which are requesting advice have to collaborate with FAO/WHO in identifying sources of funds for a meeting. Funding for JEMNU is now available to initiate an expert meeting to provide advice to CCNFSDU on appropriate nitrogen to protein conversion factors.

3. Nitrogen to Protein Conversion Factors Questions

The Committee was reminded at the 38th Session of CCNFSDU that Step 1 of the Terms of Reference and Rules of Procedures of JEMNU states the need for the Codex body or Member Countries requesting information or scientific advice from JEMNU to formulate the PICO questions necessary for JEMNU to respond to specific requests. To ensure the questions asked to JEMNU provide the Committee with the appropriate advice, draft questions have been developed for consideration by the Committee.

When determining the protein content of soy-based ingredients used in infant formula and follow-up formula, what is the appropriate science-based nitrogen to protein conversion factor to use when comparing protein content derived from nitrogen based methods to amino acid based methods?

When determining the protein content of milk-based ingredients used in infant formula and follow-up formula, what is the appropriate science-based nitrogen to protein conversion factor to use when comparing protein content derived from nitrogen based methods to amino acid based methods?

**PICO Questions**

- **P** Soy-based or milk-based ingredients for infant formula and follow-up formula
- **I** Determining the protein content from nitrogen content using a conversion factor (of milk-based and soy-based ingredients)
- **C** Amino acid based methods (gold standard)
- **O** Determination of science-based nitrogen to protein conversion factor(s) for soy-based and milk-based ingredients.
Ingredients list:

<table>
<thead>
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<td>Milk protein concentrate</td>
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<tr>
<td>full fat</td>
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<tr>
<td>Modified milk ingredients</td>
<td></td>
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<tr>
<td>Organic milk and organic skim milk</td>
<td></td>
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</tbody>
</table>

* Milk based-ingredients could originate from cow, goat, sheep, among other mammals

Proposed Inclusion Criteria:

- Published or in press in a peer-reviewed journal, or unpublished data or reports
- Original research paper or systematic review paper
- All languages
- All publication years
- Primary or secondary objective was to determine protein content of one or more of the ingredients from the ingredient list above

For original research papers:

- Determined protein content from nitrogen content using a conversion factor and/or using amino acid-based methods

Research papers and unpublished papers must include

a. description of samples including type (e.g. isolate, concentrate, powder) and source(s) of analyzed materials;

b. reference (if previously published) and/or description of method procedures;

c. description of quality control procedures and must have some replicate analysis;

d. results sections will include experimental results, including some determination of precision and the calculation of factors, including formulas and/or reference to formulas used.
For review papers:

- Looked at papers determining protein content from nitrogen content using a conversion factor and/or using amino acid-based methods

**Proposed Exclusion Criteria:**

- Published in a non-peer-reviewed source (magazine, website, etc.)
- Abstract, short communication, opinion letter, authoritative statements, oral/poster presentation

For original research papers:

- 

For review papers:

- Reference current national and international standards or trade agreements as justification for a specific conversion factor

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DRAFTED: US and Canada, Nov 2017
Edits: WHO, FAO, US, Canada during CCNFSDU, 12/6/2017
Questions agreed by CCNFSDU plenary, 12/7/17
Edits by US and Canada on Ingredient list, inclusion/exclusion criteria: March/April 2018
**ANNEX 5
THE FINAL GRADE EVIDENCE PROFILES**

**GRADE evidence profile 1 – Dairy-based ingredients**

**Question:** When using the equation $\text{amount of protein (P)} = \text{nitrogen to protein conversion factor (K)} \times \text{amount of nitrogen (N)}$ to estimate the protein content of dairy-based ingredients used in infant formula and follow-up formula, which value of K most closely estimates the true amount of protein (P), where “protein” is defined as amino acid content only?

**Population:** Infant formula and follow-up formula

<table>
<thead>
<tr>
<th>Quality assessment</th>
<th>No. of studies</th>
<th>Study design</th>
<th>Risk of bias</th>
<th>Inconsistency</th>
<th>Indirectness</th>
<th>Imprecision</th>
<th>Other considerations</th>
<th>No. of independent measurements</th>
<th>Mean (95% CI)</th>
<th>Certainty</th>
<th>Importance</th>
<th>Risk of bias</th>
<th>Inconsistency</th>
<th>Indirectness</th>
<th>Imprecision</th>
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<th>Mean (95% CI)</th>
<th>Certainty</th>
<th>Importance</th>
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</thead>
<tbody>
<tr>
<td><strong>Nitrogen to protein conversion factor $K^*$ (unitless) – all dairy foods</strong></td>
<td>5</td>
<td>Mixed</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Very serious</td>
<td>Not serious</td>
<td>None</td>
<td>10</td>
<td>6.32 (6.26, 6.38)</td>
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<tr>
<td><strong>Nitrogen to protein conversion factor $K_A$, with amide/acid ratio of 50/50 (unitless) – all dairy foods</strong></td>
<td>13</td>
<td>Amino acid analysis</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Not serious</td>
<td>None</td>
<td>31</td>
<td>6.06 (6.00, 6.12)</td>
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<tr>
<td><strong>Nitrogen to protein conversion factor $K_A$, with amide/acid ratio of 75/25 (unitless) – all dairy foods</strong></td>
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<td>Serious</td>
<td>Not serious</td>
<td>None</td>
<td>14</td>
<td>5.83 (5.77, 5.89)</td>
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<tr>
<td><strong>Nitrogen to protein conversion factor $K_{AB}$, with directly calculated amide/acid ratio (unitless) – all dairy foods</strong></td>
<td>12</td>
<td>Amino acid analysis</td>
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<td>Not serious</td>
<td>Serious</td>
<td>Not serious</td>
<td>None</td>
<td>16</td>
<td>6.03 (5.98, 6.07)</td>
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<tr>
<td><strong>Nitrogen to protein conversion factor $K_{AB}$ (unitless) – all dairy foods</strong></td>
<td>3</td>
<td>Amino acid analysis</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Serious</td>
<td>Very serious</td>
<td>None</td>
<td>3</td>
<td>5.55 (5.31, 5.78)</td>
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<td>Study</td>
<td>Risk of bias</td>
<td>Inconsistency</td>
<td>Indirectness</td>
<td>Imprecision</td>
<td>Other considerations</td>
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<tr>
<td>Design</td>
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<td></td>
<td>Analysis</td>
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<td>Not serious</td>
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<tr>
<td></td>
<td>1</td>
<td>6.38 (single measurement)</td>
<td>VERY LOW CRITICAL</td>
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<td></td>
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</tr>
<tr>
<td>Design</td>
<td>Amino acid analysis</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Not serious</td>
<td>None</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1</td>
<td>6.08 (6.05, 6.12)</td>
<td>MODERATE CRITICAL</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

CI: confidence interval.

1 Importance for decision-making. In this case, conversion factors are the only "outcome" and thus each of the variations is critical for decision-making.

2 Amino acid sequencing and measurement (weighing) of crude protein with total nitrogen analysis. Because this factor was derived primarily from studies using amino and sequencing, it was started at "high" certainty.

3 There was no indication of systematic measurement error or reporting error (i.e. selective reporting of K' values). Not downgraded.

4 Inconsistency was assessed by considering the level of variance around the mean. The 95% CI suggests very little variation around the mean. Not downgraded.

5 Only a single K' value for infant and follow-up formulas was identified in the literature review. The mean conversion factor for infant and follow-up formulas. In addition, calculation of K' includes proteins groups in the protein mass and the PICO question specifically defines protein as amino acids only. Downgraded twice for very serious indirectness.

6 The minimum of 10 measurements is satisfied. Not downgraded.

7 Because these factors were derived primarily from studies using amino acid composition analysis, they were started at "moderate" certainty.

8 Of the 31 measurements, 11 come from infant and follow-up formulas. Although K' 50/50 and 75/25 values are based on setting the ratio of amide to acid at 50/50 or 75/25, it was expected that this would not result in significant enough difference from values derived via direct adjustment. Also, unlike K B, KA values do not explicitly take into consideration non-protein nitrogen; however, non-protein nitrogen can vary considerably across food samples. It is therefore difficult to assess the level of indirectness using a conversion factor that does not take non-protein nitrogen into consideration in estimating protein content in a variety of formulas with different formulations. There was no indication of reporting error (i.e. selective reporting of K' values). Not downgraded.

9 In addition to having greater confidence in the K' 50/50 value relative to K' 75/25, because the former includes a significant number of studies that derived conversion factors directly from infant and follow-up formulas, there was greater confidence in this value because a is in line with that of the K' derived from directly adjusted amide to acid ratio. No studies reported values for infant or follow-up formulas. The mean conversion factor was therefore derived from dairy sources other than infant and follow-up formulas. Although K' 50/50 and 75/25 values are based on setting the amide to acid ratio at 50/50 or 75/25, it was expected that this would not result in significant enough difference from values derived via direct adjustment. Also, unlike K B, KA values do not explicitly take into consideration non-protein nitrogen; however, non-protein nitrogen can vary considerably across food samples. It is therefore difficult to assess the level of indirectness using a conversion factor that does not take non-protein nitrogen into consideration in estimating protein content in a variety of formulas with different formulations. Downgraded once for serious indirectness.

10 No studies reported values for infant or follow-up formulas. The mean conversion factor was therefore derived from dairy sources other than infant and follow-up formulas. Although K' 50/50 and 75/25 values are based on setting the amide to acid ratio at 50/50 or 75/25, it was expected that this would not result in significant enough difference from values derived via direct adjustment. Also, unlike K B, KA values do not explicitly take into consideration non-protein nitrogen; however, non-protein nitrogen can vary considerably across food samples. It is therefore difficult to assess the level of indirectness using a conversion factor that does not take non-protein nitrogen into consideration in estimating protein content in a variety of formulas with different formulations. Downgraded once for serious indirectness.
No studies reported values for infant or follow-up formulas. The mean conversion factor was therefore derived from dairy sources other than infant and follow-up formulas (e.g. milk, milk proteins, etc.) and was thus considered an indirect assessment of the conversion factor for infant and follow-up formulas. Also, unlike $K_A$, $K_A$ values do not explicitly take into consideration non-protein nitrogen; however, non-protein nitrogen can vary considerably across food samples. It is therefore difficult to assess the level of indirectness in using a conversion factor that does not take non-protein nitrogen into consideration to estimate protein content in a variety of formulas with different formulations. Downgraded once for serious indirectness.

No studies reported values for infant or follow-up formulas. The mean conversion factor was therefore derived from dairy sources other than infant and follow-up formulas (e.g. milk, milk proteins, etc.) and was thus considered an indirect assessment of the conversion factor for infant and follow-up formulas. Downgraded once for serious indirectness.

Fewer than 5 studies contributing to the mean. Downgraded twice for very serious imprecision.

Because this factor was derived from a study using amino acid sequencing, it was started at “high” certainty.

Conversion factors were derived from infant and follow-up formulas. However, calculation of $K'$ includes prosthetic groups in the protein mass and the PICO question specifically defines protein as amino acids only. Downgraded once for serious indirectness.

Conversion factors were derived from infant and follow-up formulas. Although $K_A$ 50/50 and 75/25 values are based on setting the ratio of amide to acid arbitrarily at 50/50 or 75/25, it was expected that this would not result in significant enough difference from values derived via direct adjustment. Also, unlike $K_A$, $K_A$ values do not explicitly take into consideration non-protein nitrogen; however, non-protein nitrogen can vary considerably across food samples. It is therefore difficult to assess the level of indirectness in using a conversion factor that does not take non-protein nitrogen into consideration in estimating protein content in a variety of formulas with different formulations. There was no indication of reporting error (i.e. selective reporting of $K_A$ values). Not downgraded.

Annex 4 in the systematic review by Tome et al. provides information on which studies provided data for each mean conversion factor shown in GRADE evidence profile 1 above.

---

**GRADE evidence profile 2 – Soy-based ingredients**

**Question:** When using the equation \( \text{amount of protein (P)} = \text{nitrogen to protein conversion factor (K)} \times \text{amount of nitrogen (N)} \) to estimate the protein content of soy-based ingredients used in infant formula and follow-up formula, which value of K most closely estimates the true amount of protein (P), where “protein” is defined as amino acid content only?

**Population:** Infant formula and follow-up formula

<table>
<thead>
<tr>
<th>Nitrogen to protein conversion factor K* (unitless) – all soy foods</th>
<th>Quality assessment</th>
<th>No. of independent measurements</th>
<th>Mean (95% CI)</th>
<th>Certainty</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total protein and nitrogen</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Very serious</td>
<td>Very serious</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrogen to protein conversion factor K A, with amide/acid ratio of 50/50 (unitless) – all soy foods</th>
<th>Quality assessment</th>
<th>No. of independent measurements</th>
<th>Mean (95% CI)</th>
<th>Certainty</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>Amino acid analysis</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Serious</td>
<td>Not serious</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrogen to protein conversion factor K A, with amide/acid ratio of 75/25 (unitless) – all soy foods</th>
<th>Quality assessment</th>
<th>No. of independent measurements</th>
<th>Mean (95% CI)</th>
<th>Certainty</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Amino acid analysis</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Serious</td>
<td>Not serious</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrogen to protein conversion factor K A, with directly calculated amide/acid ratio (unitless) – all soy foods</th>
<th>Quality assessment</th>
<th>No. of independent measurements</th>
<th>Mean (95% CI)</th>
<th>Certainty</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>Amino acid analysis</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Serious</td>
<td>Not serious</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrogen to protein conversion factor K B (unitless) – all soy foods</th>
<th>Quality assessment</th>
<th>No. of independent measurements</th>
<th>Mean (95% CI)</th>
<th>Certainty</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Amino acid analysis</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Serious</td>
<td>Not serious</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrogen to protein conversion factor K A, with amide/acid ratio of 50/50 (unitless) – formulas only</th>
<th>Quality assessment</th>
<th>No. of independent measurements</th>
<th>Mean (95% CI)</th>
<th>Certainty</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Amino acid analysis</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Very serious</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrogen to protein conversion factor K A, with amide/acid ratio of 75/25 (unitless) – formulas only</th>
<th>Quality assessment</th>
<th>No. of independent measurements</th>
<th>Mean (95% CI)</th>
<th>Certainty</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amino acid analysis</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Not serious</td>
<td>Very serious</td>
</tr>
</tbody>
</table>
### REPORT OF THE MEETING: NITROGEN TO PROTEIN CONVERSION FACTORS FOR SOY-BASED AND MILK-BASED INGREDIENTS

<table>
<thead>
<tr>
<th>Nitrogen to protein conversion factor K&lt;sub&gt;A&lt;/sub&gt;, with directly calculated amide/amino acid ratio (unitless) – formulas only</th>
<th>Study design</th>
<th>Risk of bias</th>
<th>Inconsistency</th>
<th>Indirectness</th>
<th>Imprecision</th>
<th>Other considerations</th>
<th>Certainty</th>
<th>Importance</th>
<th>Quality assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino acid analysis</td>
<td>No. of studies</td>
<td>Mean (95% CI)</td>
<td>None</td>
<td>2</td>
<td>5.42</td>
<td>(5.40, 5.44)</td>
<td>VERY LOW CRITICAL</td>
<td></td>
</tr>
</tbody>
</table>

**Amino acid analysis**

1. **Amin...**

2. **Regarding K′ and inclusion of prosthetic groups for soy, an additional conversion factor with a value of 5.91 was calculated...**

3. **Because this factor was derived from a study using measurement of crude protein with total nitrogen, it was started at "moderate" certainty.**

4. **There was no indication of systematic measurement error or reporting error (i.e. selective reporting of K′...**

5. **Inconsistency was not formally assessed as only a single study was available.**

6. **No studies reported K′ values for infant or follow-up formulas. A single study reported K′ for soybeans and this value was then considered an indirect assessment of the conversion factor for infant...**

7. **Fewer than 5 studies contributed to the mean.**

8. **Because these factors were derived primarily from amino acid analysis,...**

9. **Because amino acid analysis was the only "outcome" and thus each of the variations is critical for decision-making.**

10. **Although the overall certainty in the evidence for K<sub>A</sub> 50/50 and K<sub>A</sub> 75/25 was assessed as low, there was greater confidence in the value for K<sub>A</sub> 50/50 because it is in line with that of the K<sub>B</sub> values derived from soy sources other than infant and follow-up formulas.**

11. **Although K<sub>A</sub> 50/50 and K<sub>A</sub> 75/25 values are based on setting the ratio of amide to acid arbitrarily at 50/50 or 75/25, it was expected...**

12. **Non-protein nitrogen can vary considerably across food samples. It is therefore difficult to assess the level of indirectness in using a conversion factor that does not take non-protein nitrogen into consideration in estimating protein content in a variety of formulas with different formulations.**
13 Only two values for formulas were identified (from one source). The mean conversion factor was therefore primarily derived from soy sources other than infant and follow-up formulas (e.g., soybean, soy isolates, etc.) and was thus considered an indirect assessment of the conversion factor for infant and follow-up formulas. Also, unlike $K_A$, $K_B$ values do not explicitly take into consideration non-protein nitrogen; however, non-protein nitrogen can vary considerably across food samples. It is therefore difficult to assess the level of indirectness in using a conversion factor that does not take non-protein nitrogen into consideration in estimating protein content in a variety of formulas with different formulations. Downgraded once for serious indirectness.

14 No studies reported $K_B$ values for infant or follow-up formulas. The mean conversion factor was therefore derived from soy sources other than infant and follow-up formulas (e.g., soybean, soy isolates, etc.) and was thus considered an indirect assessment of the conversion factor for infant and follow-up formulas. Downgraded once for serious indirectness.

15 Conversion factors were calculated directly from infant and/or follow-up formulas. Although $K_A 50/50$ and $75/25$ values are based on setting the ratio of amide to acid arbitrarily at 50/50 or 75/25, it was expected that this would not result in significant enough difference from values derived via direct adjustment. Also, unlike $K_B$, $K_A$ values do not explicitly take into consideration non-protein nitrogen; however, non-protein nitrogen can vary considerably across food samples. It is therefore difficult to assess the level of indirectness in using a conversion factor that does not take non-protein nitrogen into consideration in estimating protein content in a variety of formulas with different formulations. Not downgraded.

16 Conversion factors were calculated directly from infant and follow-up formulas. Also, unlike $K_B$, $K_A$ values do not explicitly take into consideration non-protein nitrogen; however, non-protein nitrogen can vary considerably across food samples. It is therefore difficult to assess the level of indirectness in using a conversion factor that does not take non-protein nitrogen into consideration in estimating protein content in a variety of formulas with different formulations. Not downgraded.

Annex 5 in the systematic review by Tome et al., provides information on which studies provided data for each mean conversion factor shown in GRADE evidence profile 2 above.

---

GRADE evidence profile 3 – Dairy-based ingredients

**Question:** When using the equation \( \text{amount of protein (P)} = \text{nitrogen to protein conversion factor (K)} \times \text{amount of nitrogen (N)} \) to estimate the protein content of dairy-based ingredients used in infant formula and follow-up formula, which value of K most closely estimates the true amount of protein (P), where “protein” is defined as amino acid content plus prosthetic groups?

**Population:** Infant formula and follow-up formula

<table>
<thead>
<tr>
<th>Nitrogen to protein conversion factor</th>
<th>Quality assessment</th>
<th>No. of independent measurements</th>
<th>Mean (95% CI)</th>
<th>Certainty</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>K´ (unitless) – all dairy foods</td>
<td>5 Mixed</td>
<td>Not serious³</td>
<td>Not serious³</td>
<td>Serious³</td>
<td>Not serious³</td>
</tr>
<tr>
<td>K A, with amide/amino acid ratio of 50/50 (unitless) – all dairy foods</td>
<td>13 Amino acid analysis²</td>
<td>Not serious⁴</td>
<td>Not serious³</td>
<td>Serious³</td>
<td>Not serious³</td>
</tr>
<tr>
<td>K A, with amide/amino acid ratio of 75/25 (unitless) – all dairy foods</td>
<td>12 Amino acid analysis²</td>
<td>Not serious⁴</td>
<td>Not serious³</td>
<td>Very serious¹⁹</td>
<td>Not serious³</td>
</tr>
<tr>
<td>K A, with directly calculated amide/amino acid ratio (unitless) – all dairy foods</td>
<td>12 Amino acid analysis²</td>
<td>Not serious⁴</td>
<td>Not serious³</td>
<td>Very serious¹⁹</td>
<td>Not serious³</td>
</tr>
<tr>
<td>K (unitless) – all dairy foods</td>
<td>3 Amino acid analysis²</td>
<td>Not serious⁴</td>
<td>Not serious³</td>
<td>Very serious¹⁹</td>
<td>Very serious¹¹</td>
</tr>
<tr>
<td>K´ (unitless) – formulas only</td>
<td>1 Amino acid sequencing²</td>
<td>Not serious³</td>
<td>Not serious³</td>
<td>Not serious¹³</td>
<td>Very serious¹¹</td>
</tr>
<tr>
<td>K A, with amide/amino acid ratio of 50/50 (unitless) – formulas only</td>
<td>1 Amino acid analysis²</td>
<td>Not serious³</td>
<td>Not serious³</td>
<td>Serious¹⁹</td>
<td>Not serious³</td>
</tr>
</tbody>
</table>
Importance for decision-making. In this case, conversion factors are the only "outcome" and thus each of the variations is critical for decision-making.

Amino acid sequencing and measurement (weighing) of crude protein with total nitrogen analysis. Because this factor was derived primarily from studies using amino acid sequencing, it was started at "high" certainty.

There was no indication of systematic measurement error or reporting error (i.e. selective reporting of $K_\text{am}$ values). Not downgraded.

Inconsistency was assessed by considering the level of variance around the mean. The 95% CI suggests very little variation around the mean. Not downgraded.

Only a single $K_\text{am}$ value for infant and follow-up formulas was identified in the literature review. The mean conversion factor was therefore primarily derived from dairy sources other than infant and follow-up formulas. The minimum of 10 measurements is satisfied. Not downgraded.

Because these factors were derived primarily from studies using amino acid composition analysis, they were started at "moderate" certainty.

Of the 31 measurements, 11 come from infant and follow-up formulas. Although $K_{\text{A} 50/50}$ and $K_{\text{A} 75/25}$ values are based on arbitrarily setting the ratio of amide to acid at 50/50 or 75/25, it was expected that this would not result in significant enough difference from values derived via direct adjustment. Also, calculation of $K_{\text{A}}$ does not include prosthetic groups in the protein mass and the PICO question specifically defines protein as amino acids plus prosthetic groups. Downgraded once for serious indirectness.

In addition to having greater confidence in the $K_{\text{A} 50/50}$ value relative to $K_{\text{A} 75/25}$ because the former includes a significant number of studies which derived conversion factors directly from infant and follow-up formulas (and therefore was not downgraded for serious indirectness), there was greater confidence in this value because it is in line with that of the $K_\text{am}$ derived from the directly adjusted amide to acid ratio, which is the most accurate method of assessing amide to acid ratio.

No studies reported $K_{\text{A}}$ or $K_{\text{B}}$ values for infant or follow-up formulas. The mean conversion factor was therefore derived from dairy sources other than infant and follow-up formulas (e.g. milk, milk proteins, etc.) and was started at "high" certainty.

Conversion factors were derived from infant and follow-up formulas. However, calculation of $K_{\text{A}}$ does not include prosthetic groups in the protein mass and the PICO question specifically defines protein as amino acids plus prosthetic groups. Downgraded twice for very serious indirectness.

Fewer than 5 studies contributed to the mean. Downgraded twice for very serious imprecision.

Because this factor was derived primarily from studies using amino acid sequencing, it was started at "high" certainty.

Conversion factors were derived from infant and follow-up formulas. However, calculation of $K_{\text{A}}$ does not include prosthetic groups in the protein mass and the PICO question specifically defines protein as amino acids plus prosthetic groups. Downgraded once for serious indirectness.
## GRADE evidence profile 4 – Soy-based ingredients

**Question:** When using the equation \( \text{amount of protein (P)} = \text{nitrogen to protein conversion factor (K)} \times \text{amount of nitrogen (N)} \) to estimate the protein content of soy-based ingredients used in infant formula and follow-up formula, which value of K most closely estimates the true amount of protein (P), where “protein” is defined as amino acid content plus prosthetic groups?

**Population:** Infant formula and follow-up formula

### Nitrogen to protein conversion factor \( K \) (unitless) – all soy foods

<table>
<thead>
<tr>
<th>Study design</th>
<th>Risk of bias</th>
<th>Consistency</th>
<th>Indirectness</th>
<th>Imprecision</th>
<th>Other considerations</th>
<th>No. of independent measurements</th>
<th>Quality assessment</th>
<th>Certainty</th>
<th>Quality of evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein and nitrogen ( K )</td>
<td>Not serious (^1)</td>
<td>Not serious (^3)</td>
<td>None</td>
<td>Very serious (^2)</td>
<td>None</td>
<td>1 (single measurement) (^2)</td>
<td>5.71</td>
<td>Very low</td>
<td>Very low critical</td>
</tr>
<tr>
<td>Amino acid analysis ( K )</td>
<td>Not serious (^3)</td>
<td>Not serious (^3)</td>
<td>None</td>
<td>Very serious (^2)</td>
<td>None</td>
<td>28</td>
<td>5.68 (5.66, 5.69)</td>
<td>Very low</td>
<td>Very low critical</td>
</tr>
<tr>
<td>Amino acid analysis ( K )</td>
<td>Not serious (^3)</td>
<td>Not serious (^3)</td>
<td>None</td>
<td>Very serious (^2)</td>
<td>None</td>
<td>26</td>
<td>5.60 (5.38, 5.42)</td>
<td>Very low</td>
<td>Very low critical</td>
</tr>
<tr>
<td>Amino acid analysis ( K )</td>
<td>Not serious (^3)</td>
<td>Not serious (^3)</td>
<td>None</td>
<td>Very serious (^2)</td>
<td>None</td>
<td>35</td>
<td>5.65 (5.61, 5.68)</td>
<td>Very low</td>
<td>Very low critical</td>
</tr>
<tr>
<td>Amino acid analysis ( K )</td>
<td>Not serious (^3)</td>
<td>Not serious (^3)</td>
<td>None</td>
<td>Very serious (^2)</td>
<td>None</td>
<td>16</td>
<td>5.35 (5.20, 5.51)</td>
<td>Very low</td>
<td>Very low critical</td>
</tr>
<tr>
<td>Amino acid analysis ( K )</td>
<td>Not serious (^3)</td>
<td>Not serious (^3)</td>
<td>None</td>
<td>Very serious (^2)</td>
<td>None</td>
<td>2</td>
<td>5.42 (5.42, 5.42)</td>
<td>Very low</td>
<td>Very low critical</td>
</tr>
<tr>
<td>Amino acid analysis ( K )</td>
<td>Not serious (^3)</td>
<td>Not serious (^3)</td>
<td>None</td>
<td>Very serious (^2)</td>
<td>None</td>
<td>2</td>
<td>5.42 (5.40, 5.44)</td>
<td>Very low</td>
<td>Very low critical</td>
</tr>
</tbody>
</table>

**Notes:**
- \(^1\) Nitrogen to protein conversion factor \( K \) with amide/amid acid ratio of 50/50 (unitless) – all soy foods
- \(^2\) Nitrogen to protein conversion factor \( K \) with amide/amid acid ratio of 75/25 (unitless) – all soy foods
- \(^3\) Nitrogen to protein conversion factor \( K \) with directly calculated amide/amid acid ratio (unitless) – all soy foods
- \(^4\) Nitrogen to protein conversion factor \( K \) – formulas only
- \(^5\) Nitrogen to protein conversion factor \( K \) with amide/amid acid ratio of 50/50 (unitless) – formulas only
- \(^6\) Nitrogen to protein conversion factor \( K \) with amide/amid acid ratio of 75/25 (unitless) – formulas only
- \(^7\) Nitrogen to protein conversion factor \( K \) with directly calculated amide/amid acid ratio (unitless) – formulas only

**Certainty:**
- Very low
- Very low critical
Importance for decision-making. In this case, conversion factors are the only “outcome” and thus each of the variations is critical for decision-making.

Regarding $K'$ and inclusion of prosthetic groups for soy, an additional conversion factor with a value of 5.91 was calculated specifically for the soy 7S protein $\beta$-conglycinin (Maubois J-L, Lorient D (2016). Dairy proteins and soy proteins in infant foods nitrogen-to-protein conversion factors. Dairy Sci Tech 96(1):15–25), based on the assumption that all three subunits of $\beta$-conglycinin are glycosylated. The authors further use this information to estimate factors for total soy proteins with different 11S/7S ratios, in the range 5.69–5.79. These values were not included in the final analysis as they were not directly measured but estimated, based on assumptions made in reports in the literature.

Because this factor was derived from a study using measurement of crude protein with total nitrogen, it was started at “moderate” certainty.

There was no indication of systematic measurement error or reporting error (i.e. selective reporting of $K'$ values). Not downgraded.

Inconsistency was not formally assessed as only a single study was available.

No studies reported $K'$ values for infant or follow-up formulas. A single study reported $K'$ for soybeans and this value was thus considered an indirect assessment of the conversion factor for infant and follow-up formulas. Downgraded once for serious indirectness.

Because these factors were derived primarily from studies using amino acid composition analysis, they were started at “moderate” certainty.

Only four values for formulas were identified (from two sources). The mean conversion factor was therefore derived from soy sources other than infant and follow-up formulas (e.g. soybean, soy isolates, etc.) and was thus considered an indirect assessment of the conversion factor for infant and follow-up formulas. Also, calculation of $K_A$ or $K_B$ does not include prosthetic groups in the protein mass and the PICO question specifically defines protein as amino acids plus prosthetic groups. Downgraded twice for very serious indirectness.

The minimum of 10 measurements is satisfied. Not downgraded.

Although the overall certainty in the evidence for $K_{50/50}$ and $K_{75/25}$ was assessed as very low, there was greater confidence in the value for $K_{50/50}$ because it is in line with that of the $K_s$ derived from the directly adjusted amide to acid ratio, which is the most accurate method of assessing amide to acid ratio.

Only two values for formulas were identified (from one source). The mean conversion factor was therefore derived from soy sources other than infant and follow-up formulas (e.g. soybean, soy isolates, etc.) and was thus considered an indirect assessment of the conversion factor for infant and follow-up formulas. Also, calculation of $K_A$ or $K_B$ does not include prosthetic groups in the protein mass and the PICO question specifically defines protein as amino acids plus prosthetic groups. Downgraded twice for very serious indirectness.

No studies reported $K_B$ values for infant or follow-up formulas. The mean conversion factor was therefore derived from soy sources other than infant and follow-up formulas (e.g. soybean, soy isolates, etc.) and was thus considered an indirect assessment of the conversion factor for infant and follow-up formulas. Also, calculation of $K_A$ or $K_B$ does not include prosthetic groups in the protein mass and the PICO question specifically defines protein as amino acids plus prosthetic groups. Downgraded twice for very serious indirectness.

Conversion factors were calculated directly from infant and/or follow-up formulas. However, calculation of $K_A$ or $K_B$ does not include prosthetic groups in the protein mass and the PICO question specifically defines protein as amino acids plus prosthetic groups. Downgraded once for serious indirectness.

Annex 5 in the systematic review by Tome et al. provides information on which studies provided data for each mean conversion factor shown in GRADE evidence profile 4 above.

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ANNEX 6

METHODS FOR AMINO ACID ANALYSIS — BEST PRACTICE

There are several sources of inaccuracy in traditional methods of amino acid analysis. These inaccuracies can be largely addressed, however, by adopting the best available methods.

Protein hydrolysis

A particular source of inaccuracy in the quantification is the acid hydrolysis procedure used to hydrolyse the peptide bonds within protein to liberate the amino acids. This is because amino acids are unstable to different degrees during hydrolysis. The amide and amine forms are particularly unstable, with asparagine and glutamine being deaminated to produce glutamic acid and aspartic acid. Tryptophan is largely destroyed in the presence of carbohydrates and heavy metals. Methionine and cysteine also tend to be very unstable, with cysteine being destroyed and methionine being oxidized. Furthermore, different amino acids take different lengths of time to hydrolyse. Traditionally, acid hydrolysis is conducted with hydrochloric acid over a 24-hour period, and this is recognized to be a significant source of error (+/- 20%).

There are various steps to take to address these issues:

- **Hydrolysis time:** A series of multiple hydrolysates (between 10 and 20) should be conducted, using different hydrolysis times and according to pre-defined conditions. A curvilinear model can then be applied to the data to determine the rate of release and destruction at any point in time.\(^1\),\(^2\) This method has been validated against protein sequencing and there is very good agreement.

- **For tryptophan:** Use of alkaline hydrolysis instead of acid hydrolysis is recommended.

- **For methionine/cysteine:** Oxidize before hydrolysis by incubating in freshly prepared performic acid at 4 degrees C for 16 hours.\(^3\)

- **Amines (asparagine and glutamine):** Esterification-reduction; carbodiimide modification; enzymatic hydrolysis; amide to amine conversion (rarely used).

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Lysine: This can be altered into other components during processing, and acid hydrolysis can break the bonds to produce lysine again, leading to overestimation. Methods have been developed to measure available lysine (the fluorodinitrobenzene method,\(^1\) the guanidination method\(^2\) and the furosine method\(^3\)).

For more discussion of these methods see Section 4.2.2. in the systematic review by Tomé and colleagues and Rutherfurd SM, Moughan PJ. The chemical analysis of proteins and amino acids. In: Moughan PJ, Hendriks WH, editors. Feed Evaluation Science. Wageningen: Wageningen Academic Publishers; 2018.

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